Université de Strasbourg

École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

UR 7296

THÈSE présentée par :

Laurie GHEDDAR

Soutenue le : **15 novembre 2021**

Pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : Sciences de la vie et de la santé / Toxicologie

Détection des substances de la performance dans les phanères : approches analytiques et application à des cas de dopage, des dossiers cliniques et des expertises médico-légales

THÈSE dirigée par :

M KINTZ Pascal	Professeur conventionné de l'Université de Strasbourg

RAPPORTEURS :

M GOULLE Jean-Pierre	Professeur émérite de l'Université de Rouen
M HOUZE Pascal	Professeur de l'Université de Paris

AUTRES MEMBRES DU JURY :

M MONASSIER Laurent	Professeur de l'Université de Strasbourg
M RAUL Jean-Sébastien	Professeur de l'Université de Strasbourg
M ALVAREZ Jean-Claude	Professeur de l'Université de Paris-Saclay
Mme PELISSIER Anne-Laure	Professeur de l'Université de Marseille
Mme LABAT Laurence	Maître de conférences de l'Université de Paris

Remerciements

J'aimerais tout d'abord exprimer toute ma gratitude aux membres de mon jury pour m'avoir fait l'honneur d'accepter d'évaluer mon travail de thèse et pour m'apporter leur expertise dans les domaines du dopage, de la toxicologie médico-légale, de la pharmacologie et de l'analytique : merci à mes deux rapporteurs externes, le Pr Jean-Pierre Goullé et le Pr Pascal Houzé, merci à mon examinateur interne, le Pr Laurent Monassier, et merci à mes examinateurs externes, le Pr Jean-Sébastien Raul, le Pr Jean-Claude Alvarez, le Pr Anne-Laure Pélissier et le Dr Laurence Labat.

Je tiens à remercier spécialement le Pr Jean-Sébastien Raul pour m'avoir donné l'opportunité de rejoindre l'Institut de Médecine Légale de Strasbourg et de m'avoir ouvert les portes du laboratoire de toxicologie en septembre 2017 et également de m'avoir fait confiance.

Je remercie chaleureusement le Pr Pascal Kintz, mon directeur de thèse et mentor, qui au décours d'un congrès de la SFTA, m'a parlé d'une possibilité de faire une thèse sur les produits de la performance. Merci pour le dévouement, le temps qu'il a pris pour moi, la patience dont il a fait preuve et le partage de ses connaissances. Merci de me former jour après jour au beau métier de toxicologue et de m'avoir transmis sa passion. Et enfin merci de me faire autant confiance !

Je remercie le Pr Laurent Monassier pour ses nombreux conseils et j'en profite pour remercier toute l'équipe du laboratoire de pharmacologie et toxicologie neurocardiovasculaire.

Je remercie également la SFTA pour m'avoir fait l'honneur de m'avoir sélectionnée en tant qu'oratrice à l'occasion des congrès de la SFTA, de la SFML ou encore de la journée thématique de la SFTA et de m'avoir ainsi donné l'occasion de présenter mes travaux. Merci pour nos belles collaborations et en particulier avec le Pr Anne-Laure Pélissier, le Pr Jean-Claude Alvarez, le Dr Nicolas Fabresse, le Dr Véronique Dumestre-Toulet, le Dr Jean-Michel Gaulier et le Dr Frédéric Aknouche.

J'aimerais également remercier les membres du comité de communication de la SFTA pour m'avoir fait confiance et donné l'opportunité de pouvoir m'investir dans notre société savante de toxicologie.

J'aimerais remercier particulièrement le Dr Véronique Dumestre-Toulet pour m'avoir accueillie dans son laboratoire de toxicologie, ToxGen, en 2015 pour effectuer mon stage de 2ème année de Master

et pour m'avoir donné le goût de la toxicologie. À cette occasion, j'ai pu faire de belles rencontres professionnelles et amicales. Je remercie tous mes anciens collègues de ToxGen et plus particulièrement mes anciens collègues du laboratoire de toxicologie : Laurent, Loïc, Sandrine, Alain, Sandra et Émilie sans oublier Corinne.

Je tiens également à remercier Amine et Nicolas pour nos échanges toujours agréables.

Merci à mes collègues du CHU de Strasbourg et plus particulièrement à Marie Scheffknecht pour son écoute et sa bienveillance. Et également au Dr Marie-Odile Batt pour nos collaborations scientifiques.

J'aimerais à présent remercier mes collègues et amis de l'IML de Strasbourg sans qui la vie au laboratoire ne serait pas la même.
Tout d'abord, à l'équipe de toxicologie, pour leur aide au quotidien au niveau scientifique et moral :
Alice, plus qu'une collègue, une amie avant tout. Merci de m'avoir soutenue, de m'avoir aidée et merci pour tous tes conseils. Nadia, merci d'être toujours aussi présente pour moi. Une amitié est née et restera. Je te souhaite tout le meilleur pour ta thèse, tu seras parfaite. Émilie, merci pour ton soutien sans faille, ton aide au laboratoire, et ton amitié.
Je tiens également à remercier infiniment Pauline, Morgane, Julia, Elodie, Véronique pour leur disponibilité, leur gentillesse, et surtout leur bonne humeur et leur rire si communicatif. Je remercie également tous les stagiaires avec qui j'ai pu travailler et qui m'ont fait évoluer.

Sans oublier tous les autres membres de l'IML : merci Sara, pour ta joie de vivre et nos moments détentes autour d'un café. Merci également à Cathie, Lauréline, Evelyne, Catherine, Anne pour tous les moments d'échange et de convivialité qu'on a partagée. Je n'oublie pas non plus l'équipe de paléoanthropologie : Christine, Angéla, Vincent et Jean-Luc. Merci d'avoir répondu à toutes mes questions et pour nos échanges enrichissants.
Merci à Pino pour tout ce que tu fais à l'IML mais surtout pour ton oreille attentive.

Je remercie également l'ensemble des médecins légistes de l'IML de Strasbourg pour nos collaborations : le Dr Audrey Farrugia, le Dr Annie Géraut, le Dr Laurent Berthelon, le Dr Estelle Godard, le Dr Alexis Walch et particulièrement le Dr Elisa Macoin et le Dr Adeline Blanchot avec qui j'ai développé une belle complicité et amitié.

Merci infiniment à ma famille et à mes merveilleux amis :
À Jean-Pierre et Patricia pour leur soutien et leur curiosité. À Cécile, Léo, Laura, Damien et Salvo.
À mes fantastiques amies de Lyon : Laura, Chloé et Hélo.

À mes amis de toujours : Quentin, Camille et Caroline. Merci pour votre patience.
À Romane, mon petit poussin. Merci d'être toujours là pour moi.

À la « business family ». Si j'en suis arrivée là, aujourd'hui, c'est grâce à vous, à votre soutien et à votre amour. Merci de m'avoir supportée dans les moments difficiles.
À mes parents, Martine et Yamine. Merci d'avoir tout fait pour que je puisse réaliser mes envies et mes rêves.
À mes sœurs :
Dida, merci de me guider, de me conseiller et de m'avoir encouragée tout au long de ma thèse.
Napi, merci de m'avoir soutenue tout au long de ma thèse et d'avoir été toujours présente dans les bons et les mauvais moments.

À mon Amour, Sacha. Merci d'avoir cru en moi à chaque instant, d'avoir supporté mes sauts d'humeur, de ton intérêt pour mon travail et de ton soutien inconditionnel au quotidien.

Mes trois années de thèse n'auraient pas été les mêmes sans ces belles rencontres professionnelles, et amicales et sans mes proches.

Table des matières

Liste des tableaux

Liste des figures

Liste des abréviations

AFLD : Agence Française de Lutte contre le Dopage

AMA : Agence Mondiale Anti-Dopage

AMM : Autorisation de Mise sur le Marché

CHU : Centre Hospitalier Universitaire

EI : Impact Électronique

GC : Chromatographie en phase gazeuse

HRMS : High Resolution Mass Spectrometry – Spectrométrie de Masse Haute Résolution

IML : Institut de Médecine Légale

LC : Chromatographie Liquide

LD : Limite de détection

LQ : Limite de quantification

µL : microlitre

MS : Spectrométrie de masse

MS/MS : Spectrométrie de masse en tandem

NaOH : hydroxyde de sodium

Ng/mL : nanogramme par millilitre

Pg/mg : picogramme par milligramme

RAA : Résultat Anormal Analytique

SAA : Stéroïdes Anabolisants Androgènes

SARM : Selective Androgenic Modulator Receptor – Modulateur Sélectif des Récepteurs des Androgènes

SI : Standard interne

SFTA : Société Française de Toxicologie Analytique

SPE : Extraction en Phase Solide

TAS : Tribunal Arbitral du Sport

T_R : temps de rétention

UHPLC-MS/MS : Chromatographie Ultra Haute Performante couplée à la spectrométrie de masse en tandem

Orientation générale de la thèse

On parle de substances de la performance lorsque l'action recherchée par l'utilisateur est l'amélioration des performances physiques et/ou mentales. Dans cette thèse, je me suis intéressée à trois familles pharmacologiques dont les agents anabolisants comprenant les stéroïdes anabolisants androgènes (SAA) et les modulateurs sélectifs des récepteurs des androgènes (SARMs), les diurétiques et également à deux modulateurs métaboliques associés à la prise d'agents anabolisants.

Ces substances sont utilisées dans un but de dopage (augmentation de la masse musculaire, diminution du temps de repos, masquer la prise d'autres substances dopantes) et sont interdits d'utilisation en permanence (pendant et hors compétition) par l'Agence Mondiale Anti-Dopage (AMA) mais ont été, pour la plupart, développées initialement à des fins thérapeutiques. Comme pour toute drogue, l'abus de ces substances peut provoquer des risques sur la santé physique et/ou psychologique. C'est pour cette raison qu'il est important de les caractériser dans un laboratoire de toxicologie médico-légale.

Au début de ma thèse, en 2018, il n'existait pas au laboratoire de Strasbourg de méthode dédiée à l'identification et à la quantification des agents anabolisants et des diurétiques. Trois méthodes ont donc été optimisées, développées et validées pour les stéroïdes anabolisants androgènes, les modulateurs sélectifs des récepteurs des androgènes et les diurétiques. Les paramètres des méthodes sont présentés dans ce manuscrit.

L'identification dans les matrices kératinisées permet d'élargir la fenêtre de détection des analytes. C'est pour cette raison que j'ai décidé d'orienter mes travaux de thèse sur l'analyse des phanères, incluant les cheveux, les poils du corps et les ongles.

A ce jour, le screening des agents dopants dans les cheveux permet d'identifier 30 SAA, 11 SARMs (dont deux modulateurs métaboliques associés aux SARMs) et 9 diurétiques. Ces méthodes sont désormais appliquées en routine à des cas de dopage, dans le cadre d'expertises médico-légales et de demandes cliniques.
Les cas observés et les résultats associés sont présentés sous forme d'articles publiés ou en cours d'évaluation.

Première partie - Contexte

1.1. Les substances de la performance

Un produit utilisé à des fins d'amélioration de la performance physique ou mentale est nommée « substance de la performance ». Ces substances regroupent un grand nombre de produits comme des produits naturels (dérivés de plantes, par exemple la caféine), des médicaments ou encore des produits stupéfiants. L'usage de substances aidant à l'amélioration des performances n'est pas nouveau et remonte à la Grèce antique.

On parle de dopage uniquement dans le milieu sportif. Mes travaux de thèse portent sur les analytes qui sont ceux utilisés dans le milieu du dopage et interdits par l'AMA mais également utilisés à des fins thérapeutiques et détournés par les consommateurs dans le but d'améliorer leur apparence physique.

L'AMA a vu le jour en 1999 après le scandale dans le cyclisme avec l'affaire Festina. A partir de cette situation, l'AMA a été créée afin d'harmoniser les pratiques et la liste des substances interdites dans tous les sports et dans tous les pays.

Les activités de l'AMA sont multiples et on retrouve plus particulièrement la supervision du Code Mondial anti-dopage. Le Code regroupe et classe les pratiques et les produits interdits. Les substances sont classées par famille et par période d'interdiction. En effet, certains composés sont interdits en permanence comme les stéroïdes anabolisants, d'autres sont interdits uniquement en compétition comme les amphétamines et enfin d'autres selon les sports comme les corticoïdes.

Dans le cadre d'un contrôle antidopage, toute substance détectée est considérée comme une violation du Code et il en résulte un résultat analytique anormal (RAA).

Chaque année, l'AMA publie un rapport d'activité et présente les différentes données liées au contrôle antidopage dont les chiffres de RAA par famille et par sport. En 2019, les agents anabolisants représentaient 44 % des RAA et les diurétiques 16 % (figure 1) *[rapport AMA, 2019]*.

WORLD ANTI-DOPING AGENCY
play true

2019 Anti-Doping Testing Figures
Samples Analyzed and Reported by Accredited Laboratories in ADAMS

Table 17: Summary - Substances Identified as AAFs in Each Drug Class in ADAMS (All Sports)

	Substance Group	Occurrences	% of all ADAMS reported findings
S1	Anabolic Agents	1825	44%
S6	Stimulants	611	15%
S5	Diuretics and Other Masking Agents	677	16%
S4	Hormone and Metabolic Modulators	362	9%
S9	Glucocorticosteroids	230	6%
S3	Beta-2 Agonists	153	4%
S8	Cannabinoids	130	3%
S2	Peptide Hormones, Growth Factors and Related Substances	138	3%
S7	Narcotics	30	1%
P1	Beta-Blockers	20	0.5%
M1	Enhancement of Oxygen Transfer	2	0.05%
M2	Chemical and Physical Manipulation	2	0.05%
	TOTAL*	4180	

Figure 1: Résultats analytiques anormaux par famille de substances – AMA – 2019

Au niveau national, le laboratoire accrédité AMA est l'Agence Française de Lutte contre le Dopage (AFLD), basé à Châtenay-Malabry qui, chaque année également, publie ses propres statistiques dans son rapport d'activité *[Rapport AFLD 2019, Rapport AFLD 2020]*. De même que le rapport de l'AMA, les stéroïdes anabolisants sont les produits ayant le plus gros pourcentage des RAA. En 2019, ils représentaient 34 % des RAA totaux et en 2020, 33 % (figure 2). Les diurétiques représentaient respectivement 15 et 16 % en 2019 et en 2020 (figure 2).

RÉPARTITION DES RÉSULTATS POSITIFS EN 2019
en fonction des différentes classes de substances interdites

	Dans l'urine	2019
S1	S1A SAA exogène S1.1B SAA endogène S1.2 Autres anabo	56
S2	Hormones peptidiques, facteurs de croissance et substances apparentées	7
S3	Bêta-2 agonistes	1
S4	Modulateurs hormonaux et métaboliques	8
S5	Diurétiques et agents masquants	25
S6	Stimulants	24
S7	Narcotiques	2
S8	Cannabinoïdes	14
S9	Glucocorticoïdes	26
P1	Bétabloquants	1
TOTAL		164

RÉPARTITION DES RÉSULTATS POSITIFS DE CONTRÔLE ANTIDOPAGE, EN FONCTION DES DIFFÉRENTES CLASSES DE SUBSTANCES

	2020
S1. AGENTS ANABOLISANTS	19
S2. HORMONES PEPTIDIQUES, FACTEURS DE CROISSANCE ET SUBSTANCES APPARENTÉES	1
S3. BÉTA-2 AGONISTES	3
S4. MODULATEURS HORMONAUX ET MÉTABOLIQUES	3
S5. DIURÉTIQUES ET AGENTS MASQUANTS	9
S6. STIMULANTS	5
S7. NARCOTIQUES	0
S8. CANNABINOÏDES	5
S9. GLUCOCORTICOÏDES	12
P1. BÊTA BLOQUANTS	0
TOTAL	**57**

Figure 2: Résultats analytiques anormaux par famille de substances - AFLD - 2019 et 2020

Il est intéressant de noter que chaque année, la classe des agents anabolisants représente le plus gros pourcentage de RAA. Par ailleurs, ces substances ont un tropisme clinique et médico-légal. C'est pour cette raison que le laboratoire de l'Institut de Médecine Légale (IML) de Strasbourg s'intéresse particulièrement à cette classe.

Une deuxième classe a attiré notre attention : la classe S5. Diurétiques et agents masquant. Ces substances ont quant à elles un tropisme plutôt clinique puisque utilisées par des sujets souhaitant perdre rapidement du poids.

J'ai intégré deux autres substances à mes travaux du fait de leur association aux agents anabolisants : il s'agit de la cardarine et du SR 9009 qui font partie de la classe S4. Hormone et modulateurs métaboliques. La classe S4 représentait 9 % des RAA en 2019 au niveau mondial et 5 % en 2020 au niveau national (figure 1 et figure 2).

Par ailleurs, depuis plusieurs années, de nouveaux agents anabolisants ont fait leur apparition. Il s'agit des « designer steroids » et des SARMs.

Les SARMs sont classés dans la sous-section 1.2. Autres agents anabolisants et sont utilisés dans le même but que les stéroïdes anabolisants. Leur analyse fait l'objet de la partie 3 du manuscrit.

Les « designer steroids » sont classés dans la section 1.a. Stéroïdes agents anabolisants. La liste des substances interdites n'est pas exhaustive mais L'AMA a prévu de les inclure dans la liste des substances interdites en ajoutant la phrase suivante dans le Code : « *et autres substances possédant une structure chimique similaire ou un (des) effet(s) biologique(s) similaire(s).* » *[Liste d'interdiction AMA, 2021]*.

Les « designer steroids » ne font pas l'objet de recherche dans ma thèse. Néanmoins, j'ai publié une revue de littérature qui expose les problématiques analytiques et les problèmes de santé induits par l'abus de ces nouveaux produits. Les problématiques soulevées sont superposables à celles des NPS *[Ameline, 2019]*. Cette publication est une revue de la littérature sur les nouveaux produits de la performance, les « designer steroids ». Ils sont apparus dès les années 1960 et ont été mis en lumière avec le tétrahydrogestrinone (THG) en 2003 lors du scandale de l'affaire Balco. Ils sont synthétisés à partir de stéroïdes anabolisants existants. Ces produits sont utilisés par les sportifs de haut niveau et les amateurs pour améliorer leurs performances physiques et mentales.

Par ailleurs, leur mésusage peut mener à des problèmes de santé comme des cancers des testicules, ou à des maladies neuropsychiques. Leur détection est un réel challenge pour les laboratoires anti-dopage mais également pour les laboratoires de toxicologie car il n'existe pas de standard de référence pour ces nouveaux produits. De nombreuses stratégies sont d'ailleurs mises en place pour pouvoir les détecter. Pour finir, toutes substances ayant les mêmes structures chimiques et les mêmes effets pharmacologiques sont interdits par l'AMA ***[Article 1]***.

Article 1 : Designer anabolic steroids: a challenge for toxicologists

Laurie Gheddar, Alice Ameline, Jean-Sébastien Raul, Pascal Kintz

Toxicologie Analytique et Clinique. 2019;31:293-297.

Toxicologie Analytique & Clinique (2019) 31, 293–297

Disponible en ligne sur
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com

REVIEW PAPER

Designer anabolic steroids: A challenge for toxicologists

Laurie Gheddar[a,*], Alice Ameline[a], Jean-Sébastien Raul[a], Pascal Kintz[a,b]

[a] *Institut de médecine légale, 11, rue Humann, 67085 Strasbourg, France*
[b] *X-Pertise Consulting, Mittelhausbergen, France*

Received 31 May 2019; received in revised form 30 June 2019; accepted 1st July 2019
Available online 20 July 2019

KEYWORDS
Designer steroids;
Anabolic steroids;
Dietary supplement;
NPS;
Doping

Summary Novel substances of performance have appeared since the 1960s. These substances are synthesized from anabolic androgenic steroids and are presented as designer steroids. They are used by top athletes for doping purposes as they increase lean body mass, strength, aggressiveness and lead to a shorter recovery time between workouts, but also by amateurs to improve their body aesthetic. Indeed, the use of designer steroids enables to improve performance and increase muscle mass. However, an overconsumption can cause health issues such as testicular cancer, hypogonadism or neuro-psychic disorders, including domestic violence. The designer steroids are available on the internet as dietary supplements. Their easy access and the difficulty of their detection is a big challenge for toxicological and forensic laboratories. The use of these drugs is problematic for doping, clinical and forensic aspects. As it is the case with other new psychoactive substances, obtaining reference material can be a challenge for toxicology laboratories. This short review of the literature aims to highlight some issues related to the abuse of designer steroids.

Introduction

In the 1930s, testosterone was discovered and purified for the first time. Testosterone is an endogenous anabolic androgenic steroid (AAS); it is the male sex hormone for the development of male sexual organs and secondary sexual characteristics. After its discovery, the pharmaceutical laboratories but also university groups began chemical and pharmacological studies for purpose of healthcare. This research involved the development of new exogenous anabolic androgenic steroids to be active against muscle weakness, anaemia, hormonal disorders, hormone-related cancers, and post-operative treatment [1].

In the 1960s, some endogenous and exogenous AAS were used for doping purposes by athletes, as these substances allow them to improve their sports performance,

* Corresponding author.
E-mail address: l.gheddar@gmail.com (L. Gheddar).

https://doi.org/10.1016/j.toxac.2019.07.001

increase their muscle mass and reduce the recovery time.

In 1968, AAS were banned by the International Olympic Committee and in 1975 AAS were registered on the list of banned substances. In 1998, a scandal broke out in the world of cycling, i.e. the Festina case. In order to respond to this crisis in sport, the World Anti-Doping Agency (WADA) was created in 1999. A document, harmonizing prohibited practices and substances in sport, named the CODE, was set up. It includes all the aspects of doping, including the control issues. To date, 31 laboratories are accredited by WADA. AAS drugs were classified by WADA (class S1) as prohibited anabolic agents in and out-of-competition. In 2017, 44% of adverse analytical findings were due to the consumption of anabolic androgenic steroids [2].

These substances are used by top athletes but also by amateurs and even by children. In 2000, Laure published that doping products were used by 3 to 5% of the sports children and teenagers and by 5 to 15% of adult amateurs [3]. Another study showed figures ranging from 2.2% to 4% among young consumers (13—19 years). In addition, it was shown that the drugs were more consumed by the male sex [4].

Because of extensive controls, as their detection ranked first since many years, in the early 2000s, a new family of AAS appeared, the designer steroids (DS). This review of the literature aims to highlight the issues related to the use of DS, the areas in which they are used, the pharmacological and toxicological aspects and the methods used for their detection.

Method

This review aims to describe recent trends about DS and their abuse. We searched Pubmed, Scopus, Research Gate and Google using the key words ''designer steroids'', ''AAS designer'', ''Balco'', and ''legal steroids''.

History

In 2003, the first DS which was identified was tetrahydrogestrinone (THG), during the Balco case. This case, involving several top athletes such as Marion Jones, is a sport scandal for which the laboratory Bay Area Laboratory Co-Operative (Balco) was found guilty. In June 2003, the American body of doping (USADA) was contacted anonymously, to report the use a new drug. The athletes were banned and the president of the laboratory Balco was sentenced to jail [2,5]. THG is considered as the first DS released on the black market. A DS is a drug which is derivated from an AAS known and chemically modified to mimic the effects of an AAS and circumvent the anti-doping law [6].

The DS are designed to be undetectable by the anti-doping laboratories, due to a lack of the reference standard, extensive metabolism or shorter presence in the body although they have similar androgenic effects. They share the same mechanism of action as testosterone, i.e., acting on androgen receptors. In addition, to attract customers, DS can take healthier names, such as pro-hormone, natural steroids, testosterone booster [6].

Some compounds were synthetized in the 1960s and were forgotten by the scientific community. It's the case for norboletone and desoxymethyltestosterone. However, these compounds can be considered as false DS. A true DS is a compound which is completely new and unknown, at the time it is put on the market, by the scientific community, as it was the case with THG [2,5].

Designer steroids are direct or indirect derivatives of testosterone. From a known chemical structure, several transformations are possible: 17-α-methylation, halogenation or hydroxylation [7]. Joseph et al. have published possible modifications of the testosterone structure to yield designer anabolics. For instance, Rahnema et al. had published structural similarities between methylstenbolone (designer steroid) and stenbolone, mentabolan (designer steroid) and trenstolone or epistane (designer steroid) and epitiostanol [6]. For all these reasons, DS can be considered very close to new psychoactive substances (NPS).

In comparison with testosterone, chemical structures of some DS are presented in Table 1.

Pharmacological properties and clinical issues

In the literature, there is no scientific information about the pharmacological properties and clinical issues due to the absence of controlled study. However, to get these data, some authors have compared DS to known AAS.

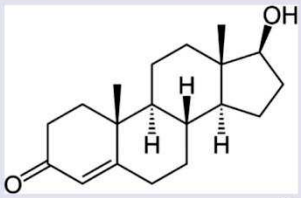
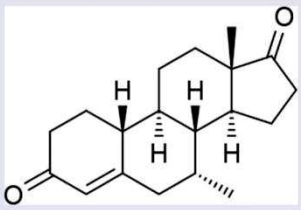
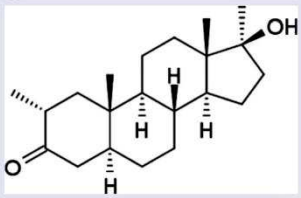
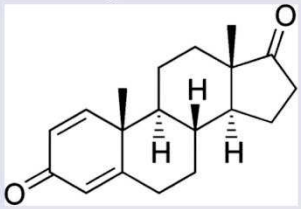
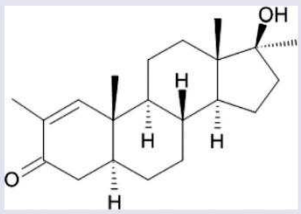
AAS have anabolic and androgenic effects. An anabolic effect facilitates the biosynthesis of proteins, favouring the growth of muscles and enables faster regeneration of red blood cells. An androgenic effect enables to develop male sexual organs and secondary sex characteristics such as body hair, aggressiveness and increase of libido. Chemical modifications can modify the anabolic: androgenic ratio. Some DS (as methylstenbolone) can be more anabolic than previously known AAS [6]. Structural modification, for example for methasteron, can also modify the oral bioavailability. Kazlauskas et al. reported that a 17-α alkylated molecule can improve the oral bioavailability but this will increase toxicity, due to the increase of the circulating blood concentrations [8].

Furthermore, a long-term exposure period over months can induce several health issues, such as vascular diseases, testicular cancer (hormone-dependent), liver cancer (hormone-dependent) [9,10] or some neuro-psychic disorders (psychological dependence and violence). More recently, Coward et al. have published that an overconsumption of AAS can cause hypogonadism in young men [11].

Dietary supplement

As it is the case with NPS, the DS are easily obtained. These drugs are sold as dietary supplements (oral tablet or injection) on the internet but also in fitness shops. The main issue is the bad labelling and the lack of control of the ingredients. Indeed, all compounds are not always cited, and the amounts quoted are often not true. This has also been observed for conventional anabolic steroids [12].

Table 1 Chemical structures of testosterone and some designer steroids.

Name of compound	Chemical structures
Testosterone	
Trestione or mentabolan	
Methasteron	
Boldione	
Methylstenbolone	

According to Geyer et al., 20% of dietary supplements have been contaminated by designer steroids since 2002 [13]. These authors have listed several DS found in dietary supplements such as methasteron, 4-hydroxytestosterone, prostanozolol [13]. Recently Lorenz et al. have identified androsta-3,5-diene-7,17-dione in a dietary supplement [14]. It appears that these compounds were not always listed in the ingredients list. As a result, due to the lack of information on the labels, consumers often do not know what they exactly ingest.

These compounds can be used by top athletes but also by amateurs. In a world where beauty has become an important criterion, the use of DS helps to get closer to this goal.

According to the literature and websites of abusers, DS are consumed daily over a period of 1 to 2 months [15—18]. Dosage depends on the nature of DS and varies generally between 4 and 10 mg [19,20]. The industry tends to find rather molecules to be taken orally vs injectable as shown by Rahnema et al. For example, boldione is oral derivatives of boldenone [6].

Case reports

Few clinical cases have been reported following ingestion of DS. In the literature, there are 4 papers dealing with DS bought in the internet. Shah et al. reported 5 cases of cholestatic liver injury associated with methasteron supplement. There were 5 males aged from 20 to 33 years. All showed jaundice, pruritus and dark urine. The period of supplement use varied between 1 and 1.5 months [15]. Singh et al. presented 3 cases of severe hepatotoxicity induced by methasteron, including 3 males aged from 25 to 51 years and the symptoms were identical to those described by Shah [16]. The period of consumption varied between 3 weeks and 2 months with a reported dosage of 10 mg for 1 case (2—3 times daily) [16]. Nasr et al. presented 1 case of cholestatic and renal failure after repetitive administration of methasteron. A 42-year-old male presented the same symptoms as the previous cases after a consumption of methasteron for 1.5 months [17]. Another DS was implicated in a cholestatic jaundice, as Agbenyefia et al. reported a case of 26-year-old male abusing methylstenbolone and dimethazine supplements [18].

For all these cases, the symptoms went stronger about 2 weeks after discontinuation of taking the DS and 1 month after stopping the symptoms completely disappeared. It can be observed from these cases that the DS are preferably abused by young men.

The use of DS has clinical impacts but can also be observed during forensic work. One of the authors has been involved in a case of a 34-year-old male, amateur bodybuilder, charged with sexual assault on a minor. He told to the judge that he used 3 times a week a THG tablet bought on the internet, which he said would have changed his libido and enhanced its aggressivity. A hair test analysis confirmed the detection of THG with a concentration of 17 pg/mg (personal data, not published).

The identification and quantification of DS are a real challenge for laboratories because of the lack of information in the literature, particularly on drug metabolism and the lack of reference standards to calibrate the analytical equipment. An interesting alternative for the forensic laboratories involved in DS detection is to buy the drugs on the internet and to verify their purity using nuclear magnetic resonance or infra-red spectroscopy, as it has been done for other NPS. As usual methods have limitations, some laboratories have implemented analytical strategies to detect DS without reference material.

Analytical strategies

The standard use of chromatography systems requires knowing the chemical structure of a molecule. Moreover, in order to quantify a drug in an authentic matrix, it is essential for a laboratory to use reference materials. In order to avoid the use of reference standards that are either too expensive, of poor quality or inexistent, specific strategies have been put in place by laboratories. The structural tools, such as NMR can be used to determine the structure of a compound. This method was applied to the identification of cathinones, synthetic opioids or cannabinoids, which have the same problem than DS, as shown by Ameline et al. for various NPS [21]. Using this approach, Cawley et al. were able to identify a new DS, i.e. 3-chloro-17α-methyl-5α-androstan-17β-ol [22]. A combination of techniques involving liquid chromatographic/electrospray ionization orthogonal acceleration time-of-flight mass spectrometry and gas chromatographic/electrospray ionization orthogonal acceleration time-of-flight mass spectrometry was used by Georgakopoulos to detect DS with accurate full scan MS data [23]. Voelker et al. proposed to use UV spectroscopy in order to compare structures and molar absorption with data from known steroid standards [24]. Another strategy was applied by Yuan and his colleagues. The idea is to identify compounds which bind to the androgen receptor [25]. Finally, the test for identical fragments with known AAS using accurate mass techniques can also be considered. The accurate mass techniques enable too to making retrospective analysis; this strategy was used by Hopker et al. for analysis of doping agents [26]. It is also possible to gain information, particularly for metabolite(s) detection to perform, in vitro, experiments using human liver microsomes, as it has been proposed for another challenging class of performance enhancing drugs very close to AAS, the SARMs, for selective androgen receptor modulators, including ligandrol, for example [27].

Conclusion

Today, the use of DS has reached a wide audience and is becoming a widespread public health problem. The easy purchase on the internet attracts new customers, such as young men. In order to protect the athletes, WADA has written in its code that AAS ''and other substances with a similar chemical structure or similar biological effect(s)'' are prohibited [2]. Despite this list of banned compounds, the abuse of these products is in constant progression. For toxicology laboratories, the identification of these new products remains a daily challenge. The combination of structural, chromatographic and high-resolution techniques should be considered as the gold standard to detect these new DS.

Disclosure of interest

The authors declare that they have no competing interest.

References

[1] Fourcroy J. Designer steroids: past, present and future. Curr Op End Diab Ob 2006;13(3):306—9.
[2] World anti-doping agency. What is prohibited; 2019 [https://www.wada-ama.org/en/content/what-is-prohibited/prohibited-at-all-times/diuretics-and-masking-agents on 29 May 2019].
[3] Laure P. Épidémiologie du dopage. Presse Med 2000;29:1365—457.
[4] LaBotz M, Grisemer BA. Use of performance-enhancing substances. Pediatrics 2016;138, e20161300-e20161300.
[5] De Groot A, Koert W. 18. Designer Steroids; 2007 [available on https://www.ergogenics.org/anabolenboek/index18en.html on 29 May 2019].

[6] Rahnema CD, Crosnoe LE, Kim ED. Designer steroids—over-the-counter supplements and their androgenic component: review of an increasing problem. Andrology 2015;3(2):150—5.
[7] Joseph JF, Parr MK. Synthetic androgens as designer supplements. Curr Neuropharmacol 2015;13(1):89—100.
[8] Kazlauskas R. Designer steroids. In: Thieme D, Hemmersbach P, editors. Doping in sport, 195. Berlin: Springer; 2009. p. 155—85.
[9] Hernandez-Nieto L, Bruguera M, Bombi JA, Camacho L, Rozman C. Benign liver-cell adenoma associated with long-term administration of an androgenic-anabolic steroid (methandienone). Cancer 1977;40:1761—4.
[10] Gorayski P, Thompson CH, Subhash HS, Thomas AC. Hepatocellular carcinoma associated with recreational anabolic steroid use. Br J Sports Med 2008;42:74—5.
[11] Coward RM, Rajanahally S, Kovac JR, Smith RP, Pastuszak AW, Lipshultz LI. Anabolic steroid-induced hypogonadism in young men. J Urol 2013;190:2200—5.
[12] Fabresse N, Larabi I-A, Knapp A, Mayer C, Alvarez J-C.Seizures of doping substances pharmaceuticals and dietary supplements—a 3 years study. 2019., http://dx.doi.org/10.1016/j.toxac.2019.03074.
[13] Geyer H, Parr MK, Koehler K, Mareck U, Schänzer, Thevis M. Nutritional supplements cross-contaminated with doping substances. J Mass Spectrom 2008;43:892—902.
[14] Lorenz LM, Toomey VM, Lanzarotta AC, Flurer RA, Falconer TM. Identification of the designer steroid Androsta-3,5-diene-7,17-dione in a dietary supplement. Drug Test Anal 2019;11(7):1109—15, http://dx.doi.org/10.1002/dta.2589.
[15] Shah NL, Zacharias I, Khettry U, Afdhal N, Gordon FD. Methasteron-associated cholestatic liver injury: clinicopathologic findings in 5 cases. Clin Gastro Hep 2008;6:255—8.
[16] Singh V, Rudraraju M, Carey EJ, Byrne TJ, Vargas HE, Williams JE, et al. Severe hepatotoxicity caused by a methasteron-containing performance-enhancing supplement. J Clin Gastroenterol 2009;43(3):287.
[17] Nasr J, Ahmad J. Severe cholestatsis and renal failure associated with the use of the designer steroid Superdrol™ (Methasteron™): a case report and literature review. Dig Dis Sci 2009;54:1144—6.
[18] Agbenyefia P, Arnold CA, Kirkpatrick R. Cholestatic jaundice with the use of methylstenbolone and dymethazine, designer steroids found in super DMZ Rx 2.0 ''Nutritional Supplement''. J Investig Med High Impact Case Rep 2014;22(2), http://dx.doi.org/10.1177/2324709614532800.
[19] BlueCloud. Superdrol: the strongest oral out there?; 2018 [https://bluecloud.org/superdrol-review/ on 29 May 2019].
[20] MySupplementstore; 2019 [https://www.mysupplementstore.com/ on 29 May 2019].
[21] Ameline A, Garnier D, Gheddar L, Richeval C, Gaulier JM, Raul JS, et al. Identification and analytical charaterization of seven NPS, by combination of H NMR spectroscopy, GC-MS and UPLC-MS/MS, to resolve a complex toxicological fatal case. Forensic Sci Int 2019;298:140—8.
[22] Cawley AT, Blakey K, Waller CC, McLeod MD, Boyd S, Heather A, et al. Detection and metabolic investigations of a novel designer steroid: 3-chloro-17α-methyl-5α-androstan-17β-ol. Drug Test Anal 2016;8(7):631—2.
[23] Georgakopoulos CG, Vonaparti A, Stamou M, Kiousi P, Lyris E, Angelis YS, et al. Preventive doping control analaysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids. Rapid Commun Mass Spectrom 2007;21(15):2439—46.
[24] Voelker SE, Lorenz LM, Litzau JJ. Semi-quantitative determination of designer steroids by high-performance liquid chromatography with ultraviolet detection in the absence of reference material. Drug Test Anal 2019;11(3):428—34.
[25] Yuan X, Forman BM. Detection of designer steroids. Nucl Recept Signal 2005;3:e002.
[26] Hopker J, Schumacher YO, Fedoruk M, Morkeberg J, Bermon S, Iljukov S, et al. Athlete performance monitoring in anti-doping. Front Physiol 2018;9:1—4.
[27] Geldof L, Pozo OJ, Lootens L, Morthier W, Van Eenoo P, Deventer K. In vitro metabolism study of a black market product containing SARM LGD-4033. Drug Test Anal 2017;9:1017—25.

1.2. Black market

La vente des substances de la performance est illégale mais est bien réelle sur le marché noir par le biais d'Internet et c'est notamment le cas pour les SAA, les diurétiques et les SARMs.

Ces dernières années, de nombreuses études ont été publiées à propos de la vente illégale des produits de la performance et discutent des multiples problèmes liés à ce phénomène. Les problématiques d'étiquetage (absence de données ou non corrélation avec les résultats d'analyse), de composition, de quantité ont été soulevées.

Par exemple, en Allemagne, une étude a eu lieu de 2010 à 2013. 337 suppléments ont été analysés et 288 correspondaient à des SAA. De plus, 55 % des suppléments SAA analysés ne correspondaient pas aux informations indiquées sur les étiquettes *[Krug, 2014]*. En Suisse, 1190 suppléments ont été analysés et 75 % étaient des stéroïdes anabolisants *[Weber, 2017]*. Et enfin, en Italie, une étude réalisée de 2017 à 2019 a montré que 64 % des produits saisis étaient des SAA *[Odoardi, 2021].*

Ces études témoignent de la dangerosité de ce phénomène car les produits et les quantités retrouvées ne correspondent pas toujours à ce qui est annoncé sur les étiquettes. Cela peut engendrer des problèmes de dopage non voulu et des effets négatifs sur la santé. De plus, il a été rapporté que les quantités utilisées par les amateurs et les sportifs étaient de 10 à 100 fois supérieures que les quantités administrées de façon thérapeutique *[Trenton, 2005].*

Par ailleurs, le fait que ces produits existent sur les chaines de production peut provoquer des contaminations lors de la fabrication d'autres suppléments diététiques et compléments alimentaires *[Geyer, 2008].*

Les mêmes observations ont été faites pour les produits vendus comme SARMs. Dans cette étude, Leaney *et al.* ont analysé des produits vendus comme SARMs et ont montré que la plupart des produits étaient contaminés par d'autres SARMs *[Leaney, 2021].*

En France, les mêmes conclusions peuvent être établies. Le marché noir des substances de la performance est un phénomène sous-estimé et un réel problème de santé publique. Cette étude a donné lieu à une publication.

En collaboration avec l'équipe du Professeur Alvarez (Garches, France), une étude a été menée de janvier 2016 à décembre 2019. Après la saisie de nombreux produits de contrebande par la justice française, le laboratoire a reçu et analysé 110 produits par chromatographie liquide couplée à un spectromètre de masse haute résolution (LC-HRMS) et par chromatographie en phase gazeuse couplé à un spectromètre de masse (GC-MS) dont 75 médicaments et 35 suppléments diététiques. Cette étude a révélé qu'une grande partie des produits était soit adultérée soit sous-dosée. 54 produits contenaient des SAA. Le manque de contrôle de qualité ainsi que l'administration par voie

intraveineuse, pour la plupart des SAA vendus sur Internet, peuvent mener à des risques accrus d'intoxication [***Article 2***].

Article 2 : Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders.

Nicolas Fabresse, Laurie Gheddar, Pascal Kintz, Adeline Knapp, Islam Amine Larabi, Jean-Claude Alvarez. Forensic Science International. 2021;doi: 10.1016/j.forsciint.2021.110771.

Forensic Science International 322 (2021) 110771

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders

Nicolas Fabresse[a,b], Laurie Gheddar[c], Pascal Kintz[c,d], Adeline Knapp[a], Islam Amine Larabi[a], Jean-Claude Alvarez[a,b,*]

[a] Laboratoire de Pharmacologie – Toxicologie, Centre Hospitalier Universitaire Raymond Poincaré, FHU Sepsis, AP-HP, 104 boulevard Raymond Poincaré, 92380 Garches, France
[b] Plateforme de Spectrométrie de Masse MassSpecLab, INSERM UMR 1173, UFR des Sciences de la Santé Simone Veil, Université Paris-Saclay (Versailles Saint-Quentin-en-Yvelines), 2 avenue de la source de la Bièvre, 78180 Montigny-le-Bretonneux, France
[c] Institut de médecine légale, Strasbourg, France
[d] X-Pertise Consulting, Mittelhausbergen, France

ARTICLE INFO

Article history:
Received 17 December 2020
Received in revised form 17 March 2021
Accepted 20 March 2021
Available online 30 March 2021

Keywords:
Anabolic androgenic steroids
Performance enhancing drugs
Doping
Toxicology
Forensics

ABSTRACT

Substandard/counterfeit drugs are a growing global problem. According to the World Health Organisation, counterfeit medicines are medicines that are mislabelled deliberately and fraudulently regarding their identity and/or source. In high income countries, drugs seized are mainly represented by performance and image enhancing drugs (PIEDs). The aim of this study was to present the qualitative and quantitative results of toxicological analyses of pharmaceutical and dietary supplements seized from the black market among bodybuilders in France. All dietary supplements and pharmaceuticals seized from the black market and addressed to the laboratory for a qualitative and quantitative analysis between January 2016 and December 2019 were included in the study. A screening was carried out by gas chromatography-mass spectrometry and liquid chromatography-high resolution mass spectrometry. Identified compounds were quantified by liquid chromatography-tandem mass spectrometry. One hundred and ten products were seized and submitted to the laboratory for identification of active compounds and quantification: 75 pharmaceuticals and 35 dietary supplements. This included 39 oily and 3 aqueous solutions for intramuscular injection, 34 tablets, 13 capsules, 14 powders, 4 liquids and 3 lyophilizates. Among the pharmaceuticals, 25/75 (33%) were substandard (dosage not on the acceptable range defined for original products), 24/75 (32%) were counterfeit (qualitative formulation does not match the label) and 14/75 (19%) were original (qualitative formulation and levels of active ingredients fully matches the declared formulation. The analysis of the 12 remaining products revealed a correct qualitative content for 11/75 (15%), but quantitation could not be carried out because of the lack of reference standards at the time of the analysis. Fifty-four pharmaceuticals contained anabolic-androgenic steroids (AAS). Four out of 54 (7.4%) AAS were found as original, 8/54 (15%) could not be quantified (one with wrong active ingredient), corresponding to 43/54 (80%) AAS being non-original. In contrast, only 1/35 dietary supplement (3%) was adulterated, with a doping substance (1,3-dimethylbutylamine, DMBA). This work allows to show that France is not spared by the trafficking of PIEDs. The use of counterfeit drugs in mainstream population is an underestimated public health issue.

1. Introduction

Counterfeit drugs are considered by the World Health Organization (WHO) as those which are substandard and falsified, and either fail to meet quality standards or are deliberately manufactured to imitate a legitimate product [1]. They may contain no active ingredient, a wrong active ingredient, the true active ingredient associated with other active substances or an incorrect amount of the correct active ingredient. These products can lead to a panel of life-threatening adverse events up to deaths due to therapeutic inefficacy or toxicity [2–4]. Counterfeit drugs affect mainly low income countries with a 10% risk of experiencing this problem, whereas this risk fall to 1% in high income countries [1,5].

* Correspondence to: Laboratoire de Pharmacologie - Toxicologie, Université Paris-Saclay (Versailles Saint-Quentin en Yvelines), Inserm U-1173, Hôpital Raymond Poincaré, AP-HP, 104, Boulevard R. Poincaré, 92380 Garches, France.
E-mail address: jean-claude.alvarez@aphp.fr (J.-C. Alvarez).

https://doi.org/10.1016/j.forsciint.2021.110771
0379-0738/

As part of the fight against drug trafficking, Interpol organized in 2018 an operation (Pangea) mobilizing police, customs and health regulatory authorities from 116 countries targeting the illicit online sale of medicines and medical products [6]. This operation resulted in 859 arrests worldwide and the seizure of potentially dangerous pharmaceuticals worth more than USD 14 millions. Focusing on delivery services manipulated by organized criminal networks, the operation saw 3671 web links closed down, including websites, social media pages, and online marketplaces. Almost one million packages were inspected during the week of action (9–16 October), with 500 tons of illicit pharmaceuticals seized worldwide. These figures allow us to appreciate the extent of the black market for drugs in the world.

In high income countries, drugs seized are mainly represented by performance and image enhancing drugs (PIEDs). These molecules are taken with the aim of improving athletic performance, body image and as a complement or even substitute for physical exercise. Focus and preoccupation on body image is increasingly common for both genders in recent decades [7]. Use of enhancement drug supplements is reportedly widespread among athletes at all ages and competitive levels [8]. Several analytical studies have been conducted on drugs and nutritional supplement seized on the black market [9–14]. The most common substances found are anabolic androgenic steroids (AAS). In France, only one testosterone ester (enanthate) is still marketed under medical prescription to treat hypogonadism. The multitude of websites selling PIEDs and the low cost offered have led to an explosion in their consumption among mainstream fitness groups or even among people who do not practice sport, and has contributed to an emergent public health issue. The use of such product poses a health risk to consumers, who have reported harms such as infections at injecting sites when counterfeit or contaminated products are used [15]. Furthermore, chronic exposure to AAS is responsible for many complications: cardiovascular, endocrine, hepatic and behavioral [16].

To our knowledge, no study carried out on the analysis of pharmaceuticals products from the black market has been conducted in France. The aim of this study was to present the qualitative and whenever possible quantitative results of toxicological analyses of pharmaceutical and dietary supplements seized from the black market among bodybuilders in France.

2. Materials and methods

2.1. Chemicals and reagents

Water, formic acid and methanol (MeOH) were MS grade and provided by Merck (Darmstadt, Germany). All other chemicals were analytical grade and provided by Sigma Aldrich (USA). 1,3-dimethylbutylamine, 4-chlorodehydromethyltestosterone (or turinabol), anastrozole, androstenedione, androstanolone (or dihydrotestosterone), boldenone, boldenone undecylenate, clenbuterol, dehydroepiandrosterone (DHEA), drostanolone propionate, epitestosterone, letrozole, lormetazepam, mesterolone, methandienone, methenolone enanthate, methoxsalen, methyltestosterone, nandrolone (19-nortestosterone), nandrolone decanoate, nandrolone phenylpropionate, nefopam, norandrostenedione, oxandrolone, sibutramine, sildenafil, stanozolol, tadalafil, tamoxifen, testosterone, testosterone-D3, testosterone benzoate, testosterone cypionate, testosterone undecanoate, testosterone isocaproate, testosterone enanthate, testosterone propionate, testosterone phenylpropionate, testosterone decanoate, testosterone undecanoate, trenbolone enanthate and trenbolone acetate were supplied by Sigma Aldrich.

Methanolic solutions of each standard were prepared from pure powders at a concentration of 1 mg/mL and stored at −20 °C. A working solution mixture, containing all the standards at a concentration of 1 µg/mL in methanol, was then prepared from the above-mentioned standard solutions and stored at −20 °C. Internal standard solution containing testosterone-D_3 at a concentration of 1 µg/mL in methanol was prepared and stored at −20 °C.

2.2. Samples

All dietary supplements and pharmaceuticals seized by the Justice in sport halls and addressed to the laboratory for a qualitative and quantitative analysis between January 2016 and December 2019 were included in the study. Samples were classified as original, counterfeit or substandard, according to the classification suggested by Neves et al. [11]:

Original:

- Qualitative formulation fully matches the one declared on the label.
- Levels of active pharmaceuticals ingredients are between 80% and 130% of the declared formulation.

- Qualitative formulation fully matches the one declared.
- Levels of active ingredients detected are not within the acceptable range defined for original products.

Substandard:

- Qualitative formulation does not match the label.
- Fake packaging, or no indication of the active ingredient included.

Counterfeit:

2.3. Sample preparation

Tablets and granules contained in the capsules were weighed and ground into a fine powder. Ten milligrams of powders were solubilized in MeOH to obtain a concentration of 1 mg/mL. The solutions were sonicated for 10 min and centrifuged for 10 min

Regarding liquid samples, 50–100 µL of aqueous solutions or 20–100 µL of oil solutions were transferred to falcon tubes, and diluted into 10 mL with MeOH. Falcon tubes were vortexed and sonicated for 10 min. When dissolution was not achieved, a first 1/10 dilution in heptane was conducted and then a second 1/10 dilution in MeOH.

Fifty µL of internal standard working solution were added to all samples prior to gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis. Identified compounds were then quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) when a commercial reference standard was available. Peptides and thyroid hormones were identified with dedicated analytical methods.

2.4. GC-MS analysis

Samples were analyzed with a simple sample preparation procedure, and no derivatization step. GC-MS analysis was carried out on a Focus GC equipped with a Triplus Duo autosampler and coupled with a DSQ II single quadrupole all supplied by Thermo Fisher (Les Ulis, France). Separation was performed using an UptiBond 5 (5% phenyl – 95% dimethylpolysiloxane) column from Interchim (Montluçon, France) (dimensions: l = 30 m, I.D. = 0.25 mm, dF = 0.25 µm) with helium as carrier gas at a constant flow of 1.2 $mL.min^{-1}$. Injection mode was splitless and injector temperature was maintained at 250 °C. The GC conditions were as follows: column temperature began at 45 °C, was held for 1 min, then increased to 115 °C at a rate of 30 °C/min, then held at 115 °C for 2 min before increasing to 300 °C at a rate of 3 °C/min and held 14 min. Total run duration was 56 min. Data acquisition was performed using the Xcalibur v2.1 software (Thermo Fisher, Les Ulis, France). The MS

was operated in full scan with a mass range 30–560 *m/z*. Samples were not derivatized and data were processed with RIHunter software (Idecos, France) using three different MS library: N.I.S.T. 17 library, Maurer, Pfleger and Weber v3 and SWGDrug v3.6.

2.5. LC-HRMS analysis

Liquid chromatography was performed on a Thermo Ultimate 3000 (Thermofisher, Les Ulis, France) pump and separation was carried out on a Hypersil GOLD column (100 × 2.1 mm × 1.9 µm, Thermo, USA) maintained at 40 °C. The device was completed with a pre-column (Hypersil GOLD, 10 × 2.1 mm, 3 µm, Thermo, USA). Elution was achieved according to Fabresse et al. method [17], the mobile phase gradient being as follow: 60% A (water/methanol 90/10 with 0.1% formic acid) for 1 min, linear gradient to 100% B (methanol with 0.1% formic acid) in 7 min, held for 5.0 min. The column re-equilibration was performed with linear gradient to 60% A in 3.0 min, held for 3.0 min. The flow rate was set at 400 µL/min. The sample injection volume was 10 µL. Ionization was performed in positive ionization mode by heated electrospray ionization (HESI). Nitrogen (N2-45 nitrogen generator, VWR International, Fontenay sous bois, France) was employed as sheath and auxiliary gas. The source parameters were as follows: ion spray voltage: 3500 V; vaporizer temperature: 150 °C; sheath and auxiliary gas pressure: 45 and 10 arbitrary units, respectively; ion transfer tube temperature: 350 °C.

Compounds were detected using an Orbitrap mass spectrometer (Q-Exactive, Thermo, USA). Data were acquired in data dependent acquisition mode according to a previously published method [18]. The masses of precursors and their related fragments ions were measured with a resolution of 70,000 and 17,500 FWHM at *m/z* 200, respectively, in the range 125–650 *m/z*. The mass isolation window was 1 *m/z*. The normalized collision energy (NCE) was set at 55%. Chromatographic data acquisition was performed using Xcalibur software (v.4.0, Thermo, USA). Raw data were processed with Compound Discoverer 2.0 software (Thermo, USA). Exact mass spectra identification was performed with the mzCloud™ data base (https://www.mzcloud.org/).

2.6. LC-MS/MS

Quantification was achieved using the same equipment (LC system, chromatographic column) and conditions as those described in the Section 2.5. for LC-HRMS. Compounds were then detected with a triple quadrupole TSQ Quantiva mass spectrometer (Thermo, USA) using multiple reaction monitoring (MRM). Argon (Messer, Puteaux, France) was used as a collision gas with a pressure set at 1.5 mTor, Q1 and Q3 resolutions were set at 0.7 FWHM and cycle time 1 s. Identified analytes were confirmed and quantified by using the corresponding reference standard solutions and product ion scan experiments. The method was validated for quantitation by LC-MS/MS in the range 10–1000 ng/mL according to EMA guidelines [19].

2.7. Peptide and thyroid hormone analysis

CJC-1295 was analyzed by LC-HRMS according to a previously published method [20]. Human chorionic gonadotrophin (HCG) was quantified on a Siemens™ dimension EXL (Siemens, Munich, Germany) automated system by enzyme-linked immunosorbent assay (ELISA) using an antibody specific for HCG. Dilutions are carried out automatically according to the concentration obtained. The analytical range is from 1 to 1000 international units per liter (IU/L). Triiodothyronine assay was performed on a fully automated ADVIA Centaur CP analyzer (Siemens Healthcare Diagnostics, Munich, Germany). The TSH3-UL assay is based upon a two-site sandwich principle.

3. Results and discussion

During the study period, a total of 110 products were seized by justice in sport halls and submitted to the laboratory for identification of active compounds and quantification: 35 dietary supplements and 75 pharmaceuticals. The pharmaceuticals included 39 oily and 3 aqueous solutions for intramuscular injection, 30 tablets or capsules and 3 lyophilizates. Dietary products were 17 tablets or capsules, 14 powders and 4 oral liquids.

All the results are detailed in "Supplemental Table 1".

All oil based injectables contained AAS esters (n = 39). This result was consistent with previous published results [11,12,21]. Steroid esters are widely used by bodybuilders, since they allow a slow release of steroids from the injection site for periods of up to 4 weeks. Two substances should not be found among injectable products: stanozolol and methandrostenolone since they are 17-alpha-methyled steroids, and designed to be taken orally since they did not have a first-pass hepatic effect. However, since they present hepatotoxicity, parenteral form could limit this side effect, but required a daily injection rather than a daily oral intake, which seems more restrictive. Thus, one out of the 6 methandrostenolone-containing products and 2 out of the 4 containing-stanozolol products analyzed in the present study were injectable forms. The two products containing stanozolol were aqueous solution for injection. A third aqueous solution for injection contained nefopam, an analgesic, probably used to reduce pain during or after exercise. Interestingly, this substance did not belong to the WADA list of prohibited substances [22].

Pharmaceutical tablets and capsules (n = 30) contained 17-alpha-alkylated steroids (n = 12), other AAS like boldenone and DHEA both associated with oxandrolone (n = 1), non-steroid anabolic agent like clenbuterol (n = 3), anti-estrogenics (anastrozole, n = 1, letrozole, n = 2, tamoxifen, n = 1), anxiolytic (lormetazepam, n = 2), photosensitizer (methoxsalen, n = 1), amphetamine related (sibutramine, n = 1), phosphodiesterase-5 enzyme inhibitors (PDE5i, sildenafil, n = 1, tadalafil, n = 2) and thyroid hormone (triiodothyronine, n = 1). No substance was detected in two tablets: clenbuterol 0.02 mg named кленбутерол® (clenbuterol) sold originally by Софарма (Sopharma) and mesterolone 25 mg named Proviron® sold originally by Bayer.

17-alpha-alkylated steroids, clenbuterol and DHEA are commons substances reported to be abused by athletes [23] since they are administered orally. Boldenone does not belong to AAS designed to be taken orally, because it presents a weak bioavailability. Boldenone is normally a veterinary pharmaceutical marketed as a prodrug (boldenone undecylenate, Equipoise®) [24]. Tamoxifen is a Selective Estrogen Receptor Modulator (SERMs). Letrozole and anastrozole are aromatase inhibitors, blocking the transformation of testosterone into estrogen. The athletes could illicitly use SERMs or aromatase inhibitors to increase endogenous testosterone levels, with the aim to by-pass the specific testing regimens for known synthetic androgens including exogenous testosterone, and to balance the feminizing adverse effects of an extensive abuse of AAS [25,26]. Methoxsalen is a pharmaceutical drug marketed under the brand name Meladinine®, prescribed in several skin diseases (psoriasis, vitiligo, and dermatitis). It is a photosensitizing agent probably used for tanning by bodybuilders, however it provides a high risk of skin burns. PDE5i are usually used by bodybuilders in order to compensate the erectile dysfunction caused by long term use of AAS.

CJC-1295 (n = 1) and HCG (n = 2) were identified in the 3 lyophilisates. CJC-1295 is an analog of growth hormone releasing hormone (GHRH), developed by ConjuChem Biotechnology Inc. (Montreal, Canada) and in its original form, it utilizes a novel bioconjugation technology referred to as Drug Affinity Construct (DAC™). A maleimidopropionamide derivative of lysine at the C-terminus allows this compound to bind covalently to albumin in vivo thereby significantly

extending its half-life [27]. However, the substance identified in this study is lacking the DAC™ feature, and should therefore have a shortened half-life [20]. HCG is used in male athletes to stimulate testosterone production and normalize suppressed testosterone concentrations due to prolonged use of anabolic steroids [28,29]. Analysis of the two samples described as HCG revealed the presence of the hormone at the concentrations indicated on the label.

Among the 75 pharmaceuticals, 25/75 (33%) were substandard, 24/75 (32%) were counterfeit and 14/75 were original (19%). For the remaining 12 products, 11/75 (15%) revealed a correct qualitative content, but quantitation could not be carried out because we did not have the standard at the time of the analysis. The last one had the true active ingredient (4-chlorodehydromethyltestosterone) but was associated with methandrostenolone (not mentioned on the label). According to the qualitative composition of the samples, 51/75 (68%) were accurately labeled, showing the interest in quantification of the compounds in these kinds of studies.

Fifty-four pharmaceuticals were anabolic agents (72%), 22/54 (41%) were counterfeit, 20/54 (37%) were substandard, 8/54 (15%) were not quantified (7/54 were qualitatively accurately labeled) and solely 4/54 (7%) were original. In most cases, steroid esters were substituted by another steroid ester (e.g. boldenone undecylenate by nandrolone decanoate or drostanolone propionate by testosterone enanthate). Similarly, a steroid ester could be replaced by another ester (e.g. testosterone cypionate substituted by testosterone propionate) (Fig. 1).

Several qualitative and quantitative studies have been realized on doping substances seized from the black market, and are summarized in Table 1. The proportion of products accurately labeled varies between 13% and 58.9%. These observations are in accordance with the results of our study, which showed a high-rate of non-original products (at least 25/75 of pharmaceuticals, 66%), and an even higher rate among AAS (at least 80%) seized from the black market among bodybuilders in France. As suggested by Coomber et al., adulteration does occur with substances that mimic or enhance the drug being supplied [30]. These adulterations do not seem to follow a rule, they are probably related to the good availability and low cost of one product rather than another at a given time [21].

Only 1/35 dietary supplement (3%) was adulterated with a doping substance (capsule containing 1,3-dimethylbutylamine, DMBA) in a product named "Thermo Shock" sold by Sci Labs Nutrition. DMBA is an analog of dimethylamylamine. DMBA has been found in a number of dietary supplements labeled as sport supplements, weight loss supplements, and supplements that claim to enhance brain function [31,32]. The rate of adultered dietary supplements is very low in comparison with previous studies showing rates over 50% [11,33–35]. This could reflect an improvement in the quality of the products. However, we cannot exclude the presence of new doping substances absent from the mass spectral libraries used in this study, although this was unlikely given the use of LC-HRMS and on-line libraries.

Most of these products are bought on the internet, and come from South East Asian countries. Fourteen products analyzed in this study were sold by the Pacific Pharmaceutical Company LTD. Malay Tiger. The French Medicines Safety Agency (ANSM) launched an alert [36] in February 2019 regarding two products analyzed in our study (Stanox-10® and Clenox®) recalling the health risks associated with exposure to these products after the death of an athlete identified by a forensic toxicologist laboratory [38]. The label of the two products mentioned stanozolol 10 mg for Stanox-10® and clenbuterol 0.04 mg for Clenox®. The forensic laboratory found a dosage of 11.5 mg and 0.073 mg, respectively, while the ANSM laboratory found 0.6 mg and 0.22 mg on other batches, respectively. In our study, these two compounds contained 6.5 mg and 0.05 mg, respectively, with the

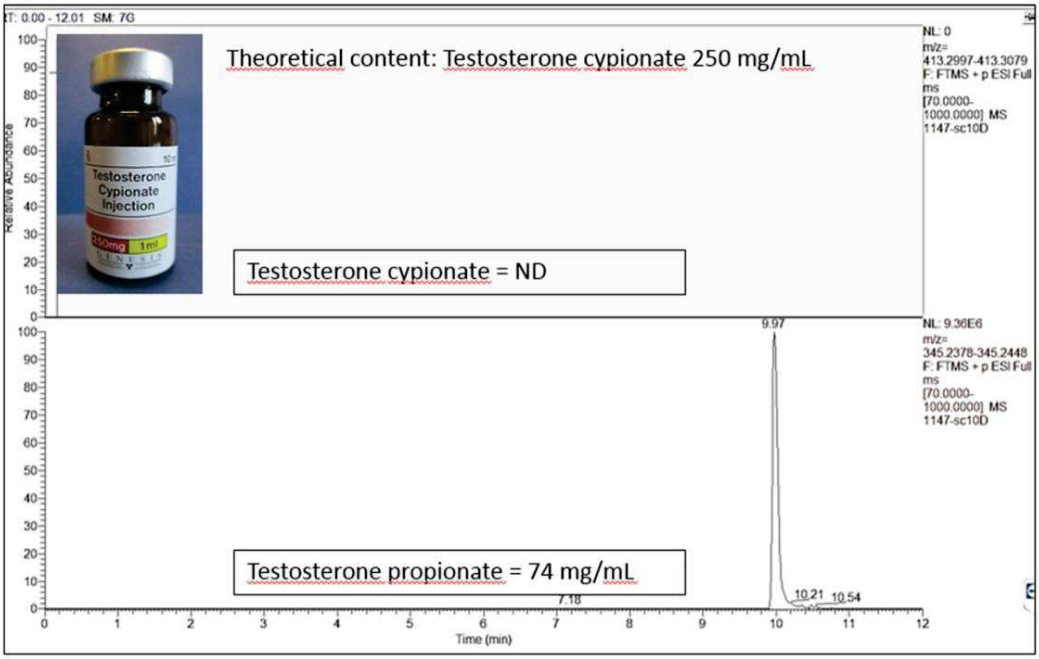


Fig. 1. Photography of sample n°55, theoretical content, experimental content and LC-HRMS extracted ion chromatograms of testosterone cypionate (m/z = 413.3050) and testosterone propionate (m/z = 345.2424) (ND: not detected).

Table 1
Published analytical studies carried out on seizures of doping products.

References	Country, study period, number of products analyzed	Results
Tircova et al. [37]	Czech Republic and Slovakia, October 2017 to January 2018, N = 358	58.9% contained the declared active substances at the declared concentrations, 15.9% no active substances, 16.8% underconcentrated, 4.5% contained pharmaceutical substance different of the label, 3.6% over concentrated
Weber et al. [21]	Switzerland, January 1st 2013 to December 31st 2014, N = 1190	AAS (n = 889): - 41% accurately labeled - 23% adultered - 31% substituted - 6% inert Peptide hormones, growth factor-related substances and mimetics (n = 146): - 66% accurately labeled - 0% adultered - 5% substituted - 30% inert Hormone and metabolic modulators (n = 113): - 88% accurately labeled - 4% adultered - 3% substituted 5% inert
Krug et al. [12]	Germany, 2010 – 2013, N = 337	AAS (n = 288): - 45% accurately labeled - 55% not labeled Others doping and non-doping agents (n = 56): - 38% accurately labeled - 62% not labeled
Coopman et al. [10]	Belgium, 2012, N = 74	33.8% contained other or did not contained the labeled active ingredients
Pellegrini et al. [38]	Italy, 2012, N = 15	13% accurately labeled 53% contained other substances 20% underconcentrated 13% did not contained any substance
Neves et al. [11]	Brazil, 2011–2016, N = 328	42.1% counterfeit 11% substandard
Shapira et al. [39]	Israel, 2018, N = 113	38.9% had labels misrepresenting content 18% contained other substances
Hullstein et al. [13]	Norway, 2011–2014, N = 296	20% did not contained any substances

same concentration claimed by the label, showing the very important variation of the concentration in the same products over time. Despite this alert, the website remains functional where users can freely continue to buy these products. This underlines the difficulty of the authorities in tracking the trafficking of PIEDs.

4. Conclusion

This study shed light on the issue related to the trafficking of anabolic products in France. The majority of the products analyzed were counterfeit or substandard. Two products were devoid of active substances. The low quality of these products associated with invasive modes of administration (injection) exposes users to risks of acute and chronic toxicity. Regarding food supplements, only one sample was adulterated with DMBA, this proportion is clearly lower than those reported in previous studies. This may reflect an improvement in the quality of the marketed products. Few studies have been carried out on anabolic steroids in France, this work allows to show that France is not spared by the trafficking of PIEDs. The use of DCs in mainstream population is an underestimated public health.

Funding

No funding for this study.

CRediT authorship contribution statement

All authors have participated to this study and to the writing of this manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2021.110771.

References

[1] World Health Organization, Definitions of Substandard and Falsified (SF) Medical Products, 2017. https://www.who.int/medicines/regulation/ssffc/definitions/en/. (Accessed 2 November 2020).
[2] N. Peyraud, F. Rafael, L.A. Parker, M. Quere, G. Alcoba, C. Korff, M. Deats, P.B. Esteve, J.-C. Cabrol, M. Serafini, I. Ciglenecki, M. Rull, I.A. Larabi, F. Baud, F. Grandesso, B.K. Ilunga, J.-C. Alvarez, P.N. Newton, An epidemic of dystonic reactions in central Africa, Lancet Glob. Health 5 (2017) e137–e138, https://doi.org/10.1016/S2214-109X(16)30287-X
[3] M.-Y. Low, Y. Zeng, L. Li, X.-W. Ge, R. Lee, B.-C. Bloodworth, H.-L. Koh, Safety and quality assessment of 175 illegal sexual enhancement products seized in red-light districts in Singapore, Drug Saf. 32 (2009) 1141–1146, https://doi.org/10.2165/11316690-000000000-00000
[4] P.N. Newton, F.M. Fernández, A. Plançon, D.C. Mildenhall, M.D. Green, L. Ziyong, E.M. Christophel, S. Phanouvong, S. Howells, E. McIntosh, P. Laurin, N. Blum, C.Y. Hampton, K. Faure, L. Nyadong, C.W.R. Soong, B. Santoso, W. Zhiguang, J. Newton, K. Palmer, A collaborative epidemiological investigation into the criminal fake artesunate trade in South East Asia, PLOS Med. 5 (2008) e32, https://doi.org/10.1371/journal.pmed.0050032
[5] Growing threat from counterfeit medicines, Growing threat from counterfeit medicines, Bull. World Health Organ. 88 (2010) 247–248, https://doi.org/10.2471/BLT.10.020410
[6] INTERPOL, Operation Pangea – shining a light on pharmaceutical crime, 2019. https://www.interpol.int/fr/Infractions/Marchandises-illicites/Operations-en-matiere-de-criminalite-pharmaceutique#. (Accessed 15 December 2020).
[7] T. Cash, T. Pruzinsky, Body image: A handbook of Theory, Research and Clinical Practice. NY: Guilford Press, New York, 2002.

[8] R. Calfee, P. Fadale, Popular ergogenic drugs and supplements in young athletes, Pediatrics 117 (2006) e577–e589, https://doi.org/10.1542/peds.2005-1429
[9] H. Bonny-Noach, R. Berkovitz, B. Shapira, Evaluation of performance-enhancing drugs seized by Israeli enforcement agencies 2012-2017: implications for policy and regulatory change, Isr. J. Health Policy Res. 9 (2020) 14, https://doi.org/10.1186/s13584-020-00369-2
[10] V. Coopman, J. Cordonnier, Counterfeit drugs and pharmaceutical preparations seized from the black market among bodybuilders, Ann. Toxicol. Anal. 24 (2012) 73–80, https://doi.org/10.1051/ata/2012012
[11] D.B. da, J. Neves, E.D. Caldas, GC–MS quantitative analysis of black market pharmaceutical products containing anabolic androgenic steroids seized by the Brazilian Federal Police, Forensic Sci. Int. 275 (2017) 272–281, https://doi.org/10.1016/j.forsciint.2017.03.016
[12] O. Krug, A. Thomas, K. Walpurgis, T. Piper, G. Sigmund, W. Schänzer, T. Laussmann, M. Thevis, Identification of black market products and potential doping agents in Germany 2010-2013, Eur. J. Clin. Pharmacol. 70 (2014) 1303–1311, https://doi.org/10.1007/s00228-014-1743-5
[13] I.R. Hullstein, H. Malerod-Fjeld, Y. Dehnes, P. Hemmersbach, Black market products confiscated in Norway 2011-2014 compared to analytical findings in urine samples, Drug Test. Anal. 7 (2015) 1025–1029, https://doi.org/10.1002/dta.1900
[14] M. Kohler, A. Thomas, H. Geyer, M. Petrou, W. Schänzer, M. Thevis, Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the cologne doping control laboratory 2009, Drug Test. Anal. 2 (2010) 533–537, https://doi.org/10.1002/dta.186
[15] C. Grodner, C. Bernigaud, S. Lapadula, H. Beringuer, P.A. Billiet, P.L. Woerther, R. Billon, H. Derhy, B. Haye, C. Hotz, C. Pressiat, D. Vodovar, C. Rodriguez, N. Fabresse, C. Hua, N. De Prost, O. Chosidow, Fasciite nécrosante abdominale secondaire à des auto-injections de produits amincissants achetés sur internet, Ann. Dermatol. Vénéréologie 146 (2019) A273, https://doi.org/10.1016/j.annder.2019.09.440
[16] S. Basaria, Androgen abuse in athletes: detection and consequences, J. Clin. Endocrinol. Metab. 95 (2010) 1533–1543, https://doi.org/10.1210/jc.2009-1579
[17] N. Fabresse, S. Grassin-Delyle, I. Etting, J.-C. Alvarez, Detection and quantification of 12 anabolic steroids and analogs in human whole blood and 20 in hair using LC-HRMS/MS: application to real cases, Int. J. Leg. Med. 131 (2017) 989–999, https://doi.org/10.1007/s00414-017-1552-3
[18] N. Fabresse, I.A. Larabi, T. Stratton, R. Mistrik, G. Pfau, G. Lorin de la Grandmaison, I. Etting, S. Grassin Delyle, J.-C. Alvarez, Development of a sensitive untargeted liquid chromatography-high resolution mass spectrometry screening devoted to hair analysis through a shared MS2 spectra database: a step toward early detection of new psychoactive substances, Drug Test. Anal. (2018), https://doi.org/10.1002/dta.2535
[19] European Medicines Agency, Guideline on Bioanalytical Method Validation. 2011.
[20] N. Fabresse, S. Grassin Delyle, I. Etting, J.C. Alvarez, Identification d'un analogue peptidique de la GHRH, le CJC-1295, par CL-SM/SMHR, Toxac 29 (2017) 205–211, https://doi.org/10.1016/j.toxac.2016.10.004
[21] C. Weber, O. Krug, M. Kamber, M. Thevis, Qualitative and semiquantitative analysis of doping products seized at the Swiss border, Subst. Use Misuse 52 (2017) 742–753, https://doi.org/10.1080/10826084.2016.1263665
[22] WADA, 2020, Prohibited List, 2020. https://www.wada-ama.org/en/what-we-do/the-prohibited-list. (Accessed 30 November 2020).
[23] F.R. de, S. Nogueira, A. de, F. Brito, C.V.C. de Oliveira, T.I. Vieira, R.L.B. Gouveia, Anabolic–androgenic steroid use among Brazilian bodybuilders, Subst. Use Misuse 49 (2014) 1138–1145, https://doi.org/10.3109/10826084.2014.912062
[24] U.S.D. Food and Drug Administration, CFR - Code of Federal Regulations Title 21, 2014. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=522.204. (Accessed 30 November 2020).
[25] D.J. Handelsman, Indirect androgen doping by oestrogen blockade in sports, Br. J. Pharm. 154 (2008) 598–605, https://doi.org/10.1038/bjp.2008.150
[26] D.J. Handelsman, Clinical review: the rationale for banning human chorionic gonadotropin and estrogen blockers in sport, J. Clin. Endocrinol. Metab. 91 (2006) 1646–1653, https://doi.org/10.1210/jc.2005-2569
[27] J. Henninge, M. Pepaj, I. Hullstein, P. Hemmersbach, Identification of CJC-1295, a growth-hormone-releasing peptide, in an unknown pharmaceutical preparation, Drug Test. Anal. 2 (2010) 647–650, https://doi.org/10.1002/dta.233
[28] D. Knorr, D. Beckmann, F. Bidlingmaier, F.J. Helmig, W.G. Sippell, Plasma testosterone in male puberty. II. hCG stimulation test in boys with hypospadia, Acta Endocrinol. 90 (1979) 365–371.
[29] U.-H. Stenman, K. Hotakainen, H. Alfthan, Gonadotropins in doping: pharmacological basis and detection of illicit use, Br. J. Pharm. 154 (2008) 569–583, https://doi.org/10.1038/bjp.2008.102
[30] R. Coomber, A. Pavlidis, G.H. Santos, M. Wilde, W. Schmidt, C. Redshaw, The supply of steroids and other performance and image enhancing drugs (PIEDs) in one English city: fakes, counterfeits, supplier trust, common beliefs and access, Perform. Enhanc. Health 3 (2014) 135–144, https://doi.org/10.1016/j.peh.2015.10.004
[31] P.A. Cohen, J.C. Travis, B.J. Venhuis, A synthetic stimulant never tested in humans, 1,3-dimethylbutylamine (DMBA), is identified in multiple dietary supplements, Drug Test. Anal. 7 (2015) 83–87, https://doi.org/10.1002/dta.1735
[32] R.S. Pawar, E. Grundel, Overview of regulation of dietary supplements in the USA and issues of adulteration with phenethylamines (PEAs), Drug Test. Anal. 9 (2017) 500–517, https://doi.org/10.1002/dta.1980
[33] V. Abbate, A.T. Kicman, M. Evans-Brown, J. McVeigh, D.A. Cowan, C. Wilson, S.J. Coles, C.J. Walker, Anabolic steroids detected in bodybuilding dietary supplements - a significant risk to public health, Drug Test. Anal. 7 (2015) 609–618, https://doi.org/10.1002/dta.1728
[34] S. Odoardi, E. Castrignanò, S. Martello, M. Chiarotti, S. Strano-Rossi, Determination of anabolic agents in dietary supplements by liquid chromatography-high-resolution mass spectrometry, Food Addit. Contam. Part A 32 (2015) 635–647, https://doi.org/10.1080/19440049.2015.1014868
[35] C. Van Poucke, C. Detavernier, R. Van Cauwenberghe, C. Van Peteghem, Determination of anabolic steroids in dietary supplements by liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta 586 (2007) 35–42, https://doi.org/10.1016/j.aca.2006.09.050
[36] ANSM, 'ANSM alerte sur les risques pour la santé des produits à visée anabolisante ou amaigrissante CLENOX® et STANOX-10® de Pacific Pharmaceutical Company LTD. Malay Tiger, vendus sur internet - Point d'information, 2019. https://www.ansm.sante.fr/S-informer/Points-d-information-Points-d-information/L-ANSM-alerte-sur-les-risques-pour-la-sante-des-produits-a-visee-anabolisante-ou-amaigrissante-CLENOX-R-et-STANOX-10-R-de-Pacific-Pharmaceutical-Company-LTD.-Malay-Tiger-vendus-sur-internet-Point-d-information. (Accessed 4 December 2020).
[37] B. Tircova, Z. Bosakova, P. Kozlik, Development of an ultra-high performance liquid chromatography-tandem mass spectrometry method for the determination of anabolic steroids currently available on the black market in the Czech Republic and Slovakia, Drug Test. Anal. 11 (2019) 355–360, https://doi.org/10.1002/dta.2541
[38] M. Pellegrini, M.C. Rotolo, R. Di Giovannadrea, R. Pacifici, S. Pichini, A simple toxicological analysis of anabolic steroid preparations from the black market, Ann. Toxicol. Anal. 24 (2012) 67–72, https://doi.org/10.1051/ata/2012011
[39] B. Shapira, A. Poperno, M. Arieli, R. Berkovitz, Label misrepresentation in seized anabolic steroids and performance-enhancing substances, Eur. J. Public Health 28 (2018), https://doi.org/10.1093/eurpub/cky213.374

1.3. Intérêt de l'analyse des phanères

Les phanères regroupent les cheveux, les poils du corps et les ongles. Ce sont des matrices kératinisées présentant comme intérêt majeur d'augmenter la fenêtre de détection.

Les xénobiotiques s'incorporent dans les cheveux par 4 voies : le sang, le sébum, la sueur ou encore par contamination environnementale *[Kronstrand, 2007].*

C'est la seule matrice, à ce jour, permettant de caractériser une contamination environnementale ou une contamination par un supplément alimentaire.

Depuis une quarantaine d'années, l'analyse des cheveux est pratiquée en médecine légale mais ce n'est qu'en 2000 que l'analyse des cheveux a été utilisée pour la recherche de xénobiotiques dans le domaine du dopage. Dans cette étude, Gaillard *et al.* ont montré que les cheveux permettaient d'identifier autant voire plus de molécules que les urines. C'était le cas principalement pour les anabolisants *[Gaillard, 2000].*

Les laboratoires accrédités anti-dopage analysent les produits dopants dans les urines et le sang pour l'identification des hormones peptidiques. Dans l'urine, la majorité les produits présents et détectables sont des produits de transformation ou métabolites. Les métabolites ne sont pas toujours connus et/ou commercialisés. De plus, le coût est assez élevé pour un laboratoire hospitalo-universitaire pour se les procurer.

20 ans après, les matrices usuelles utilisées dans les laboratoires anti-dopage n'ont pas changé. Néanmoins, les phanères présentent de nombreux avantages. Dans les phanères, la molécule cible est généralement la molécule parent ce qui permet de discriminer deux molécules ayant le même métabolite.

Il semble important de caractériser l'incorporation des substances de la performance dans les phanères et d'appliquer une approche médico-judiciaire pour interpréter au mieux les résultats.

Les matrices kératinisées présentent néanmoins certaines limites comme une mauvaise incorporation des molécules acides et neutres. Toutes ces limites sont à prendre en compte lors de l'interprétation des résultats. L'article 3 présente les limites et les intérêts de l'analyse des matrices kératinisées pour l'identification des substances de la performance ***[Article 3]***.

Article 3 : Hair testing for doping agents. What is known and what remains to do

Pascal Kintz, Laurie Gheddar, Alice Ameline, Nadia Arbouche, Jean-Sébastien Raul

Drug Testing and Analysis. 2020;12:316-322.

Received: 14 November 2019 | Revised: 9 January 2020 | Accepted: 10 January 2020

DOI: 10.1002/dta.2766

PERSPECTIVE

WILEY

Hair testing for doping agents. What is known and what remains to do

Pascal Kintz[1,2] | Laurie Gheddar[2] | Alice Ameline[2] | Nadia Arbouche[2] | Jean-Sébastien Raul[2]

[1]X-Pertise Consulting, 42 rue principale, F-67206 Mittelhausbergen, France

[2]Institut de médecine légale, 11 rue Humann, F-67000 Strasbourg, France

Correspondence
Pascal Kintz, X-Pertise Consulting, 42 rue principale, F-67206 Mittelhausbergen, France.
Email: pascal.kintz@wanadoo.fr

KEYWORDS: alternative specimens, doping, forensic, hair

1 | INTRODUCTION

Testing for drugs in hair to document exposure is receiving more and more attention by scientists and lawyers due to is long detection window, particularly when compared to blood and urine, its less embarrassing conditions of collection and its storage at ambient temperature. By providing information on exposure to drugs over time, hair analysis may be useful in verifying self-reported histories of drug use in any situation in which a history of past rather than recent drug use is desired. Hair analysis can also provide a retrospective calendar of an individual's drug use. For this, multi sectional analysis is required and involves taking a length of hair and cutting it into sections to measure drug use during shorter periods of time. Given an average growth rate of 1 cm per month, each cm of hair in the vertex region represents what has circulated in the body during the corresponding month. Therefore, hair analysis is suitable for monitoring relative changes in drug intake in the same individual, i.e. the dosage of drug, and the switch from one to another compound with the same pharmacological activity. Depending on the physico-chemical properties of the drug, some parameters are able to influence drug concentrations, including hair colour and cosmetic treatments. Researchers have demonstrated that different hair types incorporate differing amounts of drugs when exposed under identical conditions. There is a higher accumulation of alkaline substances (such as cocaine or amphetamine) in black hair when compared with blond hair. Hair is continuously subjected to natural factors, such as sunlight, weather, water, pollution, etc., which affect and damage the cuticle but hair cosmetic treatments enhance the damage. The authors have recently reviewed all these aspects.[1]

Although hair is not yet a valid specimen for the International Olympic Committee or the World Anti-Doping Agency (WADA), it is accepted in most courts of justice in the world. The Society of Hair Testing (SoHT) has established in 1999[2] during its annual meeting a consensus on hair testing for doping agents. The statement includes that hair specimens are not suitable for general routine control and that a negative hair result cannot exclude the administration of the detected drug and cannot overrule the adverse analytical finding (AAF). In the early 2000, some forensic scientists[3,4] have suggested to use hair to document drug abuse in sport. This was simply an extension to doping agents from what was possible with drugs of abuse such as cocaine, stimulants or narcotics. However, very rapidly, it became obvious that forensic toxicology is not doping control and that numerous points remained unknown in this new field of investigation. As a general rule in doping control, any doping substance which is detectable in urine (or blood) was – more or less – ingested/incorporated. It seems more complicated when using hair as a matrix to document drug exposure. Among the unanswered questions, several were of critical importance. For example, Kintz et al[5] questioned the minimal amount of drug detectable in hair after administration. They published that a single injection of nandrolone undecanoate is not detectable in hair, while the urine remained positive for more than 8 months for norandrosterone. At the same time, Rivier[6] also published that hair reference ranges for anabolic agents, including endogenous substances remain to be established. He also indicated that the incorporation rate into blond hair is poorer than that of dark coloured hair, raising the questions of individual equality against the controls, a important matter of concern for the sport's authorities. In addition, because there are different classes of restriction in- and out-competition, Thieme et al[7] reported that a differentiation between training and competition period is impossible by means of hair analysis due to the uncertainty of hair growth rate. They concluded that the most interesting target substances are certainly the anabolic agents.

Some years later, in 2005, Ambrose[8] published that any hair from the body provides a viable source for doping agent testing. Although not detailed, it must be emphasized that the window of detection of head hair is far different from the one of body hair, each anatomical location having some specificities.[1] Despite major issues in the use of hair testing to document doping practices and the lack of consideration by WADA, some papers[9] provided further evidence of the interest of hair in this particular field.

More recently, two papers have reviewed the advantages and limitations of alternative specimens in doping control. In the first one, Thieme[10] indicated that compounds prohibited at all times, such as clenbuterol, ß$_2$ agonists or estrogen-receptor modulators, may be successfully tested and clearly interpreted in hair. However, the author pointed some issues, including substances limited to time ranges, such as stimulants or those permitted according to their administration routes, such as glucocorticosteroids that cannot be properly differentiated in hair. To the best of the authors' knowledge, the discrimination in urine between systemic (forbidden) and topical use of glucocorticoids is still a daily challenge. Thieme concluded, on a more optimistic mode, that the detection of social drugs, such as new psychoactive substances represents promising applications of hair tests in doping control. In their review, Thevis et al[11] seemed less enthusiastic when discussing the potential interest of hair in doping control, as they considered that the combined use of blood and urine does provide the majority of the desired information with respect to the goals of protecting the honest athlete and identifying the cheating individuals. In their manuscript, when comparing several alternative specimens (exhaled breath, oral fluid, dried blood spots and hair) for testing doping agents, obviously hair had the less citations of applications.

Twenty years after the publication of the SoHT consensus for doping agents[2] and the regular increase of hair test requests to document AAF, it seems important to spend some lines on what is possible to detect in hair, what can be interpreted and what remains to achieve before hair testing for doping agents could be considered as a suitable specimen in doping control.

Each year WADA issues a prohibited list of compounds, which is part of the world anti-doing code. The latest version has been recently published.[12] It contains the list of substances prohibited at all times and those prohibited in-competition. With respect to the list, Table 1 presents a compendium of selected literature papers[13-24] dealing with doping agent detection in human hair. Although several compounds from the same class have been identified in hair, the aim of this table was just to indicate whether within each specific class one substance has already been identified. In addition, some animal applications have been described, including the detection of anabolic steroids in horses[25] or dogs[26] hair and glucocorticoids in racing camels hair.[27]

Obviously, substances from each WADA controlled class have been identified in human hair, except from the S2 class, i.e. peptide hormones, growth factors, related substances, and mimetics. This is not surprizing, due to the large molecule size of the substances belonging to the S2 class that does not allow the passive diffusion from the blood capillaries to the hair follicle. To the best of the author knowledge, the biggest drug identified in hair is cyclosporin A, with a molecular weight of 1202.6 Da.[28] Although one could consider that the absence of detection of hormones is a major limitation of hair testing for doping agents, one should focus on the fact that it is possible to test for all the other drug classes. However, if a lot of work has been done for the detection and the interpretation of drugs of abuse, such as cocaine, amphetamines, narcotics or cannabis, considerable improvements are necessary for other classes, particularly for anabolic agents and diuretics and other masking agents, which represented in 2017, 44 and 15% of all ADAMS reported findings.[29] Unfortunately, due to their neutral (steroids, glucocorticoids) or acidic (diuretics) chemical properties, these drugs are badly incorporated in hair, which makes challenging their detection. At this time, testing for new compounds in hair (i.e. not yet published), irrespective they are approved

TABLE 1 Substance identification in human hair within each class of the WADA list

Class	In- & out-competition	Substance	Reference
S1 exogenous anabolic agents	All time	Clostebol	13
S1 endogenous anabolic agents	All time	DHEA	14
S1 other anabolic agents	All time	Ostarine	15
S2 peptide hormones, GF	All time	-	
S3 ß-2-agonists	All time	Clenbuterol	16
S4 hormone & metabolic modulators, aromatase inhibitors	All time	Letrozole	17
S4 hormone & metabolic modulators, SERMs	All time	Tamoxifen	18
S5 diuretics and masking agents	All time	Hydrochlorothiazide	19
S6 stimulants, non-specified	In-	Cocaine	20
S6 stimulants, specified	In-	Cathinones	21
S7 narcotics	In-	Fentanyl	22
S8 cannabinoids	In-	Synthetic cannabis	23
S9 glucocorticoids	In-	Methylprednisolone	24
P1 beta-blockers	Specific sport	Sotalol	16

or non-approved substances (S0 class), should be considered feasible, given the available analytical instruments. Of course, the analytical procedure has to follow the international criteria, as per the SoHT recommendations.[2] However, if establishing a new method is one thing, interpreting the generated data is another one, much more complicated, as it requires careful evaluation of numerous parameters. For such drugs, it is very difficult, with a single detection, to put any suitable discussion as the toxicological significance of the measured concentrations is difficult to establish given the lack of suitable reference. It is therefore not possible to interpret the data in terms of dosage and frequency of use. In particular, little or even nothing (for example for SARMs) is known about the incorporation into the keratin matrix after intake and the correlation between dose and hair concentrations. One should also considered different scenarios such as passive exposure vs. active consumption, mindful vs. unaware intake, and sporadic vs. chronic use.

Since 1997, the authors have been asked to test for doping agents in hair specimens to document AAFs. In all these cases, care was taken to collect the hair specimens several weeks after the doping control in order to cover the period when the AAF occurred. When head hair was not available, body hair can be an alternative source of keratin matrix. However, with body hair, due to irregular hair growth, it is not possible to achieve segmental analyses. Most body hair specimens (axillary, pubic, chest) have a window of detection of 4 to 8 months.

During these years, 3 major situations have occurred:

- situation 1: the hair specimen is positive for the drug challenged in urine
- situation 2: a. the hair specimen is positive at very low concentration(s) with a specific pattern of distribution for the drug challenged in urine or b. the hair specimen is positive for another substance with common metabolite(s)
- situation 3: the hair test result is negative for the drug challenged in urine

In the first case (situation 1), the interpretation is simple. The donor of the hair has used the substance and segmental analyses allowed establishing the pattern of drug use, and, for some drugs, the frequency and rough estimate of the dosage.[1] In this situation, there is no possibility claiming for no doping and in most cases, and the defence, even do not use the results. In the author's experience, this occurred in one third of the cases and some findings have been published.[30,31]

Situation 2 is close to forensic science and was observed in less than 5% of the submitted requests. The hair test results will help interpretation. Scientists have accepted[1] that the major analyte found in hair is the parent drug and not the metabolite(s). This has been used[32] to discriminate in hair the parent compound (nandrolone, norandrostenediol and norandrostenedione) after an AAF involving their common metabolite, 19-norandrosterone (19-NA). Although it documented the source of exposure, it was with no consequence on the doping offence as all 3 substances are banned. Unlike in urine, the interpretation of testosterone concentrations in hair can be difficult. The range between physiological concentrations of testosterone and those found in abusers is small. Therefore, to complement testosterone determination, the identification of unique testosterone esters in hair enables an unambiguous determination of doping because the esters are exogenous substances and therefore could not come from endogenous testosterone. It must be emphasized that isotope ratio mass spectrometry (IRMS) was never used to test for endogenous compounds in hair, such as testosterone or DHEA, probably due to a lack of instrument sensitivity. Another application-based on parent drug detection is the discrimination between zeranol, a S1 banned substance and zearalenone, a mycotoxin, which share the same metabolite(s). This is possible with hair tests and has been used to demonstrate no significant fault or negligence.[33] In theory, this should also be used to discriminate the prohibited S4 metabolic modulator trimetazidine from the metabolic conversion of the permitted drug lomerizine, a drug used for the treatment of migraines. Testing human hair for clenbuterol, a ß-adrenergic agonist with anabolic properties allows discriminating therapeutic use from contamination, based on the measured concentrations.[16]

External contamination is a controversial issue for hair testing, as it has been demonstrated that there is no technique which allows to fully eliminated the contaminant(s), even after tedious decontamination procedures, including complex washes.[1] Table 2 reports the hair test results of a young athlete who failed a doping control when benzoylecgonine, a cocaine metabolite was identified in his urine. Cocaine and benzoylecgonine, but not norcocaine nor cocaethylene, 2 hepatic metabolites, were identified in hair, at low concentrations, far from the SoHT positive cut-off at 500 pg/mg. The specific pattern of cocaine (linear increase of concentrations from the root to the tip) is consistent with external cocaine contamination, the older hair being in contact with the drug for a longer period of time. In addition, it was possible, by other tests, to demonstrate that the athlete was living in a place where cocaine was being used. His international sport federation accepted his claims and he was dismissed of any doping offence.[34] Situation 2 requires complex investigations and close discussions between all the parts (athlete, lawyers, sport authorities and toxicologists) involved in the case, where hair testing results are only one of the pieces of evidence. This is also the case for salbutamol, a threshold substance according to WADA TD2019DL, with a valid threshold concentration for urine samples. Establishing the pattern of drug use can be of interest in subject with occasional medical receipts to avoid tedious discussions.[35]

Situation 3 is the more frequent situation, and also the more controversial, as a negative hair test can be interpreted in 2 different ways: for the athlete, a negative hair test means no doping, while for the sport authorities, it means that there is nothing in hair but this is not enough to be qualified as no doping, due to hair limitations. These limitations include a major issue linked to the minimal detectable dosage in hair. Indeed, the key point is to know if the analytical method is sensitive enough to identify traces of drugs, when the urine specimen of the athlete was positive and the hair sample turned back negative. It has been accepted that a negative hair result cannot exclude the administration of a specific drug and the negative findings should not

TABLE 2 Segmental hair test results indicating environmental contamination

Item	Cocaine	Benzoylecgonine	Cocaethylene	Norcocaine
Segment 0–1 cm	15	Not detected	Not detected	Not detected
Segment 1–2 cm	36	12	Not detected	Not detected
Segment 2–3 cm	60	14	Not detected	Not detected
Segment 3–4 cm	77	19	Not detected	Not detected
Segment 4–5 cm	130	33	Not detected	Not detected
Segment 5–6 cm	122	30	Not detected	Not detected
Segment 6–7 cm	145	41	Not detected	Not detected
Segment 7–8 cm	135	37	Not detected	Not detected

All concentrations are in pg/mg
The limit of quantitation for each drug is 10 pg/mg

overrule a positive urine result. However, the negative hair findings can, on occasion, cast doubt on the positive urine analysis, resulting in substantial legal debate and several consequences for the athlete.

The concept of minimal detectable dosage in hair is of major importance to document the negative hair test results, but limited data is currently available in the scientific literature.[36] This is particularly true for doping agents, as most studies have focused on drugs of abuse or popular pharmaceuticals, such as benzodiazepines or hypnotics, with a focus on drug-facilitated crimes.[1] The sensitivity of the analytical procedure is very important for such purposes. For example, it has been published that a 4 mg dose of cocaine is not detectable in hair (with a LOD at 10 pg/mg),[5] while a single 35 mg is detectable, (with a LOQ at 0.1 ng/mg).[37] As a consequence, minute amounts of cocaine (1 or 2 mg), with no pharmacological effect, are undetectable in hair, while the major metabolite, benzoylecgonine, can peak up to 2800 ng/mL in urine.[38] Contamination of an athlete by kissing a partner using cocaine was supported by negative hair tests and accepted by the Court of Arbitration for Sport (CAS) in Lausanne during the hearing in the Richard Gasquet case.[39] At this time, the limit of detection of the laboratory was 10 pg/mg. Nevertheless, because laboratories can buy more sensitive equipment, the published data about method performance are not always those of the current method that was run for a specific case. With respect to doping agents, it has been published that a single dose of nandrolone undecanoate,[5] testosterone esters,[40] betamethasone[41] or hydrochlorothiazide[19] is not detectable in hair, even when using the most sophisticated instruments, such as high resolution mass spectrometry. Although it is always possible to claim that anabolic steroids have to be administered on a long-term basis to be active, meaning that they should be present in hair, the absence of detection of a single dose is a key point during discussion with a panel at the time of the hearing. It has been advocated that anabolic steroids are mostly detectable in hair collected from bodybuilders or powerlifters,[42] which is an over-estimated statement of the authors. Several anabolic steroids have been detected in "standard" athletes, including clostebol[13] in two boxers and one runner, methanedienone[30] in one football player, norandrostenedione in a runner[32] or nandrolone and testosterone undecanoate[43] in two cyclists. Obviously, there is a default in controlled studies involving the incorporation into hair of performance-enhancing drugs. For some drugs, such as selective androgen receptor modulators (SARMs), the data are also missing for urine. For example, very few (if any) data are available for andarine, ligandrol or ostarine. These studies should address the current known limitations of hair analysis, i.e. dose necessary to give a positive result, variable incorporation into hair, external factors (cosmetic treatment) that may have an impact on drug concentration. Although one can easily document a negative hair test result for cocaine, stimulants or narcotics, no data is available for several classes of the WADA prohibited list. As a consequence, the interpretation of isolated results from single cases appears tedious. To circumvent this problem, controlled administration study seems to be the unique possibility, but this must be achieved under secured conditions. Given the lack of therapeutic use of some of the WADA prohibited drugs, achievement of such evaluations will probably never occur.

It has been advocated that hair testing has little interest for testing substances only prohibited in-competition, due to a lack of retrospective accuracy as prohibition is limited to time ranges. It has been repetitively published[7,10,11] that stimulants, narcotics or cannabinoids testing in hair has particular significance in forensic toxicology but limited utility in doping control. Although this would be true if hair testing would be used as a screening matrix for doping control, such as urine or blood, this statement is inappropriate in practice. For example, testing for the social drug cocaine, which represents about 25% of the authors' requests, has demonstrated strong importance to document the AAFs as for this drug (and some others from the S6, S7 and S8 classes). Indeed, a lot of published data allows estimating the moment of drug exposure, its dosage and the conditions of exposure, particularly when the hair test result is negative.

When dealing with an unusual drug (for example, a drug which has gain no interest in clinical or forensic toxicology), one should encourage the laboratory performing the hair test to address the following questions:

- is a single pharmacological dose detectable in hair?
- what is the minimal detectable dose?
- what are the expected hair concentrations of patients using the same drug for therapeutic purposes?
- what (if any) is the influence of hair colour?
- what (if any) is the influence of hair cosmetic treatment?

In 2019, these points have been published for letrozole,[17] a S4 aromatase inhibitor and for hydrochlorothiazide,[19] a S5 diuretic, allowing to document the negative hair findings and it was concluded by the authors that hair analysis turned to be a possibility for the discrimination of drug repetitive use vs inadvertent exposure.

Even after complete analytical validation using the same standards as WADA accredited laboratories, including estimation of interference and contamination risks, the key point of hair analysis for doping agents remains the final interpretation of the results. Segmental analyses are also recommended, as the owner of the hair is his/her own control. It must be noticed that segments should be no longer than 1 cm to avoid dilution of a single dose over larger segments (of 3 or 6 cm), which would facilitate obtaining a negative result.

An important issue of concern for drug analysis in hair is the change in the drug concentration induced by cosmetic treatment of hair. Hair is continuously subjected to natural factors, such as sunlight (UV), weather, water, pollution, etc., which affect and damage the cuticle but hair cosmetic treatments enhance that damage. The chemicals used for cosmetic treatments (bleaching, permanent waving, dyeing or relaxing) are generally strong bases and will cause hair damage and affect drug content (by loss) or affect directly drug stability.[1] It has been published many times[1] that after cosmetic treatments, drug concentrations decline dramatically by decreasing from 50–80% the original concentration. Although repeated shampooing was found to have no significant action on the drug content of hair,[44] it has been advocated that shampoos like Ultra Clean can reduce drug concentrations in hair.[45] When the expected hair concentrations are very low, such as after a single administration, these effects can lead to a negative result, which has to be considered.

Taking into consideration the above mentioned limitations and those pointed in previous reviews (overlap in the window of detection for drugs only prohibited in competition, lack of discrimination about the route of administration for corticoids), hair analysis remains a fantastic tool to document drug exposure.

A negative hair test result is also a result.[46] However, this can be interpreted in two different ways: 1, the owner of the hair did not take or was not exposed to the specific drug, or 2, the procedure is not sensitive enough to detect the drug. Interpretation is difficult in case of suspected single exposure, but on the opposite, repeated drug use will favour identification by hair analysis.

Hair is a unique and challenging matrix for drug testing in that detailed information on historical use is recorded over time as compared to traditional blood and urine matrices. In order to make best use of hair testing in doping casework, a thorough understanding of the advantages and limitations of this matrix is essential.

Due to the recent development of imaging instruments and their availability in toxicological laboratories, it has been proposed to use these tools to test for drugs using a single hair. Matrix-assisted laser desorption/ionization (MALDI) combined with imaging is an innovative and powerful tool used since few years, especially in forensic research. Some authors have proposed innovative methods to monitor drugs consumption through direct mapping of the compounds with a high spatial distribution in human hair samples by MALDI-MS, including for example, cocaine,[47] synthetic cannabinoids,[48] zolpidem[49] or methamphetamine.[50] In addition to higher temporal resolution, this approach can be used to document potential external contamination.

However, mass spectrometry imaging techniques are very expensive and do not seem to be useful for routine analysis during the next few years. Since a dozen of years, it has been published that drug(s) can be detected in a single hair using highly sensitive conventional LC–MS/MS or LC-HRMS. Alibe et al[51] have recently reviewed such possibilities. For example, using GC/MS, cocaine and heroin[52] or methamphetamine and amphetamine[53] were identified in segmented hair. Using LC–MS/MS, other compounds, such as zuclopenthixol,[51] fentanyl, quetiapine or doxylamine[54] and chlorpheniramine[55] were also detected. It seems that the use of a single segmented hair in comparison with a complete lock of hair may be more informative to obtain a retrospective timeline of drug exposure. To exclude the risk of using hair fibres in different growth cycles (anagen vs catagen + telogen), one has to test several single hairs. The variations due to hair thickness between individual hairs can be limited by expressing the results in pg per length unit, such as mm. Segmental analysis of a single hair can overcome the issues of poor alignment and irregular hair growth which are sometimes observed after segmentation of a complete hair lock.

Because hair testing for drugs increases the window of detection and permits the differentiation of long-term use from a single exposure when performing segmental analyses, this matrix should be considered as a suitable complement, and not an alternative, to standard investigations. In addition, hair testing can significantely contribute to exculpation of athletes by demonstrating alternative administration patways with contamination risks, for example with ecstasy (MDMA) or clenbuterol.[10,11,42] Under all the above mentionned conditions, the application of hair testing, which is a non-invasive and a non-intrusive matrix, is a viable approach to document the case after an AAF. Finally, given hair is not proposed to replace urine for general routine control, aesthetic consequences of repetitive hair collection do not exist as hair will not be collected on a regular basis.

ORCID

Pascal Kintz https://orcid.org/0000-0002-4174-5362
Laurie Gheddar https://orcid.org/0000-0002-7804-3946
Alice Ameline https://orcid.org/0000-0002-1117-0887

REFERENCES

1. Kintz P. Hair analysis in forensic toxicology: an update review with a special focus on pitfalls. *Curr Pharm des*. 2017;23(36):5480-5486.
2. Sachs H, Kintz P. Consensus of the Society of Hair Testing on hair testing for doping agents. *Forensic Sci Int*. 2000;107:3.
3. Kintz P. Hair testing and doping control in sport. *Toxicol Lett*. 1998; 102–103:109-113.
4. Midio AF, de Moraes Moreau RL, Silva OA. The possibilities of hair analysis in the determination of involuntary doping in sports. *Sports Med*. 2001;31(5):321-324.
5. Kintz P, Cirimele V, Ludes B. Pharmacological criteria that can affect the detection of doping agents in hair. *Forensic Sci Int*. 2000;107(1-3): 325-334.

6. Rivier L. Is there a place for hair analysis in doping controls? *Forensic Sci Int*. 2000;107(1-3):309-323.
7. Thieme D, Grosse J, Sachs H, Mueller RK. Analytical strategy for detecting doping agents in hair. *Forensic Sci Int*. 2000;107(1-3): 335-345.
8. Ambrose P. Hair follicle testing another option for controlling drug use in sports. *J am Pharm Assoc*. 2005;45:6.
9. Petoczi A, Uvacsek M, Nepusz T, et al. Incongruence in doping related attitudes, beliefs and opinions in the context of discordant behavioural data: in which measure do we trust? *PLoS ONE*. 2011;6(4): e18804.
10. Thieme D. Potential and limitations of alternative specimens in doping control. *Bioanalysis*. 2012;4(13):1613-1622.
11. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complimentary matrices: advantages and limitations. *J Pharm Biomed Anal*. 2016;130:220-230.
12. https://www.wada-ama.org/sites/default/files/-wada_2020_english_prohibited_list_0.pdf, consultation 12 November 2019
13. Salomone A, Gerace E, Di Corcia D, Allardio E, Vincenti M, Kintz P. Hair analysis can provide additional information in doping and forensic cases involving clostebol. *Drug Test Anal*. 2019;11(1):95-101.
14. Kintz P, Cirimele V, Ludes B. Physiological concentrations of DHEA in human hair. *J Anal Toxicol*. 1999;23(6):424-428.
15. Kintz P, Ameline A, Gheddar L, Raul JS. LGD-4033, S-4 and MK-2866 – testing for SARMs in hair: about 2 doping cases. *Toxicol Anal Clin*. 2019;31:56-63.
16. Krumbholz A, Anielski P, Gfrerer L, et al. Statistical significance of hair analysis of clenbuterol to discriminate therapeutic use from contamination. *Drug Test Anal*. 2014;6(11-12):1108-1116.
17. Favretto D, Snenghi R, Pertile R, et al. Hair analysis to discriminate voluntary doping vs inadvertent ingestion of the aromatase inhibitor letrozole. *Drug Test Anal*. 2019;11(6):762-771.
18. Drooger JC, Jager A, Lam MH, et al. Development and validation of an UPLC-MS/MS method for the quantification of tamoxifen and its main metabolites in human scalp hair. *J Pharm Biomed Anal*. 2015; 114:416-425.
19. Gheddar L, Raul JS, Kintz P. First identification of a diuretic, hydrochlorothiazide, in hair: application to a doping case and interpretation of the results. *Drug Test Anal*. 2019;11(1):157-161.
20. Musshoff F, Thieme D, Schwarz G, Sachs H, Skopp G, Franz T. Determination of hydroxyl metabolites of cocaine in hair samples for proof of consumption. *Drug Test Anal*. 2018;10(4):681-688.
21. Rotolo MC, Klein J, Pacifici R, Busardo FP, Pichini S, Marchei E. Analytical strategies to disclose repeated consumption of new psychoactive substances by hair analysis. *Curr Pharm Biotechnol*. 2017;18(10): 834-839.
22. Salomone A, Palamar JJ, Bigiarini R, Gerace E, Di Corcia D, Vincenti M. Detection of fentanyl analogs and synthetic opioids in real hair samples. *J Anal Toxicol*. 2019;43(4):259-265.
23. Arbouche N, Raul JS, Garnier D, Kintz P, Ameline A. Testing for AB-PINACA in human hair: distribution in head hair versus pubic hair. *Drug Test Anal*. 2019;11(4):610-616.
24. Bévalot F, Gaillard Y, Lhermitte M, Pépin G. Analysis of corticosteroids in hair by liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr B Biomed Appl*. 2000;740(2): 227-236.
25. Choi TLS, Kwok KY, Kwok WH, Tsoi YYK, Wong JKY, Wan TSM. Detection of seventy-two anabolic and androgenic steroids and/or their esters in horse hair using ultra-high performance liquid chromatography-high resolution mass spectrometry in multiplexed targeted MS^2 mode and gas chromatography-tandem mass spectrometry. *J Chromatogr a*. 2018;1566:51-63.
26. Devi JL, Zahra P, Vine JH, Whittem T. Determination of testosterone esters in the hair of male greyhound dogs using liquid chromatography-high resolution mass spectrometry. *Drug Test Anal*. 2018;10(3):460-473.
27. Shah I, Haddow JD, Ibrahim HA, Sheikh MVA, Alhemeiri FSA. A novel and innovative hair test to determine glucocorticoid levels in racing camels for use in assessment of doping, health, and disease. *Drug Test Anal*. 2018;10(4):742-749.
28. Müller A, Jungen H, Iwersen-Bergmann S, Sterneck M, Andresen-Streichert H. Analysis of cyclosporine a in hair samples from liver transplanted patients. *Ther Drug Monit*. 2013;35(4):450-458.
29. https://www.wada-ama.org/sites/default/files/resources/-files/2017_anti-doping_testing_figures_en_0.pdf, consultation 12 November 2019
30. Bresson M, Cirimele V, Villain M, Kintz P. Doping control for methandienone using hair analysed by gas chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006; 836(1-2):124-128.
31. Gheddar L, Raul JS, Kintz P. Recherche d'hydrochlorothiazide dans les phanères après deux contrôles anti-dopage. *Toxicol Anal Clin*. 2018; 30:268-272.
32. Kintz P, Cirimele V, Ludes B. Discrimination of the nature of doping with 19-norsteroids through hair analysis. *Clin Chem*. 2000;46(12): 2020-2022.
33. Kintz P, Ameline A, Raul JS. Discrimination between zeranol and zearalenone exposure using hair analysis. Application to an adverse analytical finding case. *Drug Test Anal*. 2018;10(5):906-909.
34. https://www.tennisworldusa.org/tennis/news/-Tennis_Stories/81536/argentinian-tennis-player-luciano-tacchi-cleared-by-itf-of-doping-charges/, consultation 16 December 2019
35. Kintz P, Dumestre-Toulet V, Jamey C, Cirimele V, Ludes B. Doping control for beta-adrenergic compounds through hair analysis. *J Forensic Sci*. 2000;45(1):170-174.
36. Kintz P. Value of the concept of minimal detectable dosage in human hair. *Forensic Sci Int*. 2012;218(1-3):28-30.
37. Henderson GL, Harkey MR, Zhou C, Jones RT, Jacob P. Incorporation of isotopically labelled cocaineand metabolites into human hair: 1. Dose-response relationships. *J Anal Toxicol*. 1996;20(1):1-12.
38. Jackson GF, Saady JJ, Poklis A. Urinary excretion of benzoylecgonine following ingestion of health Inca tea. *Forensic Sci Int*. 1991;49(1): 57-64.
39. https://www.wada-ama.org/en/resources/legal/cas-wada-itf-v-gasquet, consultation 12 November 2019
40. Segura J, Pichini S, Peng SH, de la Torre X. Hair analysis and detectability of single dose administration of androgenic steroid esters. *Forensic Sci Int*. 2000;107(1-3):347-359.
41. Raul JS, Cirimele V, Ludes B, Kintz P. A single therapeutic treatment with betamethasone is detectable in hair. *J Anal Toxicol*. 2002;26(8): 582-583.
42. Thieme D, AnielskiP. Doping, application of hair analysis. In, Kintz P, Salamone A and Vincenti M, eds. *Hair analysis in clinical and forensic toxicology*, Academic Press, London, 2015, pp275–299.
43. Gaillard Y, Vayssette F, Pépin G. Compared interest between hair analysis and urinalysis in doping controls. Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists. *Forensic Sci Int*. 2000;107(1-3):361-379.
44. Baumgartner WA, Hill VA. Hair analysis for drugs of abuse: decontamination issues. In: Sunshine I, ed. *Recent Developments in Therapeutic Drug Monitoring and Clinical Toxicology*. New York: Marcel Dekker; 1992:577-597.
45. Röhrich J, Zömtlein S, Pötsch L, Skopp G, Becker J. Effect of the shampoo ultra clean on drug concentrations in human hair. *Int J Leg Med*. 2000;113(2):102-106.
46. Kintz P, Ameline A, Gheddar L, Feisthauer E, Eibel A, Raul JS. The significance of a negative hair test result. *Toxicol Anal Clin*. 2019;31:S15.
47. Musshoff F, Arrey T, Strupat K. Determination of cocaine, cocaine metabolites and cannabinoids in single hairs by MALDI Fourier

transform mass spectrometry – preliminary results. *Drug Test Anal*. 2013;5(5):361-365.
48. Kernalléguen A, Enjalbal C, Alvarez JC, et al. Synthetic cannabinoid isomers characterization by MALDI-MS^3 imaging. Application to single scalp hair. *Anal Chim Acta*. 2018;1041:87-93.
49. Erne R, Bernard L, Steuer AE, Baumagartner MR, Kraemer T. Hair analysis: contamination versus incorporation from the circulatory system-investigations on single hair samples using time-of-flight secondary ion mass spectrometry and matrix-assisted laser desorption/-ionization mass spectrometry. *Anal Chem*. 2019;91(6):4132-4139.
50. Wang H, Wang Y. Matrix-assisted laser desorption/ionization mass spectrometric imaging for the rapid segmental analysis of methamphetamine in a single hair using umbelliferone as a matrix. *Anal Chim Acta*. 2017;975:42-51.
51. Alibe N, Kintz P, Faure A, et al. Interest of single hair analysis to document drug exposure: literature review and a case report involving zuclopenthixol. *Curr Pharm des*. 2017;23:5502-5510.
52. Wainhaus SB, Tzanani N, Dagan S, Miller ML, Amirav A. Fast analysis of drugs in a single hair. *J am Soc Mass Spectrom*. 1998;9(12):1311-1320.
53. Nishida M, Yashiki M, Namera A, Kimura K. Single hair analysis of methamphetamine and amphetamine by solid phase microextraction coupled with in matrix derivatization. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006;84:106-110.
54. Thieme D, Baumer C, Sachs H, Teske J. Screening and long-term retrospection for psychoactive drugs in presumptive drug-facilitated crimes using segmented single hairs. *Drug Test Anal*. 2013;5(9-10):736-740.
55. Kuwayama K, Miyaguchi H, Iwata YT, et al. Three-step drug extraction from a single sub-millimeter segment of hair and nail to determine the exact day of drug intake. *Anal Chim Acta*. 2016;948:40-47.

How to cite this article: Kintz P, Gheddar L, Ameline A, Arbouche N, Raul J-S. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal*. 2020;12:316–322. https://doi.org/10.1002/dta.2766

1.4. Intérêt de la recherche des substances de la performance dans un laboratoire de toxicologie

L'émergence de nouveaux produits de la performance ainsi que la facilité d'accès à des produits dopants par Internet attire le grand public et ne s'arrête plus aux sportifs de haut niveau.
En effet, les amateurs veulent également perdre du poids et/ou se muscler rapidement pour plaire à un monde où l'apparence est devenue très importante. Ce public naïf est très mal averti en comparaison des sportifs de haut niveau qui sont suivis, entourés et conseillés par une équipe médicale. Ces produits présentent un tropisme médico-légal et/ou clinique. C'est le cas pour les SAA, les SARMs et les diurétiques.

Certains produits sont commercialisés à des fins thérapeutiques. La testostérone est commercialisée sous la forme d'ester « énanthate » et est vendue sous la dénomination Androtardyl®. Ce médicament est utilisé dans le traitement de l'hypogonadisme.
Les diurétiques sont quant à eux associés à des hypotenseurs dans le traitement contre l'hypertension. Les SARMs n'ont aucune indication thérapeutique à ce jour.
Il semble donc important, pour un laboratoire de toxicologie, d'identifier et de quantifier ces molécules pour le suivi thérapeutique de patients.

Par ailleurs, de nombreux effets secondaires ont été décrits dans la littérature après exposition sur le long terme d'agents anabolisants. En effet, à haut dosage et ou sur une longue période, les SAA sont connus pour provoquer des risques sur la santé. Ils sont connus pour leur toxicité cardiaque, hépatique, rénale, et ils peuvent également provoquer une augmentation de la pression artérielle ou encore un dysfonctionnement du système sexuel *[Van Amsterdam, 2010 ; Hernandez-Guerra, 2019]*.
Des problèmes d'ordre dermatologique ont également été décrits dans la littérature avec l'apparition de nécrose au point d'injection *[Friedman, 2016].*
L'usage répété de SAA peut également engendrer des problèmes sur la santé psychologique comme des modifications dans le comportement et peuvent augmenter l'agressivité des individus pouvant mener à des cas d'extrêmes violences comme des agressions sexuelles et des homicides. En effet, il a été démontré dans la littérature que l'abus de ces substances provoquait des effets psychologiques sévères comme des délires, des comportements maniaques, des dépressions *[Ganson, 2019 ; Khoodoruth, 2020].* Une altération de la densité des récepteurs sérotinergiques HHT1B et 5HT2 a été établi. Un lien entre la testostérone et la sérotonine a été démontré, ce qui pourrait expliquer des modifications dans le comportement et dans le système émotionnel *[Kindlundh, 2003 ; Anderson 2012]*. Une autre étude a montré que l'abus de SAA peut moduler l'activité des récepteurs de la

sérotonine car celle-ci est associée à une diminution de la neurotransmission de la sérotonine *[Birger, 2003].*

Les diurétiques ont également des effets négatifs connus. Il a été observé de l'hyponatrémie, de l'hypovolémie, de l'hypotension ou encore de l'hyperkaliémie à la suite d'un traitement prolongé par diurétiques *[Laurent, 2017].*

L'intérêt d'identifier de façon formelle les produits de la performance réside également dans la nécessité d'analyser des produits de saisie dans des affaires médico-judiciaires.

Pour les nouveaux agents dopants, les « designer steroids » et les SARMs, il y a un manque de connaissance important sur les effets secondaires après un usage régulier.

Pour finir, certains cas de dopage sont dus à des contaminations. Les affaires peuvent alors se transformer en affaire médico-judicaire impliquant des dépôts de plainte comme cela a été le cas pour Richard Gasquet qui a été contrôlé positif à la cocaïne après un échange de baisers avec une journaliste *[CAS WADA, 2009].* Il s'agit de la première affaire de dopage pour lesquels les cheveux ont été acceptés par le Tribunal Arbitral du Sport (TAS).

L'activité privée existante au laboratoire de Strasbourg a permis de prendre en charge des défenses de sportifs dans des cas de contamination par suppléments diététiques.

Les objectifs de ma thèse étaient les suivants. Le premier objectif a été de développer des méthodes d'analyse dans les cheveux, puis d'appliquer une approche médico-judiciaire en utilisant des matrices alternatives pour expliquer au mieux les résultats, d'établir les critères d'interprétation dans les phanères lorsque cela était réalisable et enfin d'appliquer les méthodes développées à des cas de dopage, des expertises médico-légales et des cas cliniques.

Durant ces trois années, je me suis intéressée à trois catégories de substances interdites en permanence, ayant un tropisme médico-légal et clinique et pour lesquels il y a peu de données dans les phanères dans la littérature et notamment pour les diurétiques et les SARMs.

La suite du manuscrit décrit la mise en place des méthodes analytiques et les applications de ces méthodes pour les stéroïdes anabolisants androgènes (partie 2), pour les substances classées comme autres agents anabolisants (partie 3) et enfin pour les diurétiques (partie 4).

Deuxième partie – Les stéroïdes anabolisants androgènes

2.1. Présentation

Les stéroïdes anabolisants androgènes sont des hormones dérivées de la testostérone sécrétée par les gonades chez les hommes et par les ovaires, en petites quantités, chez les femmes.
Les SAA exogènes sont soit des dérivés de la testostérone soit de la nandrolone et accessibles facilement sur Internet. Ils se consomment par voie orale (mestérolone, méthandiénone) ou par voie intra-musculaire (esters de SAA come la testostérone énanthate).
Du fait de leurs propriétés anabolisantes, les sportifs de haut niveau ainsi que les sportifs amateurs les utilisent pour augmenter la quantité de masse musculaire ou réduire le temps de récupération entre les entraînements.
Les SAA sont les produits les plus utilisés par les sportifs et sont interdits en permanence par l'AMA. En France, ces produits n'ont pas d'autorisation de mise sur le marché (AMM) hormis l'Androtardyl® (testostérone énanthate) - utilisé comme traitement substitutif -, le Danazol® - utilisé dans les cas d'endométriose - , le Nilevar® (noréthandrolone) et l'Androgène® (testostérone) - crème utilisée comme traitement substitutif. La vente ou l'achat de ces produits même pour sa consommation personnelle est interdits.
Il peut être important dans un laboratoire de toxicologie de savoir les identifier et de les quantifier pour deux raisons : l'utilisation sur le long terme provoque des risques sur la santé physique et psychologique et l'importance de suivre les taux des SAA endogènes pour surveiller le profil stéroïdien dans le traitement contre l'hypogonadisme, par exemple, et de répondre ainsi aux demandes des services cliniques du CHU de Strasbourg.
Dans la littérature, l'analyse des SAA dans les phanères existent depuis 30 ans. Néanmoins, il est nécessaire d'apporter de nouvelles données dans la littérature pour être capable d'expliquer au mieux les résultats observés en médico-légal et clinique.

2.2. Développement d'une méthode d'identification et de quantification dans les cheveux

Afin d'identifier tous les SAA couramment utilisés dans le milieu sportif et en thérapeutique, j'ai adapté les méthodes publiées *[Gaillard, 1999]* dans la littérature au sein du laboratoire de toxicologie de Strasbourg.
30 mg de cheveux sont nécessaires pour l'identification des analytes. Le protocole suivant a été optimisé et validé pour identifier 30 SAA. La liste des molécules, les transitions utilisées, les temps de rétention (t_R) et les limites de quantification (LQ) et de détection (LD) sont résumés dans les tableaux 2 et 3.

Les SAA sont identifiés et quantifiés soit par de la chromatographie liquide ultra haute performance couplée à la spectrométrie de masse en tandem (UHPLC-MS/MS), soit par de la chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem (GC-MS/MS). Certains analytes ne sont pas détectables par UHPLC-MS/MS comme la mestérolone.

Les méthodes de chromatographie en phase liquide et gazeuse sont des techniques séparatives. En chromatographie en phase liquide, une compétition d'affinité s'installe entre analytes-phase mobile liquide et entre analytes-phase stationnaire (colonne de chromatographie liquide). En chromatographie gazeuse, il y a également une compétition d'affinité avec la phase stationnaire (colonne de chromatographie gazeuse).

Le spectromètre de masse est un détecteur permettant de détecter et d'identifier les molécules d'intérêt selon leur rapport masse/charge. Les acquisitions se font en mode « Multiple Reaction Monitoring ».

Protocole d'extraction

Le type d'extraction choisi est une extraction liquide-liquide suivie d'une purification par extraction sur phase solide (SPE).

30 mg de matrices kératinisées (cheveux coupés finement à l'aide d'une paire de ciseaux /ongles pulvérisés par un broyeur à billes Retsch MM200) sont pesés. Les phanères sont incubés dans 1 mL de méthanol pendant 90 min dans un bain à ultrasons en présence de 1 ng d'un mélange de standards internes (SI) (testostérone-D_3, clenbutérol-D_9, nandrolone-D_3, stanozolol-D_3 et 19-norandrostérone-D_4) afin d'extraire les esters de SAA.

Après centrifugation, la phase organique est recueillie et évaporée sous un flux d'azote puis reprise avec un tampon phosphate 1 M pH 7,0 (partie A). Le reste de la matrice du tube, est recueilli et hydrolysé avec 1 mL d'hydroxyde de sodium (NaOH) à 1M (partie B) afin d'extraire les autres SAA non-esters. Une extraction liquide-liquide est réalisée sur les parties A et B avec 2,5 mL d'acétate d'éthyle. Les extraits sont combinés et évaporés. Le résidu sec est dissous dans 1 mL de dichlorométhane, qui est soumis à de la SPE en utilisant des colonnes Isolute C18. Les colonnes sont conditionnées avec 3 mL de dichlorométhane, puis la séquence suivante est réalisée :

- 1 mL de l'échantillon dans le dichlorométhane
- 1 mL de dichlorométhane
- 2 mL de dichlorométhane/acétate d'éthyle (3/1)

Les 3 phases (1, 2, et 3) sont collectées et évaporées en utilisant un concentrateur SpeedVac (Thermo Scientific SPD121P) et solubilisées dans 30 µL de méthanol.

2 µL sont ensuite injectés sur le système LC-MS/MS (Waters TQS micro).

Après analyse, le résidu est évaporé. 35 µL de l'agent dérivant (MSTFA/NH4I/2-mercaptoéthanol, 1000:2:5) sont ajoutés. Les échantillons sont dérivés pendant 20 minutes à 60°C puis 1 µL est injecté sur le système GC-MS/MS (Agilent 7010B).

Paramètres du système UHPLC-MS/MS

Les échantillons sont injectés sur un système UHPLC-MS/MS (Waters Acquity UPLC (Waters Corporation, Milford, MA, USA)) muni d'une colonne Acquity™ UPLC BEH C18 (100 × 2,1 mm, 1,7 µm) (Waters), thermostatée à 50 °C, permettant de travailler en phase inverse.

Le débit de travail est fixé à 0,4 mL/min. Une élution en gradient a été réalisée en utilisant un tampon acétate à 1,0 mM et de l'acétonitrile de qualité LCMS acidifié à 0,1 % d'acide formique (HCOOH). Le temps d'analyse est de 11,0 min. Le gradient a été optimisé et est présenté dans le tableau 1.

Temps (min)	Tampon acétate d'ammonium (%)	Acétonitrile + 0,1 % HCOOH (%)
0	80	20
1	80	20
6	5	95
7,5	5	95
7,6	80	20
11	80	20

Tableau 1 : Gradient d'analyse UHPLC – méthode des SAA

La détection a été effectuée à l'aide d'un triple quadripôle (XEVO™ TQ-S Micro, Waters Corporation, Milford, MA, USA) équipé d'une source d'ionisation Z-spray-TM-électrospray (ESI) et utilisé en mode positif et négatif (ES+/ES-). L'azote a été utilisé comme gaz de nébulisation. Les paramètres optimisés sont les suivants : température de la source : 150°C ; débit du gaz de désolvatation : 800 L/h ; température de désolvatation : 500°C ; gaz de collision : argon. La tension capillaire est réglée à 3,0 kV. Les transitions ainsi que les temps de rétention et les LD et LQ pour chaque SAA ont été optimisés durant la période de développement et sont décrits dans le tableau 2.

Paramètres du système GC-MS/MS

Les analyses par GC-MS/MS ont été réalisées en mode impact électronique (EI) à ions positifs sur un Agilent 8890 GC System équipé d'une colonne capillaire à phase liée HP-5MS ultra inerte (30 m, 25 mm, 0,25 µm d'épaisseur de film). Les conditions de fonctionnement étaient les suivantes : temps pour l'injection en mode splitless : 1 min ; température initiale fixée à 80°C pendant 2 min et augmentation jusqu'à 300°C avec une pente de 30°C/min. La température de 300°C est maintenue

14 min pour permettre l'élution des esters de SAA. Les échantillons ont été analysés sur un spectromètre de masse en tandem quadripolaire 7010B avec une source High Efficiency Source de chez Agilent, fonctionnant en mode EI+ (70 eV). Les analytes sont élués en 24 min. Les transitions ainsi que les temps de rétention et les LD et LQ pour chaque SAA ont été optimisés durant la période de développement et sont décrits dans le tableau 3.

UHPLC-MS/MS					
Analytes	Transition de quantification	Transition de confirmation	T_R (min)	LD (pg/mg)	LQ (pg/mg)
Androstèdione	287,2 > 97,0	287,2 > 109,0	4,83	0,5	1
Boldenone	287,2 > 121,1	287,2 > 135,1	4,32	0,2	1
Clenbuterol	277,1 > 203,0	277,1 > 1319	1,6	5	10
DHEA	289,4 > 97,1	289,4 > 109,0	4,62	0,5	1
DHT	290,2 > 109,3	290,2 > 97,2	4,62	0,5	1
Epitestosterone	289,2 > 97,1	289,2 > 109,0	4,95	0,5	1
Gestrinone	309,1 > 241,2	309,1 > 199,1	4,76	0,5	1
Méthandienone	301,2 > 121,1	301,2 > 149,1	4,52	0,2	1
Méthénolone	303,2 > 187,3	303,2 > 83,0	4,85	0,5	1
Méthyltestostérone	303,2 > 97,0	303,2 > 108,9	4,85	0,5	1
Nandrolone	275,2 > 109	275,2 > 82,9	4,40	0,5	1
Oral turinabol	335,2 > 155,0	335,2 > 91,0	5,09	0,5	1
Stanozolol	329,3 > 81,1	329,3 > 95,1	4,68	0,2	1
Testostérone	289,2 > 97,0	289,2 > 109,0	4,63	0,5	1
THG	313,3 > 241,2	313,3 > 159,1	5,25	0,5	1
Trenbolone	271,2 > 199,1	271,2 > 253,2	4,18	0,4	1
Testostérone benzoate	393, 1 > 104,9	393, 1 > 97,0	6,84	5	10
Testostérone propionate	345,1 > 97,0	345,1 > 109,0	6,35	5	10
Testostérone acétate	331,3 > 109,0	331,3 > 97,0	5,92	5	10
Testostérone décanoate	443,5 > 97,0	443,5 > 109,0	8,45	5	10
Testostérone isocaproate	387,4 > 97,0	387,4 > 81,0	7,20	5	10
Nandrolone décanoate	429,4 > 155,1	429,4 > 71,0	8,24	5	10
Testostérone 17β-cypionate	413,2 > 97,0	413,2 > 78,9	7,61	5	10
Testostérone enanthate	401,4 > 97,0	401,4 > 113,1	7,52	5	10
SI					
Clenbuterol-D_9	286,1 > 168,9	/	1,62	/	/
Nandrolone-D_3	278,2 > 109,0	/	4,41	/	/
Stanozolol-D_3	332,3 > 81,1	/	4,67	/	/
Testostérone-D_3	292,2 > 109,0	/	4,62	/	/

Tableau 2 : Liste des SAA analysés – transitions – temps de rétention – LD et LQ

GC-MS/MS					
Analytes	Transition de quantification	Transition de confirmation	T_R (min)	LD (pg/mg)	LQ (pg/mg)
19-Norandrostenedione	416,4 > 234,2	416,4 > 401,4	10,50	5	10
Mesterolone	448,0 > 141,0	448,0 > 156,0	10,80	0,6	1
Drostanolone	448,0 > 141,0	448,0 > 156,0	10,90	0,5	1
Drostanolone enanthate	488,4 > 141,0	488,4 > 156,1	15,50	3	5
Drostanolone propionate	432,4 > 141,1	432,4 > 156,1	11,80	3	5
SI					
Testostérone-D_3	453,4 > 209,1	/	10,89	/	/
19-Norandrostérone-D_4	409,4 > 229,3	/	9,95	/	/

Tableau 3 : Liste des SAA analysés – transitions – temps de rétention – LD et LQ – SUITE

2.3. Applications à des cas de dopage et des expertises médico-judiciaires

Les méthodes développées au laboratoire ont été appliquées à des affaires de dopage dans des cas de suspicion de contamination par le biais de l'activité privée menée au laboratoire.
Elles ont également été appliquées à des affaires médico-légales pour lesquelles il y avait des suspicions d'abus d'agents anabolisants qui auraient provoqués des accès de violence et/ou des décès.
Cette méthode est également applicable à des demandes hospitalières pour le suivi des profils stéroïdiens et à l'analyse de produits de saisie dans le cadre d'affaires médico-judiciaires.
Les applications de cette méthode d'identification ont donné lieu à 8 publications nationales et internationales et sont décrites ci-dessous.

Cas de dopage et autres matrices :
Les 3 publications suivantes montrent l'intérêt de l'analyse des cheveux et des autres matrices kératinisées (ongles, poils du corps) en comparaison à l'analyse urinaire :

Article 4 : Pendant une période de compétition, une athlète de haut niveau s'est faite contrôlée par un laboratoire anti-dopage. Ses urines ont révélé un résultat anormal pour le stanozolol et ses métabolites.
L'athlète a contesté avoir consommé délibérément du stanozolol et a pensé à une contamination par un supplément diététique. Afin de prouver qu'il n'y a pas eu consommation, son avocat, en discussion avec l'expert, a demandé une analyse complémentaire dans les cheveux et les poils du corps.
Des cheveux, des poils de jambes, des poils de bras, axillaires et pubiens ont été reçus au laboratoire. Une méthode et une extraction spécifique ont été développées et appliquées. Le stanozolol est le seul SAA à avoir un noyau d'azote dans sa structure chimique. Une extraction dans des conditions alcalines est donc préférable.
Le stanozolol a été identifié et quantifié dans tous les poils de l'athlète : 73 pg/mg (cheveux), 454 pg/mg (poils pubiens), 238 pg/mg (poils de bras), 244 pg/mg (poils de jambes) et 7100 pg/mg (poils axillaires).
Pour la première fois, le stanozolol a été observé simultanément dans cinq types de poils prélevés sur cinq régions anatomiques différentes chez un même sujet avec un large éventail de concentrations, ce qui est cohérent avec les vitesses de pousse et les taux d'incorporation différents de chaque poil. Ces résultats montrent que le stanozolol s'incorpore mieux dans les poils du corps que dans les cheveux.

Article 5 : La recherche des agents anabolisants dans les cheveux n'est pas nouvelle et a plusieurs applications dans les domaines du dopage, d'expertise médico-légale et clinique. Néanmoins, il y a un certain nombre de limites à prendre en compte : l'indisponibilité de la matrice ou encore les traitements cosmétiques pouvant altérer les concentrations dans les cheveux. Pour remédier à ces problématiques, il est possible d'utiliser d'autres poils du corps. Il est à noter que les taux d'incorporation ainsi que les vitesses de pousse sont différents de celle des cheveux. Ces paramètres seront pris en compte lors de l'interprétation. Dans cet article, plusieurs SAA sont mis en évidence dans les poils. Les concentrations sont souvent plus hautes dans les poils que dans les cheveux.

Article 6 : Les SAA sont généralement analysés dans les urines et rarement dans les cheveux dans le cadre antidopage. Dans le cadre médico-légal, les cheveux sont utilisés pour documenter les profils de consommation.

Lorsque les cheveux ou les autres poils ne sont pas disponibles, il reste une matrice kératinisée qui permet d'avoir une fenêtre de détection importante : les ongles. Les fenêtres de détection sont de 3 à 8 mois pour les ongles de mains et de 4 à 12 mois pour les ongles de pieds. L'analyse des ongles, dans un cadre de dopage, est une nouvelle approche pour documenter une exposition répétée.

Le stanozolol (7 et 24 pg/mg), la nandrolone (6 pg/mg), la trenbolone (26, 67, 81 et 89 pg/mg), la drostanolone (8 et 11 pg/mg) et la testostérone enanthate (14 pg/mg) ont été identifiés dans des ongles dans 6 affaires de dopage, et d'expertises médico-légales (décès lié à l'abus de stéroïdes, violence).

L'analyse des phanères dans des cas de dopage permet d'apporter des informations sur le profil de consommation et permet d'obtenir des informations rétrospectives.

Cas de décès liés à l'abus de SAA :

L'abus et l'utilisation répétée des SAA peuvent provoquer des problèmes hépatiques et cardiaques comme décrits en partie 1. De plus, les consommateurs utilisent la plupart du temps plusieurs SAA et à des cycles de consommation différents.

Des cas de décès ont été observés à l'IML de Strasbourg. Les analyses complémentaires demandées par les magistrats ont permis de mettre en évidence un usage régulier de SAA.

Deux cas mettent en évidence l'importance de rechercher les SAA dans les phanères suite à l'observation de problèmes cardiaques, à l'autopsie :

Article 7 : Un homme de 59 ans a été retrouvé mort chez lui. A côté de lui, 2 flacons vides de solutions huileuses sans étiquette ont été retrouvés. L'homme était connu pour s'entrainer dans une salle de musculation et comme consommateur de SAA. Le procureur a ordonnée une levée de corps et a estimé que des analyses toxicologiques supplémentaires suffisaient. Il n'y a donc pas eu d'autopsie.

Dans le sang, uniquement de la testostérone a été identifiée à une concentration inférieure à 1 ng/mL. Dans les urines, le ratio T/E était de 1,56 et de la boldenone a été quantifiée à 9 ng/mL. Enfin, dans les cheveux, plusieurs SAA ont été identifiés : testostérone (140 pg/mg), testostérone propionate (605 pg/mg), testostérone décanoate (249 pg/mg), boldenone (160 pg/mg), trenbolone (143 pg/mg) et méthandiénone (60 pg/mg).

L'analyse des cheveux a permis dans ce cas de mettre en évidence une consommation multiple et répétée de SAA, contrairement à l'urine et au sang. Les experts ont attribué le décès à une insuffisance cardiaque due à l'abus de SAA.

Article 8 : Un homme de 60 ans est retrouvé décédé à son domicile par sa femme. Cette dernière rapporte qu'il s'entrainait régulièrement dans une salle et qu'il consommait des SAA de manière quotidienne. L'autopsie a révélé une cardiomégalie ainsi qu'une congestion multi viscérale. Les analyses toxicologiques ont mis en évidence de la trenbolone dans le sang fémoral (3,9 ng/mL), dans le sang cardiaque (3,2 ng/mL), dans la bile (231 ng/mL), dans l'humeur vitrée (< 0,5 ng/mL) et dans les cheveux (9 pg/mg).

Les experts ont conclu que la prise répétée des SAA pouvait expliquer les problèmes cardiaques avancés.

Cas d'agressivité lié à l'abus de SAA :

L'usage répété des stéroïdes anabolisants peut avoir un impact négatif sur le comportement des consommateurs (partie 1). Durant ma thèse, plusieurs cas de violence induits par l'abus d'agents anabolisants ont été observés et étudiés dans le cadre médico-légal. Trois cas ont donné lieu à des publications dont une chez l'animal et sont décrits ci-dessous :

Article 9 : Un homme de 32 ans s'est rendu aux forces de police pour dénoncer son acte, celui d'avoir tué sa petite amie après lui avoir infligé plusieurs coups de couteau. L'homme était très agité et agressif. Il était inconnu de la police et était videur de boîte de nuit. Deux tubes de sang ont été prélevés au commissariat. Trois semaines après les faits, 3 mèches de cheveux ont été prélevées (brunes, 3 cm, orientées). La recherche d'AAS a mis en évidence dans le sang de la méthandiénone (32 ng/mL) et de la trenbolone (9 ng/mL). Les cheveux étaient également positifs pour la méthandienone (11 et 3 pg/mg) et pour la trenbolone (14 pg/mg).
Ce cas met en évidence la dangerosité psychique de l'usage répétée d'agents anabolisants.

Article 10 : Un homme de 21 ans est arrêté et placé en prison après avoir tué son rival suite à une crise de jalousie. Plusieurs mois après sa condamnation, l'homme annonce qu'il est un ancien consommateur de SAA. Pour documenter cette exposition, seule l'analyse de cheveux permet de réaliser une analyse rétrospective. Une mèche de 9 cm segmentée en 3 segments de 3 cm a été analysée et l'analyse a mis en évidence de la drostanolone et son ester, la drostanolone énanthate. Seul le segment correspondant au moment de l'homicide était positif avec des concentrations de 3 et 14 pg/mg respectivement pour la drostanolone et son ester.

Article 11 : Trois chiens sont devenus de jour en jour plus agressifs envers leurs maitres mais également entre eux. Inquiets, les maîtres ont décidé de se tourner vers un laboratoire de toxicologie pour savoir si un produit pharmaceutique pouvait modifier les comportements de leurs chiens. En discussion avec l'expert, les poils des chiens ont été analysés. La méthandiénone, un SAA exogène, a été identifiée à 42, 13 et 32 pg/mg. Afin de rechercher la source de contamination, 16 produits pharmaceutiques et compléments alimentaires ont été analysés. Parmi ces produits, deux suppléments diététiques étaient contaminés par de la méthandiénone à 452 et 25 ng/g. Les chiens ont retrouvé un comportement qualifié de normal par leur maîtres quatre mois après arrêt complet de la consommation de ces suppléments.

Les différentes publications décrivent les dangers liés à l'utilisation répétée des stéroïdes anabolisants et montrent également l'importance d'identifier les composés dans les phanères afin d'identifier les molécules mises en cause dans des cas de violence, d'homicide et de décès.

Article 4 : Testing for stanozolol, using UHPLC-MS/MS and confirmation by UPLC-q-TOF-MS, in hair specimens collected from five different anatomical regions

Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz

Journal of Analytical Toxicology. 2020;44(8):834-839.

Journal of Analytical Toxicology, 2020;44:834–839
doi: 10.1093/jat/bkaa023
Advance Access Publication Date: 03 March 2020
Special Issue

OXFORD

Special Issue

Testing for Stanozolol, Using UPLC–MS-MS and Confirmation by UPLC–q-TOF-MS, in Hair Specimens Collected from Five Different Anatomical Regions

Laurie Gheddar[1,*], Jean-Sébastien Raul[1] and Pascal Kintz[1,2]

[1]Institut de Médecine Légale, 11 rue Humann, Strasbourg 67000, France, and [2]X-Pertise Consulting, 42 rue Principale, 67206 Mittelhausbergen, France

*Author to whom correspondence should be addressed. Email: l.gheddar@gmail.com

Abstract

An athlete challenged the result from an in-competition doping test which returned with an adverse analytical finding for stanozolol, claiming it was due to supplement contamination. Her lawyer asked the laboratory to analyze several hair specimens simultaneously collected from five different anatomical regions, head, arm, leg, pubis and armpit, to document the pattern of drug exposure. A specific UPLC–MS-MS method was developed. After decontamination with dichloromethane, stanozolol was extracted from hair in the presence of stanozolol-d_3 used as internal standard, under alkaline conditions, with diethyl ether. Linearity was observed for concentrations ranging from 5 pg/mg to 10 ng/mg. The method has been validated according to linearity, precision and matrix effect. Concentrations of stanozolol in head hair, pubic hair, arm hair, leg hair and axillary hair were 73, 454, 238, 244 and 7,100 pg/mg, respectively. The concentration of stanozolol in head hair is in accordance with data published in the literature. When comparing the concentrations, body hair concentrations were higher than the concentration found in head hair. These results are consistent with a better incorporation rate of stanozolol in body hair when compared to head hair. The simultaneous positive concentrations in different hair types confirm the adverse analytical finding in urine of the top athlete, as the measured concentrations do not support the theory of contamination. For the first time, an anabolic agent was simultaneously tested in hair collected from five different anatomical regions from the same subject, with a large distribution of concentrations, due to anatomical variations, and these findings will help interpretation in further doping cases when documented with hair.

Introduction

The widespread use of anabolic in sports causes numerous problems of integrity and sport ethics. That's why, in 1999, the World Anti-Doping Agency (WADA) has created a document, called the CODE, to harmonize prohibited practices and substances in and out of competition. Despite the ban of some drugs by the WADA, the use of anabolic steroids continues to grow. For example in 2017, they represent 44% of adverse analytical findings (AAFs). Among the anabolic steroids, stanozolol accounted for 20% of AAFs (1).

Stanozolol is marketed as Stanozolol®, Stromba® and Winstrol®, and it is available as 2, 10 and 50 mg tablets for oral administration and at 50 and 75 mg/mL for injectable administration (2). It has been prohibited by the International Olympic Committee since 1974 and is classified in the S1.1.a class as exogenous anabolic androgenic steroid (3). Stanozolol is a derivative of testosterone and the only anabolic drug containing two nitrogen atoms. It has been established since many years that the presence of a nitrogen atom allows a better incorporation into hair, due to electric interaction with the negatively

charged melanin, which is the major protein in the hair matrix. In addition, a basic drug (stanozolol has a pKa 2.9) is more easily incorporated into hair because it has a better penetrating ability to break through the membrane, based on pH gradient between blood and the acidic hair matrix (2, 4–5).

In the literature, analysis of stanozolol in human head hair has been seldom reported, mainly by the same groups. In addition, there is a major lack of information about stanozolol identification in body hair. Only Kintz et al. (6) and Thieme et al. (7) reported concentrations in the leg, chest and axillary, but they did not compare the data with head hair. To the best of the knowledge of the authors, there is no publication where hair specimens collected from five different anatomical regions from the same subject was described. This is of importance when the standard head hair test cannot be performed (bald subject, cosmetic treatment, religious reasons, aesthetic issues, etc.). The aim of this report is to document the distribution of stanozolol in different hair collected from different anatomical regions for the same subject.

Case report and hair specimens

During in-competition period, an anti-doping laboratory tested the urine of a top athlete (woman) and returned an adverse analytical finding for stanozolol and its metabolites (3-OH-stanozolol, 4-OH-stanozolol and 16β-OH-stanozolol). The athlete challenged the result and denied any voluntary intake of the drug. Her lawyer requested our laboratory for further analysis because of possible contamination by food or supplements. Contamination of supplements by stanozolol has been previously reported in literature. Geyer et al. (8) and Odoardi et al. (9) reported concentrations of stanozolol in nutritional supplements between 10 and 40 ng/g and in one supplement at 25 µg/g. In addition, Campos et al. presented during the 2019 TIAFT meeting that 90% of the supplements from a seizure were contaminated by stanozolol (analysis of seizure formulations of stanozolol in south Brazil by high-resolution mass spectrometry).

The athlete confirmed that she did not cosmetically treat her head hair for at least 6 months. In addition, she also confirmed no leg and axillary hair shaving for the last 2 months. At the request of her lawyer, the laboratory received five hair specimens collected from five different anatomical regions to establish the pattern of stanozolol use at the time of the urinary control. Hair specimens included head hair, leg hair, arm hair, pubic hair and axillary hair. The hair was collected by a US point of collection with suitable chain of custody about 1 month after the urinary control. The head hair was 4 cm in length, curvy and black in color. No segmentation of the head hair was possible because of its curvy nature. All body hair specimens were 0.5–1.5 cm in length and black in color.

Each hair specimen was received in a separate envelope and stored at ambient temperature in the laboratory before analysis.

Blank hair for method validation was obtained from laboratory staff.

Chemicals and reagents

Stanozolol and stanozolol-d_3 were obtained from Cerilliant (Texas, USA). Ammonium acetate, dichloromethane and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). Methanol for HPLC LC–MS grade (MeOH), acetonitrile for HPLC LC–MS grade (ACN) and formic acid were purchased from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Diethyl ether was purchased from Honeywell Riedel-de Haën (Seelze, Germany).

The standard solutions, prepared in ACN, were stored at −20°C.

Sample treatment

The hair strands were decontaminated twice with 5 mL of dichloromethane for 1 min at room temperature and dried.

Thirty milligrams of decontaminated hair was incubated in 1 mL 1 M NaOH for 10 min at 95°C, in the presence of 300 pg of stanozolol-d_3 used as internal standard (IS). After cooling, 5 mL of diethyl ether were added. After agitation for 15 min and centrifugation (15 min, 3,000 rpm), the organic phase was collected and evaporated to dryness under a nitrogen flow. The dry residue was dissolved in 50 µL of MeOH, and 5 µL was injected into the UHPLC-system.

Instrumentation

Chromatographic separation was performed with a liquid chromatography Waters Acquity UPLC™ system (Waters Corporation, Milford, MA, USA) using an Acquity UPLC HSS C18 column (150 × 2.1 mm, ID 1.8 µm particle size). The column temperature was set to 50°C and the flow rate at 0.3 mL/min. The mobile phase consisted of 1.0 mM ammonium acetate in water (eluent A) and ACN, 0.1% formic acid (eluent B). Analysis time was 6.0 min, and the following gradient pattern (eluent A) was used for the separation of stanozolol and the IS, 0 min, 95%; 2.00 min, 95%; 4.00 min, 0%; 4.10 min, 0%; 4.30 min, 95%; 6.00 min, 95%. Stanozolol and stanozolol-D_3 were eluted both in 4.93 min.

Detection was performed using a triple quadrupole (XEVO™ TQD, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionization source (ESI) and used in the positive mode (ES+). Nitrogen was used as drying gas as well as nebulizing gas. The desolvation gas flow was set to 800 L/h and the desolvation temperature was 400°C. The source temperature was 150°C. The capillary voltage was set at 1.50 kV. Cone voltage and collision energy were adjusted to optimize the signal of the two most abundant product ions of stanozolol, m/z 329.3 > 81.1 and 329.3 > 121.1 and for stanozolol-d_3: m/z 332.3 > 81.1. MassLynx 4.1 software was used for quantification.

To confirm the head hair result, an UPLC-q-TOF-MS system was used. Chromatographic separation was performed with an identical equipment as per UPLC–MS-MS. The applied parameters (column temperature, mobile phase, gradient pattern, flow) were the same compared to UPLC–MS-MS.

Detection was performed using a high-resolution (XEVO™ G2XS Q-TOF, Waters Corporation, Milford, MA, USA) mass spectrometer operating in positive ion mode and in sensitivity mode. The desolvation gas flow was set to 800 L/h at a temperature of 600°C with the cone gas set to 20 L/h and the source temperature set to 150°C. The capillary voltage and the cone voltage were set to 1,500 and 30 V, respectively. Leucine-enkephalin was used as the lock mass (m/z 556.2766) for accurate mass calibration and introduced using the LockSpray interface at 10 µL/min and a concentration of 200 pg/µL in 50% aqueous ACN, 0.1% formic acid. In MS scanning, data were acquired from 70 to 800 m/z. As for MS-MS

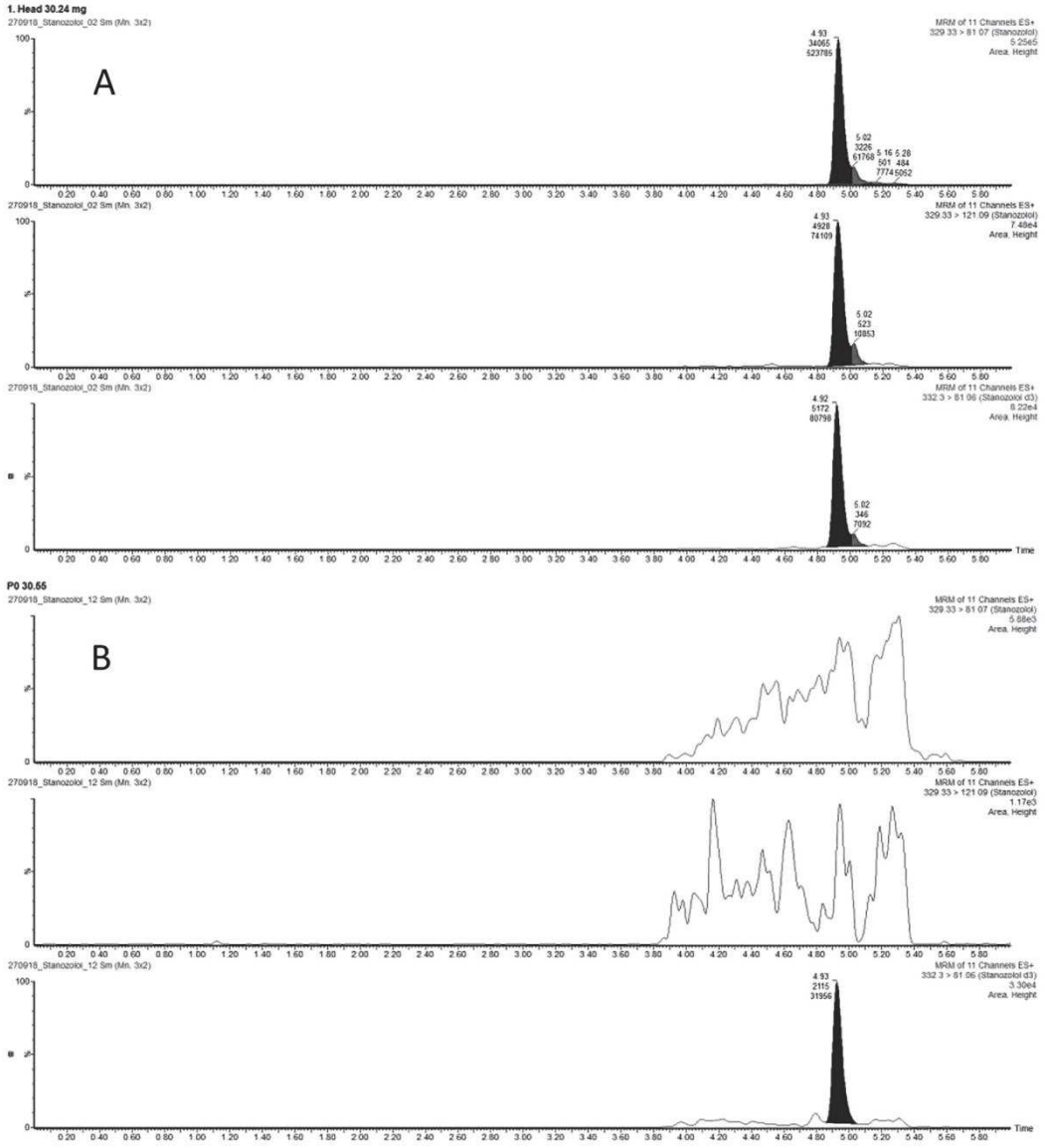


Figure 1. Chromatograms of head hair specimen and blank sample. (A) Head hair; (B) blank hair sample. From top to bottom: first transition of stanozolol, second transition of stanozolol, quantification transition of stanozolol-d_3.

fragmentation of target ions, collision energy ranging from 10 to 40 V was applied. Unifi software was used for data, chromatograms and spectra acquisition.

Validation method

A nine-point calibration curve ($n = 3$) was obtained by spiking blank hair at 5, 10, 50, 100, 150, 200, 500, 1,000 and 10,000 pg/mg. The limit of quantification (LOQ) was defined as the first point of calibration. The limit of detection (LOD) was the lowest concentration of analyte that could be detected with signal-to-noise ratio superior than 3. For the repeatability, two calibration points at 10 and 500 pg/mg were evaluated eight times. With respect to matrix effect, 15 different hair blank specimens were extracted, and 50 µL of standard solution of stanozolol (1 mg/L) was added at the end of extraction. These specimens were compared to 50 µL of standard solution of stanozolol in MeOH (1 mg/L).

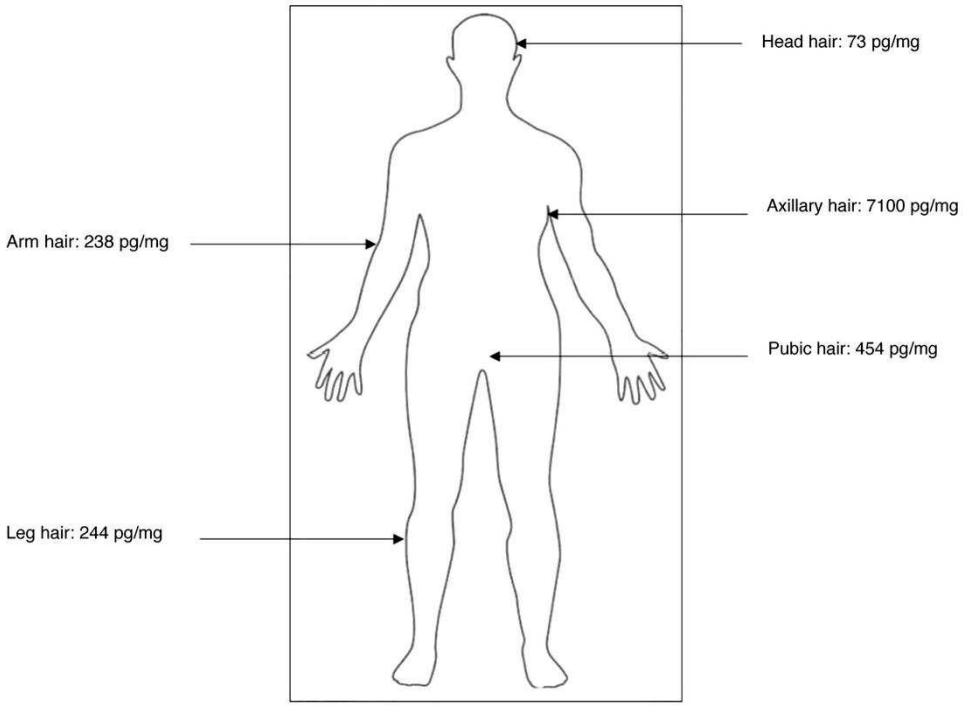


Figure 2. Body distribution of stanozolol hair concentrations.

Results and discussion

Validation

Three calibration curves based on analyte/internal standard peak area ratios were obtained on three separate days and analyzed by weighted linear regression to validate the linearity of the method. The assay was linear in the concentration range 5–10,000 pg/mg ($r^2 > 0.990$) with a LOQ of 5 pg/mg. The LOD was 2 pg/mg. QC samples (10 and 500 pg/mg), analyzed in duplicate in eight independent experimental assays, were used to determine a coefficient of variation for precision. These CVs were lower than 20% (8.7 and 5.2% for intra-assay and 15 and 18% for inter-assay, for 10 and 500 pg/mg respectively). The matrix effect was less than 20%.

Hair test results

Figure 1 shows the chromatograms obtained after extraction of head hair specimen and blank hair sample. The use of UPLC–MS-MS allowed to measure the following concentrations: head (73 pg/mg), arm (238 pg/mg), leg (244 pg/mg), pubis (454 pg/mg) and axillary (7,100 pg/mg) hair. It is also possible to represent this distribution according to the representation in Figure 2. Moreover, the decontamination wash was analyzed and was negative.

More and more often, WADA-accredited laboratories use high specificity properties of HRMS method to document their positive findings. As indicated in the consensus of the Society of Hair Testing for doping agents (10), it is suggested for additional data using hair analysis in the field of doping control to use the same sophisticated equipments. Therefore, the concept of testing anabolic drugs in hair using HRMS has emerged, and recently Fabresse et al. (11) have published some interesting results. The UPLC–q-TOF-MS system therefore confirmed the presence of stanozolol in head hair (m/z 329.2580) with a mass error of −2.2 ppm, when compared to a reference standard (m/z 329.2588). A common fragment is also identified (81.0447 for the reference standard and 81.0441 for head hair). Figure 3 presents the chromatogram and high and low spectra obtained with UPLC–q-TOF-MS.

When compared, the concentrations of body hair showed a large variation. This variation can be explained by the difference in growth rate and the incorporation rate for each type of hair. Xenobiotics are only incorporated into hair during the anagen stage (12). It appears that the concentration in head hair was the lowest. A similar observation was already published by Kintz et al. (13), who presented in one article that the concentrations of DHEA, another anabolic steroid, are much higher in axillary and pubic hair when compared to head hair. Concentrations measured in arm hair and in leg hair are quite similar. These results are consistent with a close growth rate (0.6 and 0.9 cm/month for leg and arm) and similar duration of the anagen stage (25 and 30% for leg and arm) (12). The highest concentrations were found in the pubic and axillary hair. This can be explained by the incorporation routes, given that a xenobiotic can be incorporated in hair by three body routes: blood, sweat and sebum and by external contamination (5, 12). In the case of axillary hair and pubic hair, contribution by sweat and contamination by urine, respectively, are possible.

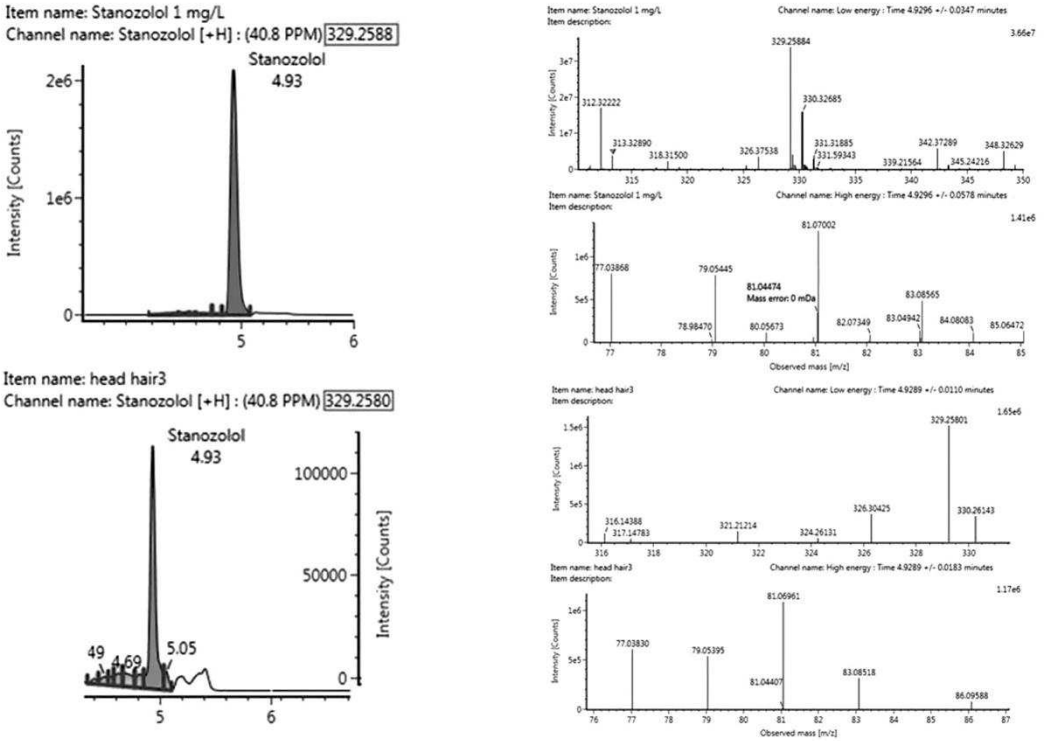


Figure 3. Comparison of responses between standard reference (left) and head hair (right).

Table I. Concentrations of Stanozolol in Head Hair, Data of Literature

Head hair concentration (pg/mg)	Sport practice	Reference
135 and 156	Bodybuilding	(14)
180	Bodybuilding	(15)
15	Bodybuilding	(16)
2–84	Bodybuilding	(17)
5–86.3	Bodybuilding and other	(18)
112	Bodybuilding	(6)
1–60	Bodybuilding and other	(7)
17–1,992	Bodybuilding	(19)
11	Fitness	(20)

The concentration found in head hair (73 pg/mg) is consistent with the data from the scientific literature. In various papers, mostly written by few groups, concentrations ranged from 1 to 1,992 pg/mg. It is interesting to note that the concentration found for the case involving fitness practice (11 pg/mg) is part of the low range when compared to what can be measured in specimens from bodybuilders. In view of the results reported in Table I, it is conceivable to conclude that the measured concentration in head hair from the athlete (73 pg/mg) is highly indicative of repeated abuse and does not support the claim of contamination by supplement(s).

Conclusion

This is the first report of simultaneous identification of stanozolol in hair collected from five different anatomical regions from the same subject. The presence of stanozolol in all hair specimens supports the adverse analytical finding in the urine and is consistent with repeated abuse of stanozolol. In addition, the results seem to show that stanozolol incorporates better into body hair than in head hair. As a consequence, it appears that the analysis of the body hair can be performed when head hair is not available. According to the consensus of the Society of Hair Testing, hair analysis can be performed as a complement to urinalysis (10).

Conflict of Interest statement

The authors declare no conflict of interest.

References

1. World Anti-Doping Agency. (2017) *2017 Anti-Doping Testing Figures. Laboratory Report*. https://www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures-report (accessed Sept 12, 2019).
2. Baselt, R.C. (ed) *Disposition of Toxic Drug and Chemicals in Man*, 11th edition. Biomedical Publications: Seal Beach, CA, 2017; pp1970–1971.
3. USADA. (2015) *What is Stanozolol?* https://www.usada.org/spirit-of-sport/education/what-is-stanozolol/ (accessed Sept 12, 2019).

4. Kintz, P. (2012) Value of the concept of minimal detectable dosage in human hair. *Forensic Science International*, 218, 28–30.
5. Kronstrand, R., Scott, K. Analytical and practical aspects of drug testing in hair, Chapter 1. In: Kintz P. (ed). *Forensic Science Series*. Taylor and Francis: Boca Raton, Fl, 2007; pp1–23.
6. Kintz, P., Vayssette, F., Deveaux, M. (2014) Compendium of results from hair tested for anabolics. *Toxicologie Analytique et Clinique*, 26, 197–200.
7. Thieme, D., Anielski, P. Doping, applications of hair analysis, Chapter 10. In: *Hair Analysis in Clinical and Forensic Toxicology*. Academic Press: Elsevier, London UK, 2015; pp275–299.
8. Geyer, H., Parr, M.K., Koehler, K., Mareck, U., Schänzer, W., Thevis, M. (2008) Nutritional supplements cross-contaminated and faked with doping substances. *Journal of Mass Spectrometry*, 43, 892–902.
9. Odoardi, S., Castrignano, E., Martello, S., Chiarotti, M., Strano-Rossi, S. (2015) Determination of anabolic agents in dietary supplements by liquid chromatography-high-resolution mass spectrometry. *Food Additives and Contaminants—Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 32, 635–647.
10. Sachs, H., Kintz, P. (2019) Consensus of the Society of Hair Testing on hair testing for doping agents. https://www.soht.org/consensus/9-nicht-kategorisiert/88-consensus-on-doping-agents (accessed Sept 12, 2019).
11. Fabresse, N., Grassin-Delyle, S., Etting, E., Alvarez, A. (2017) Detection and quantification of 12 anabolic steroids and analogs in human whole blood and 20 in hair using LC-HRMS/MS: application to real cases. *International Journal of Legal Medicine*, 131, 989–999.
12. Cooper, G.A.A. Anatomy and physiology of hair, and principles for its collection. In: *Hair Analysis in Clinical and Forensic Toxicology*. Academic Press: Elsevier, London, UK, 2015; pp1–22.
13. Kintz, P., Cirimele, V., Ludes, B. (2000) Pharmacological criteria that can affect the detection of doping agents in hair. *Forensic Science International*, 107, 325–334.
14. Kintz, P., Cirimele, V., Sachs, H., Jeanneau, T., Ludes, B. (1999) Testing for anabolic steroids in hair from two bodybuiders. *Forensic Science International*, 17, 209–216.
15. Thieme, D., Grosse, J., Sachs, H., Mueller, R.K. (2000) Analytical strategy for detecting doping agents in hair. *Forensic Science International*, 107, 335–345.
16. Cirimele, V., Kintz, P., Ludes, B. (2000) Testing of the anabolic stanozolol in human hair by gas chromatography-negative ion chemical ionization mass spectrometry. *Journal of Chromatography B*, 740, 265–271.
17. Dumestre-Toulet, V., Cirimele, V., Ludes, B., Gromb, S., Kintz, P. (2002) Hair analysis of seven bodybuilders for anabolic steroids, ephedrine, and clenbuterol. *Journal of Forensic Sciences*, 47, 211–214.
18. Desmukh, N., Hussain, I., Barker, J., Petroczi, A., Naughton, D.P. (2010) Analysis of anabolic steroids in human hair using LC-MS/MS. *Steroids*, 75, 710–714.
19. Kintz, P. (2017) A new series of hair test results involving anabolic steroids. *Toxicologie Analytique et Clinique*, 29, 320–324.
20. Kintz, P., Gheddar, L., Ameline, A., Dumestre-Toulet, V., Verschoore, M., Comte, J., *et al.* (2019) Complete post-mortem investigations in a death involving clenbuterol after long-term abuse. *Journal of Analytical Toxicology*, 43, 660–665.

Article 5 : Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic location has several advantages when compared with the standard head hair analysis.

Pascal Kintz, Laurie Gheddar, Jean-Sébastien Raul

Drug Testing and Analysis. 2021;13(7) :1445-1451.

Received: 19 January 2021 | Revised: 23 February 2021 | Accepted: 23 February 2021
DOI: 10.1002/dta.3020

SHORT COMMUNICATION

WILEY

Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis

Pascal Kintz[1,2] | Laurie Gheddar[2] | Jean-Sébastien Raul[2]

[1]X-Pertise Consulting, Mittelhausbergen, France
[2]Laboratory of Toxicology, Institut de medecine légale, Strasbourg, France

Correspondence
Pascal Kintz, Laboratory of Toxicology, Institut de medecine légale, 11 rue Humann, Strasbourg F-67000, France.
Email: pascal.kintz@wanadoo.fr

Abstract

Since the late 90s, hair testing for anabolic steroids in humans has found numerous forensic, clinical, and anti-doping applications. In most cases, analyses were performed on head hair, collected in the vertex regions. However, for various reasons (shaved subject, bald subject, religious belief, cosmetic treatment and aesthetic reason), hair collectors can face the lack of head hair, and therefore, body hair can be the unique alternative choice. Although there is no possibility to perform segmental analyses with body hair, their use has two major advantages: (1) In most cases, anabolic steroids are more concentrated in body hair when compared with head hair, which allows detecting abuse at lower frequency and for lower dosages; and (2) the window of drug detection is generally much longer in body hair when compared with head hair, particularly in male athlete presenting short head hair. To document the relevance of simultaneous collection of head and body hair, the authors present eight authentic cases of anabolic steroids abuse, including clostebol (one case), drostanolone (one case), metandienone (one case), 19-norandrostenedione (one case), stanozolol (two cases) and trenbolone (three cases). In all cases, body hair concentrations were higher than head hair concentrations. Even in three cases, no steroid was identified in head hair, although present in body hair.

KEYWORDS
anabolic steroids, body hair, doping, forensic toxicology, hair

1 | INTRODUCTION

In the recent years, hair specimens have been proposed to document drug exposure in any situation in which a history of past rather than recent drug use is requested. In addition, keratinous matrices are the unique matrices allowing to document long-term exposure to drugs. Finally, testing head hair by segmentation allows establishing a retrospective calendar of an individual's drug use and the distinction between single and repetitive use.

Testing for anabolic steroids in human hair was not an initial priority for scientists involved in hair analysis. The first series of publications, in the 80s and early 90s, were devoted to drugs of abuse, mainly opiates and cocaine. It is only late in the 90s that pioneers presented the first procedures for the detection of anabolic steroids, all using gas chromatography coupled to single mass spectrometry (GC–MS).[1–4] Rapidly, Gaillard et al.[5] and Thieme et al.[6] proposed methods with higher selectivity, using tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry, respectively. Ten years later, analytical approaches using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS)[7] and liquid chromatography coupled to high-resolution mass spectrometry[8,9] were described. Initially, these procedures were

proposed for anti-doping analyses when the athlete challenged an adverse analytical finding. Later, they have been used in forensic science to document changes in behaviour, enhanced libido and the associated risk of sexual assault, aggressiveness and cardiac side effects in sudden deaths.

However, all these publications were based on the identification of anabolic steroids in hair collected from head hair (=scalp hair). In some special cases, head hair can be missing or is not available for sampling. This can happen with shaved or bald subjects or subject with religious belief. Aesthetic issues and heavy cosmetic treatment, such as bleaching or colouring, can also prevent head hair collection, as these treatments will dramatically decrease the concentrations of the drugs already incorporated.[10] Alternatively, body hair can be collected. To date, only Gheddar et al.[11] compared stanozolol concentrations in hair specimens collected from five different anatomical regions obtained from a repetitive abuser. Stanozolol was identified in head hair (73 pg/mg), pubic hair (454 pg/mg), arm hair (238 pg/mg), leg hair (244 pg/mg) and axillary hair (7100 pg/mg). Obviously, concentrations measured in body hair were much higher than the one measured in head hair. According to the authors, these variations were explained by differences in the hair growth cycle, which is different for each type of hair.

Indeed, in a comprehensive review, Pianta et al.[12] reported several publications dealing with hair growth cycles, which are summarized in Table 1. Obviously, the growth rate is variable for each type of hair, but more important, the duration and the number of hair in the anagen state (the only state where drugs are incorporated) are highly variable.

Although the detection of anabolic steroids in human hair has some limitations,[13–15] mostly due to very low incorporation rate and therefore an unknown minimal detectable dose, there is a considerable interest in forensic,[16] clinical[17] and anti-doping[18] situations to be able to document their use or abuse.

The objective of this publication is to present the advantages of collecting several body hair specimens from the same donor and to compare the concentrations with head hair. This series of results will produce new data about anabolic steroids detection in an alternative matrix, which can be a basis for routine analysis in various situations (forensic, clinical or doping) where their identification is needed.

2 | MATERIALS AND METHOD

2.1 | Materials/standards

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99–100%, AnalaR NORMAPUR was purchased from VWR Prolabo (Fontenay-sous-Bois, France). Acetonitrile for LC–MS grade, ethyl acetate, methanol and dichloromethane were purchased from Merck (Molsheim, France). Steroids and the deuterated standards (testosterone-d_3, nandrolone-d_3 and stanozolol-d_3) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Isolute C_{18} columns were purchased from Interchim (Montlucon, France). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), 2-mercaptoethanol and ammonium iodide were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

The drugs were diluted to appropriate concentrations with methanol, using a working 1 mg/L solution.

2.2 | Hair specimen

Hair specimens were collected for forensic purposes (drug trafficking, aggressiveness and fatality) or after challenging an adverse analytical finding during an anti-doping control. Clients were lawyers, judges or solicitors, who always directly requested tests for anabolic steroids, given the specific context, that is, challenging a doping violation, steroids trafficking, death of an abuser or intrafamily violence. There was no additional request for other drugs, including recreational ones. Demographic data of the eight subjects are summarized in Table 2. All subjects were male, aged from 17 to 42 years. Their claims (or the claims of relatives) with respect to steroids abuse are presented in Table 2.

In all cases, the hair specimens were collected by experienced professional collectors by cutting them with scissors close to the skin and sent to the laboratory in a hair collection kit. The specimen was stored at room temperature until analysis.

2.3 | Toxicological analyses

Anabolic steroids were tested according to our screening procedure, already published.[19] Briefly, 30 mg of finely cut hair, previously

TABLE 1 Hair growth cycle (summarized from Pianta et al.[12])

Hair	Growth rate (cm/month)	Anagen (months)	Anagen (%)	Catagen + telogen (months)	Catagen + telogen (%)
Head	0.7–1.5	Up–96	85	3–4	5–15
Chest	0.8–1.1	4	40	4–6	50–70
Leg	0.4–0.8	4–7	20	3–9	70–80
Arm	0.6–1.2	1–4	30	1–4	60–80
Axillary	0.8–1.1	4–18	30–40	3–18	50–70
Beard	0.6–0.9	4–22	60–70	9–12	30–40
Pubic	0.6–0.9	9–18	30–40	12–18	50–70

decontaminated by two dichloromethane baths (5 mL, 2 min) were weighted. Internal standards (1 ng of testosterone-d_3, nandrolone-d_3 and stanozolol-d_3) were added together with 1 mL of methanol for a 90-min ultrasonic bath at room temperature. After centrifugation, the organic phase was collected and evaporated and diluted with phosphate buffer pH 7.0 (part A). The remaining hair, at the bottom of the tube, was collected and hydrolyzed with 1-mL NaOH 1 M (part B). Liquid–liquid extractions were operated on parts A and B with 2-mL ethyl acetate. Extracts were combined and evaporated to dryness. The residue was dissolved in 1-mL dichloromethane, which was submitted to solid phase extraction, using Isolute C_{18} columns. Columns were conditioned with 3 mL of dichloromethane and then, the following sequence was used: 1 mL of the specimen in dichloromethane (phase 1), 1 mL of dichloromethane (phase 2) and finally 2 mL dichloromethane/ethyl acetate (3/1) (Phase 3). All three phases (1–3) were collected and evaporated using a SpeedVac Concentrator (Thermo Scientific SPD121P) and solubilized in 30-µL methanol to be divided in two parts: for injection onto LC–MS/MS apparatus (Waters TQS micro) and onto GC–MS/MS apparatus (Agilent 7010B) after evaporation

TABLE 2 Demographic data of the subjects involved in the study

Case	Reason of test	Description of the specimens	Claims about abuse
1	Child custody	Head hair: 3 cm, brown Arm hair	Very occasional
2	Doping	Head hair: 1.5 cm, black Leg hair + arm hair	No abuse
3	Doping	Head hair: 2.6 cm, black Leg hair + arm hair	No abuse
4	Workplace	Head hair: 2 cm, brown Chest hair + arm hair	Occasional
5	Aggressiveness	Head hair: 1 cm, brown Chest hair + arm hair	Regular
6	Trafficking	Head hair: 3 cm, black Leg hair + arm hair	Regular
7	Trafficking	Head hair: 1 cm, black Pubic hair + axillary hair	Regular
8	Death	Head hair: 1 cm, blond Pubic hair + axillary hair + chest hair	Occasional

TABLE 3 Summary of the validation parameters

Drug	Linearity	LOD (pg/mg)	LOQ (pg/mg)	Precision (20 pg/mg, %)
Androstenedione	1–200 pg/mg	0.5	1	14.6
Testosterone	1–200 pg/mg	0.5	1	13.5
Boldenone	1–200 pg/mg	0.2	1	17.1
Tetrahydrogestrinone	1–200 pg/mg	0.5	1	16.9
Metandienone	1–200 pg/mg	0.2	1	15.1
Metenolone	1–200 pg/mg	0.5	1	11.6
Methyltestosterone	1–200 pg/mg	0.5	1	12.4
Nandrolone	1–200 pg/mg	0.5	1	16.1
Chlorodehydromethyl-testosterone	1–200 pg/mg	0.5	1	14.8
Oxandrolone	5–200 pg/mg	2	5	17.5
Stanozolol	1–200 pg/mg	0.2	1	13.6
Trenbolone	1–200 pg/mg	0.4	1	13.9
Dehydroepiandrosterone	1–200 pg/mg	0.5	1	14.7
Dihydrotestosterone	1–200 pg/mg	0.5	1	14.7
Drostanolone	1–200 pg/mg	0.5	1	16.2
Mesterolone	1–200 pg/mg	0.6	1	15.8

Abbreviations: LOD, limit of detection; LOQ, limit of quantification.

and fully automated trimethylsilyl (TMS) derivatization (MSTFA/NH_4I/2-mercaptoethanol, 1000:2:5) for 20 min at 60°C.

Androstenedione, testosterone, boldenone, tetrahydrogestrinone, metandienone, metenolone, methyltestosterone, nandrolone, chlorodehydromethyltestosterone, oxandrolone, stanozolol and trenbolone were tested by LC–MS/MS. Dehydroepiantrosterone, dihydrotestosterone, drostanolone, mesterolone and all esters (testosterone, nandrolone and drostanolone) were tested by GC–MS/MS.

A summary of the validation parameters is presented in Table 3. In all cases, matrix effects (tested at 20 pg/mg) were less than 25%. In case of very concentrated specimens, less hair material was used to fit the linearity range.

3 | RESULTS AND DISCUSSION

The method was validated according to the 2012 recommendations of the Society of Hair Testing.[20] Given there is no manufacturer of certified reference material for anabolic steroids in hair, it is not possible to establish a true measurement uncertainty of the procedure. In addition, there is no interlaboratory proficiency test for this class of drugs.

At the initial stages of the developpement of a screening method for the most common anabolic steroids, LC–MS/MS and GC–MS/MS analyses were compared. Given anabolic steroids are badly incorporated into hair, it was decided to obtain the more sensitive method possible. Blank hair was spiked with the different anabolic steroids at 20 pg/mg, extracted, injected in the 2 apparatus and the signal to noise ratio (S/N) was measured for each drug. For some compounds (dehydroepiantrosterone, dihydrotestosterone, drostanolone, mesterolone and all esters), a much higher S/N was obtained using GC–MS/MS. Drostanolone and mesterolone signals were very low using LC–MS/MS, quite in the background noise at 20 pg/mg. This is the reason why it was decided to split the screening method into two different approaches. Once this was acted, the method was validated separately using LC–MS/MS for androstenedione, testosterone, boldenone, tetrahydrogestrinone, metandienone, metenolone, methyltestosterone, nandrolone, chlorodehydromethyltestosterone, oxandrolone, stanozolol and trenbolone and GC–MS/MS for dehydroepiantrosterone, dihydrotestosterone, drostanolone, mesterolone and all esters. One can anticipate that the differences in S/N with LC–MS/MS and GC–MS/MS are issues in ionization.

In all the eight cases, the request was only to test the hair specimens for anabolic steroids, without specifying which drug(s) could be involved. In routine, when the compound has been previously identified, a simplified method is always preferred.[11]

The cases were selected because several hair specimens, from various body locations, were requested for testing, and positive results were obtained. These cases were obtained within the last 2 years. In the routine activity of the laboratory for anabolic steroids, in about 30%, the solicitors have submitted body hair in addition to head hair. In about 10% of the requests, only body hair was submitted to analysis.

Results are presented in Table 4. Clostebol (one case), drostanolone (one case), metandienone (one case), 19-norandrostenedione (one case), stanozolol (two cases) and trenbolone (three cases) were identified and quantified in multiple hair specimens from the same donor. In case n° 4, the subject was using simultaneously trenbolone and 19-norandrostenedione. The results from cases n° 5 and n° 6 are in agreement with Gheddar et al.,[11] as body hair concentrations were higher than head hair concentration and also because in case n° 6, the concentration in arm hair was close to the one in leg hair.

Figure 1 is representative of what can be expected as chromatographic signals. Although concentrations were generally low, a

TABLE 4 Concentrations of anabolic steroids in various body hair specimens

Case	Steroid	Results
1	Trenbolone	Head hair (0–1.5 cm): 14 pg/mg
		Head hair (1.5–3 cm): 42 pg/mg
		Arm hair: 165 pg/mg
2	Trenbolone	Head hair (0–1.5 cm): Traces (<5 pg/mg)
		Leg hair: 20 pg/mg
		Arm hair: 15 pg/mg
3	Metandienone	Head hair (0–1.3 cm): Not detected
		Head hair (1.3–2.6 cm): Traces (<1 pg/mg)
		Leg hair: 19 pg/mg
		Arm hair: 11 pg/mg
4	19-Norandrostenedione	Head hair (0–2 cm): Not detected
		Chest hair: 6 pg/mg
		Arm hair: 2 pg/mg
4	Trenbolone	Head hair (0–2 cm): 121 pg/mg
		Chest hair: 2964 pg/mg
		Arm hair: 258 pg/mg
5	Stanozolol	Head hair (0–2 cm): 6 pg/mg
		Chest hair: 41 pg/mg
		Arm hair: 23 pg/mg
6	Stanozolol	Head hair (0–1.5 cm): Traces (<1 pg/mg)
		Head hair (1.5–3 cm): 5 pg/mg)
		Leg hair: 38 pg/mg
		Arm hair: 43 pg/mg
7	Clostebol	Head hair (0–1 cm): Not detected
		Pubic hair: 56 pg/mg
		Axillary hair: 31 pg/mg
8	Drostanolone	Head hair (0–1 cm): 3 pg/mg
		Pubic hair: 66 pg/mg
		Axillary hair: 14 pg/mg
		Chest hair: 29 pg/mg

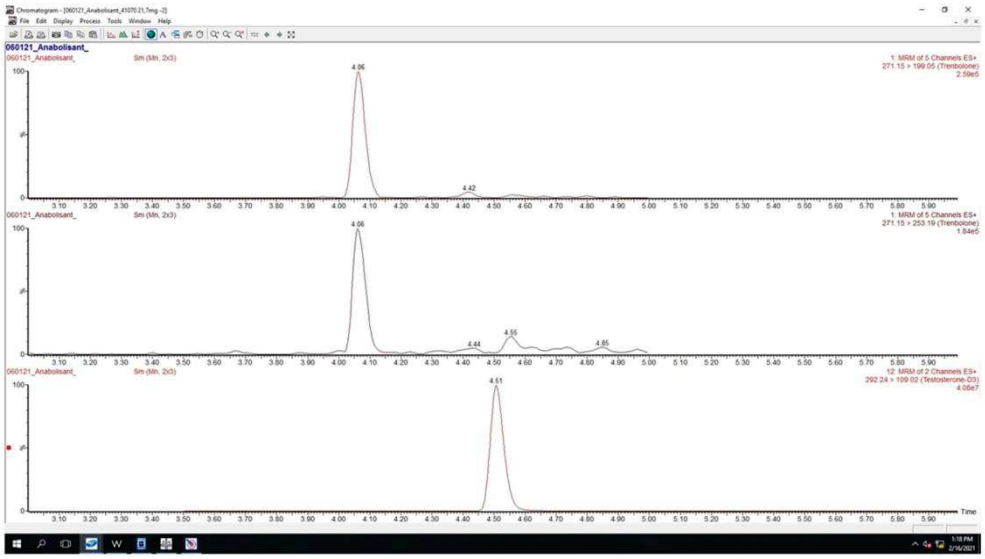


FIGURE 1 Chromatogram obtained after extraction of the arm hair specimen of subject n° 2. Trenbolone concentration was 15 pg/mg. From top to bottom: Two transitions for trenbolone and one transition for testosterone-d_3 (IS) [Colour figure can be viewed at wileyonlinelibrary.com]

suitable chromatographic signal was obtained in each hair specimen. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in hair. Other chromatograms are presented as Supporting Information.

Obviously, irrespective of its anatomical location, body hair testing has produced higher drug concentrations when compared with head hair. This has a direct implication on the possibility of detection as the chromatographic signal to noise is always in favour of body hair versus head hair, influencing the sensitivity and the selectivity of the test. At this time, in the scientific literature, hair concentrations after a controlled administration of a specific steroid for a given period of time are unknown. In addition, nothing has been published about the concentrations in various hair specimens from the same donor after administration of a given dosage. In particular, no one has promoted the use of body hair instead of head hair to document steroid administration. Based on the results the authors are presenting, this possibility should be taken into consideration, as in many hearings at the Court of Arbitration for Sport (CAS), the interpretation of a negative head hair test was challenged by the World Anti-Doping Agency (WADA) due to a lack of sensitivity (i.e., the impossibility to detect a single administration).

The major interest of using body hair when compared with head hair is the extension of the window of detection. Using head hair, the window of detection is typically linked to the length of the submitted specimen; that is, each centimetre represents the growth of 1 month (for example, 3 cm = 3 months). It can be easily observed that the male population who uses anabolic steroids has generally short hair or even very short hair. Therefore, the window of steroid detection using head hair is generally 1–3 months before hair collection. Using body hair, standard windows of detection are generally up to 8 months before collection. Most academic and commercial laboratories are using an equivalent period of 8 months. It can be easily understood that body hair testing dramatically increases the window of detection of anabolic steroids.

Nothing has been published about the decline in steroid concentration in head hair after abstinence. It has been published that cocaine,[21] heroin,[22] methamphetamine[23] and Δ^9-tetrahydrocannabinol carboxylic acid[24] disappear within 3–6 months after discontinuation of drug abuse. The persistence of past drug use markers, due to nongrowing hair (in the catagen and telogen stages), is the most common reason why head hair segments continue to test positive after drug discontinuation. Residues can also come from tissue depots (acting as drug storage) in the scalp or fat tissues. Although not supported by any scientific study, it is the feeling of the authors that concentrations decline very rapidly after steroid discontinuation, probably because they cleared from the hair by washing as they are not firmly bound to melanin. This has been observed occasionally in cases involving trenbolone, metandienone, oxandrolone or nandrolone, perhaps because of the lack of electric charge binding possibility between neutral (and therefore not positively charged) steroids and melanin, which is negatively charged. Only stanozolol, which is the only anabolic steroid containing two nitrogen atoms, has a different physical behaviour. If anabolic steroids are rapidly cleared from head

hair after discontinuation, this will enhance the advantage of collecting body hair.

Several explanations to account for higher steroid concentrations in body hair have been published. Gheddar et al.[11] explained the variation of concentrations among the five different hair types by the difference in growth rate and the variable ratio between the anagen stage and the catagen + telogen stages (i.e., 85/15 and 20–70/80–30 for head hair and body hair, respectively). It seems usual to find the lowest concentrations in head hair when compared with body hair, as already published for endogenous compounds, such as dehydroepiandrosterone.[25] Concentrations measured in leg and arm hair are often more or less identical, which is consistent with a similar growth rate and a similar duration of the anagen phase. Contamination by sweat or urine can account for the high concentrations measured in axillary hair and pubic hair, respectively.

Voegel et al.[26] have indicated that body hair is not really suitable as alternative when scalp hair is not available. If this seems true for endogenous steroid hormones, as demonstrated back in 2000 by Kintz et al.[25] for dehydroepiandrosterone, it is not the case with synthetic derivatives of testosterone. The present report is exclusively dealing with exogenous anabolic steroids, which is fairly different from endogenous compounds with complicated excretion process in sweat and sebum.

4 | CONCLUSION

The detection of anabolic steroids in body hair has been seldom described in the literature[27,28] and is presented in isolation, with no test using head hair. Only one publication[11] has systematically compared stanozolol in hair collected from five different anatomical locations. Although there is no possibility to perform segmental analyses with body hair, testing for anabolic steroids in body hair specimens has two major advantages: (1) In most cases, anabolic steroids are more concentrated in body hair when compared with head hair, which allows detecting abuse at lower frequency and for lower dosages; and (2) the window of drug detection is generally much longer in body hair when compared with head hair, particularly in male athletes presenting short head hair.

Obviously, there is a future for anabolic steroids testing in forensic situations. There is an increased risk of developing an antisocial lifestyle, and the frequency of crimes of violence and weapon offences is increased once anabolic steroids are consumed for a long period. Abusers of steroids are more prone to be involved in criminal acts, as demonstrated by epidemiological studies, and steroids are sometimes identified as indirect cause of death.[16,29]

Finally, there are also some interesting perspectives for testing anabolic steroids to document anti-doping violation. Although some limitations do exist,[15] particularly the impossibility, at this time, to detect a single steroid administration, identification of an anabolic steroid, in human and animal, allows to better document and understand challenged cases. In that sense, controlled administration studies would be of great interest.

ORCID

Pascal Kintz https://orcid.org/0000-0002-4174-5362
Laurie Gheddar https://orcid.org/0000-0002-7804-3946

REFERENCES

1. Segura J, Ventura R, Jurado C. Derivatization procedures for gas chromatographic-mass spectrometric determination of xenobiotics in biological samples, with special attention to drugs of abuse and doping agents. *J Chromatogr B Biomed Sci Appl*. 1998;713(1):61-90.
2. Deng XS, Kurosu A, Pounder DJ. Detection of anabolic steroids in head hair. *J Forensic Sci*. 1999;44(2):343-346.
3. Kintz P, Cirimele V, Jeanneau T, Ludes B. Identification of testosterone and testosterone esters in human hair. *J Anal Toxicol*. 1999;23(5):352-356.
4. Kintz P, Cirimele V, Sachs H, Jeanneau T, Ludes B. Testing for anabolic steroids in hair from two bodybuilders. *Forensic Sci Int*. 1999;101(3):209-216.
5. Gaillard Y, Vaysette F, Balland A, Pépin G. Gas chromatographic-tandem mass spectrometric determination of anabolic steroids and their esters in hair. Application in doping control and meat quality control. *J Chromatogr B Biomed Sci Appl*. 1999;735(2):189-205.
6. Thieme D, Grosse J, Sachs H, Mueller RK. Analytical strategy for detecting doping agents in hair. *Forensic Sci Int*. 2000;107(1-3):335-345.
7. Deshmukh N, Hussain I, Barker J, Petroczi A, Naughton DP. Analysis of anabolic steroids in human hair using LC-MS/MS. *Steroids*. 75:710-714.
8. Strano-Rossi S, Castrignano E, Anzillotti L, et al. Screening for exogenous androgen anabolic steroids in human hair by liquid chromatography/orbitrap-high resolution mass spectrometry. *Anal Chim Acta*. 2013;793:61-71.
9. Fabresse N, Grassin-Delyle S, Etting I, Alvarez JC. Detection and quantification of 12 anabolic steroids and analogs in human whole blood and 20 in hair using LC-HRMS: application to real cases. *Int J Leg Med*. 2017;131(4):989-999.
10. Kintz P. Hair analysis in forensic toxicology: an update review with a special focus on pitfalls. *Curr Pharm des*. 2017;23(36):5480-5486.
11. Gheddar L, Raul JS, Kintz P. Testing for stanozolol using UPLC-MS-MS and confirmation by UPLC-q-TOF-MS in hair specimens collected from five different anatomical regions. *J Anal Toxicol*. 2020;44(8):834-839.
12. Pianta A, Liniger B, Baumgartner MR. Ethyl glucuronide in scalp and non-head hair: an intra-individual comparison. *Alcohol Alcohol*. 2013;48(3):295-302.
13. Thieme D. Potential and limitations of alternative specimens in doping control. *Bioanalysis*. 2012;4(13):1613-1622.
14. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complimentary matrices: advantages and limitations. *J Pharm Biomed Anal*. 2016;130:220-230.
15. Kintz P, Gheddar L, Ameline A, Arbouche N, Raul JS. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal*. 2020;12(3):316-322.
16. Lehmann S, Thomas A, Schiwy-Bochat KH, et al. Death after misuse of anabolic substances (clenbuterol, stanozolol and metandienone). *Forensic Sci Int*. 2019;303:109925.
17. de Ronde W, Smit DL. Anabolic androgenic steroid abuse in young males. *Endocr Connect*. 2020;9(4):R102-R111.
18. https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf (Accessed 18 January 2021)
19. Kintz P. A new series of hair test results involving anabolic steroids. *Toxicol Anal Clin*. 2017;29:320-324.
20. Cooper GAA, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci Int*. 2012;218(1-3):20-24.

21. Felli M, Martello S, Marsili R, Chiarotti M. Disappearance of cocaine from human hair after abstinence. *Forensic Sci Int*. 2005;154(2-3):96-98.
22. Shen M, Xiang P, Sun Y, Shen B. Disappearance of 6-acetylmorphine, morphine and codeine from human scalp hair after discontinuation of opiate abuse. *Forensic Sci Int*. 2013;227(1-3):64-68.
23. Wang T, Shen B, Wu H, et al. Disappearance of R/S-methamphetamine and R/S-amphetamine from human scalp hair after discontinuation of methamphetamine abuse. *Forensic Sci Int*. 2018;284:153-160.
24. Kintz P, Ameline A, Raul JS. Disappearance of tramadol and THC-COOH in hair after discontinuation of abuse. Two different profiles. *J Anal Toxicol*. 2020;44(1):65-68.
25. Kintz P, Cirimele V, Ludes B. Pharmacological criteria that can affect the detection of doping agents in hair. *Forensic Sci Int*. 2000;107(1-3):325-334.
26. Voegel CD, Hofmann M, Kraemer T, Baumgartner MR, Binz TM. Endogenous steroid hormones in hair: investigations on different hair types, pigmentation effects and correlation to nails. *Steroids*. 2020;154:108547. https://doi.org/10.1016/j.steroids.2019.108547
27. Salomone A, Gerace E, di Corcia D, Alladio E, Vincenti M, Kintz P. Hair analysis can provide additional information in doping and forensic cases involving clostebol. *Drug Test Anal*. 2019;11(1):95-101.
28. Thieme D, Anielski P. Doping, application of hair analysis. In: Kintz P, Salamone A, Vincenti M, eds. *Hair Analysis in Clinical and Forensic Toxicology*. London: Academic Press; 2015:275-299.
29. Klötz F, Garle M, Granath F, Thiblin I. Criminality among individuals testing positive for the presence of anabolic androgenic steroids. *Arch Gen Psychiatry*. 2006;63(11):1274-1279.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Kintz P, Gheddar L, Raul J-S. Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis. *Drug Test Anal*. 2021;13:1445–1451. https://doi.org/10.1002/dta.3020

Article 6 : Testing for anabolic steroids in human nail clippings

Pascal Kintz, Laurie Gheddar, Jean-Sébastien Raul

Journal of Forensic Sciences. 2021. 66(4):1577-1582

Received: 28 January 2021 | Revised: 24 March 2021 | Accepted: 25 March 2021

DOI: 10.1111/1556-4029.14729

JOURNAL OF FORENSIC SCIENCES

CASE REPORT

Toxicology

Testing for anabolic steroids in human nail clippings

Pascal Kintz PhD[1,2] | Laurie Gheddar MS[1] | Jean-Sébastien Raul PhD[1]

[1]Institut de médecine légale, Strasbourg, France

[2]X-Pertise Consulting, Mittelhausbergen, France

Correspondence
Pascal Kintz PhD, Institut de médecine légale, Strasbourg, France.
Email: pascal.kintz@wanadoo.fr

Abstract

Anabolic steroids are synthetic derivatives of testosterone, and their abuse can have numerous health consequences. Identification of this group of drugs has found applications in forensic toxicology, clinical situations, psychiatric disorders, and of course, anti-doping violations. Although anabolic steroids are generally tested in urine and very occasionally in head hair, collectors can face the lack of standard specimens, and therefore, nail clippings can be the unique alternative choice. Although there is no possibility to perform segmental analyses using nail clippings, the window of drug detection is generally much longer in nail when compared to head hair (particularly in male subjects), that is, 3–8 months and 4–12 months for finger and toenail clippings, respectively. A new method was developed, including nail pulverization in a ball mill, sonication for 90 min in methanol, and a combination of liquid–liquid and solid-phase extractions, followed by gas and liquid chromatography coupled to tandem mass spectrometry. To document the application of steroid testing in nail clippings, the authors present 6 authentic cases of abuse, involving stanozolol (7 and 24 pg/mg), nandrolone (6 pg/mg), trenbolone (26, 67, 81, and 89 pg/mg), drostanolone (8 and 11 pg/mg), and testosterone enanthate (14 pg/mg). Given concentrations were always in the low pg/mg range, the use of tandem mass spectrometry appears as a prerequisite.

KEYWORDS
anabolic steroids, clippings, doping, forensic toxicology, hair, nail

Highlights

- Violence and criminality are associated with abuse of steroids, together with serious health consequences.
- Testing for anabolic steroids in nail clippings is a new approach to document repetitive exposures.
- As concentrations of steroids in nail are very low (pg/mg range), tandem mass spectrometry is a prerequisite.
- Nail extraction is sophisticated, time-consuming, and expensive.
- Stanozolol, nandrolone, trenbolone, drostanolone, and testosterone enantate have been identified in 6 authentic cases.

1 | INTRODUCTION

Professional athletes, but also amateurs, can use anabolic steroids because it has been claimed that these drugs increase lean body mass, increase strength, increase aggressiveness, increase endurance, and lead to a shorter recovery time between workouts [1]. Today, anabolic steroids are mostly available on the Internet, at low costs, both as oral and injectable forms (generally as esters). In most cases, these compounds are used in dosages much higher than what is recommended by the manufacturers [2].

Numerous side effects after long-term anabolic steroid abuse have been described and include increase in blood pressure, alterations of the cardiovascular system, liver toxicity, acne, or sexual dysfunction [3]. When abuse is prolonged, subjects can develop psychiatric effects [4–6], and epidemiological studies, both in Europe [7] and in the United States of America [8], have demonstrated that the abuse of anabolic steroids constitutes an increased risk for violence.

Nails can accumulate substances for long periods of time, therefore providing retrospective information with respect to drug(s) use. When compared to blood and urine, nails have several advantages over these standard matrices, including a longer detection window, non-invasive specimen collection, easy storage at room temperature, and easy transport in plastic tubes or envelopes (collection kit). These practical aspects make nails a very interesting alternative matrix for clinical and forensic toxicology [9]. Because of the very low concentrations of drug(s) present in nails and the solid status (versus the liquid one of blood and urine) of this keratinized matrix, analytical methods need to be very sensitive, and sample preparation is critical, in order to extract all the incorporated substance(s) and to avoid destruction or hydrolysis of the compound(s) of interest.

Incorporation of substances into nails mainly occurs through diffusion from the blood supply, which deposits substances to both the germinal matrix and the nail bed on the underside of the nail plate, thus allowing incorporation in both a horizontal and a vertical direction during nail formation [10].

Substance incorporation can be influenced by several factors, including nail-specific, individual-specific, and substance-specific parameters (e.g., growth rate and physical state of the nail, age, and gender of the individual, physicochemical properties of the substance, use of varnish, and remover). For example, seasonal variations in growth rate can lead to variation in incorporation rates of abused substances. While this effect may be minor due to the relatively slow growth rate of nails per se, it can bias the window of detection during interpretation. On the opposite of hair, nails grow at a continuous rate. The average growth rate of fingernails is 3.0 mm per month, while toenails grow at an average rate of 1.1 mm per month [11]. It is generally considered by laboratory personnel that fingernail clippings have a window of detection of 3–8 months. Toenail clippings typically represent what was incorporated 4–8 up to 12 months ago (sometimes even longer). In both specimens, the contribution of the most recent period (germinal matrix) is essential. There is no agreement on the exact time frame nail clipping can represent and each laboratory has its own window of detection [11].

With respect to steroids, data about identification in nails of endogenous compounds, such as dehydroepiandrosterone, androstenedione, and testosterone, are available in the scientific literature [12–15], but there is a critical lack of reports dealing with synthetic derivatives of testosterone.

Because anabolic steroids are seldom tested in forensic laboratories, and even less in alternative specimens such as nail clippings, the present publication presents data obtained from routine casework during the last 18 months.

2 | MATERIALS AND METHOD

2.1 | Materials/standards

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99%–100%, AnalaR NORMAPUR, was purchased from VWR Prolabo (Fontenay-sous-Bois, France). Acetonitrile for LC-MS grade, ethyl acetate, methanol, and dichloromethane were purchased from Merck (Molsheim, France). Sodium hydrogenophosphate and potassium dihydrogenophosphate for the phosphate buffer were also purchased from Merck (Molsheim, France). Steroids and the deuterated standards (testosterone-d_3, nandrolone-d_3, and stanozolol-d_3) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Isolute C_{18} columns were purchased from Interchim (Montlucon, France). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), 2-mercaptoethanol, and ammonium iodide were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

The drugs were diluted to appropriate concentrations with methanol, using a working 1 mg/L solution.

2.2 | Nail specimens

Nail specimens were collected by professional collectors or by a forensic pathologist (case 6) for forensic purposes (drug trafficking, aggressiveness, fatality) or after challenging an adverse analytical finding during an anti-doping control. Just before collection, nails from the hands and feet were cleaned with an isopropanol wipe. Left and right specimens were put together before analysis. However, fingernails and toenails are always separated. After each subject, the nail clippers were washed with alcohol. Demographic data of the 6 subjects are summarized in Table 1. All subjects were male, aged from 24 to 42 years.

In all cases, the nail clippings (from finger or toe) were collected by experienced collectors by cutting them with a nail clipper and sent to the laboratory in a plastic tube. The specimens were stored at room temperature until analysis.

2.3 | Toxicological analyses

Anabolic steroids were tested according to our screening procedure, already published for hair [16]. Briefly, 30 mg of pulverized nail clippings (Retsch MM200 ball mill) was weighted. Internal standards (1 ng of testosterone-d3, nandrolone-d3, and stanozolol-d3) were added together with 1 ml of methanol for a 90-min ultrasonic bath. After centrifugation, the organic phase was collected and evaporated and diluted with 1 M phosphate buffer pH 7.0 (part A). The remaining nail, at the bottom of the tube, was collected and hydrolyzed with 1 ml sodium hydroxide (NaOH) 1 M (part B). Liquid–liquid extractions were operated on parts A and B with 2 ml ethyl acetate. Extracts were combined and evaporated to dryness. The residue was dissolved in 1 ml dichloromethane, which was submitted to solid-phase extraction, using Isolute C_{18} columns. Columns were conditioned with 3 ml

of dichloromethane, and then, the following sequence was used: 1 ml of the specimen in dichloromethane (phase 1), 1 ml of dichloromethane (phase 2), and finally 2 ml dichloromethane/ethyl acetate (3/1) (phase 3). All 3 phases (1, 2, and 3) were collected and evaporated using a SpeedVac Concentrator (Thermo Scientific SPD121P) and solubilized in 30 µl methanol to be divided into two parts: for injection onto a liquid chromatography tandem–mass spectrometry (LC-MS/MS) apparatus (Waters TQS micro) and onto a gas chromatography tandem–mass spectrometry (GC-MS/MS) apparatus (Agilent 7010B) after evaporation and TMS derivatization (MSTFA/NH_4I/2-mercaptoethanol, 1000:2:5) for 20 min at 60°C.

Androstenedione, testosterone, boldenone, tetrahydrogestrinone, metandienone, metenolone, methyltestosterone, nandrolone, chlorodehydromethyltestosterone, oxandrolone, stanozolol, and trenbolone were tested by LC-MS/MS. Dehydroepiandrosterone, dihydrotestosterone, drostanolone, mesterolone, and all esters (testosterone, nandrolone, drostanolone) were tested by GC-MS/MS.

2.4 | Method validation

In order to validate the method, blank fingernails were collected from laboratory staff and verified they test negative.

Given there is no manufacturer of certified reference material for anabolic steroids in nail, it is not possible to establish a true measurement uncertainty of the procedure. In addition, there is no interlaboratory proficiency test for this class of drugs.

A eight-point calibration curve was obtained by spiking blank fingernail at 1–100 pg/mg. The limit of quantification (LOQ) was defined as the first point of linearity. The limit of detection (LOD) was the lowest concentration of drug that could be detected with signal-to-noise ratio superior than 3. The precision and matrix effect were evaluated. For precision, a point at 20 pg/mg was evaluated 8 times. With respect to matrix effect, 12 different fingernail blank specimens were extracted and 10 µl of standard solution of steroids (1 mg/L) was added at the end of extraction. These specimens were compared with 10 µl of standard solution of the drugs in MeOH (1 mg/L).

Typical limits of quantitation, when processing a 30 mg specimen, were 1–5 pg/mg. Matrix effects were always less than 20%.

Individual validations are presented in Table 2.

TABLE 1 Demographic data of the subjects involved in the study

Case	Reason of test	Description of the specimens
1	Doping	Fingernail clippings
2	Aggressiveness	Fingernail + toenail clippings
3	Workplace	Fingernail clippings
4	Doping	Toenail clippings
5	Trafficking	Fingernail clippings
6	Death	Fingernail + toenail clippings

3 | RESULTS AND DISCUSSION

The standard approach to document long-term abuse is based on the identification of the drug(s) in hair, generally collected from the head. In some special cases, head hair can be missing or is not available for sampling. This can happen with shaved or bald subjects or subject with religious belief. Aesthetic issues and heavy cosmetic treatment, such as bleaching or coloring, can also prevent head hair collection, as these treatments will dramatically decrease the concentrations of the drugs already incorporated [17]. Alternatively, nail clippings can be collected but data are very limited for the identification of anabolic steroids in this matrix. To date, to the best of our knowledge, only stanozolol has been identified in fingernail clippings but no concentration was reported [18]. The authors promoted the use of nails in doping control, a possibility that was admitted by Thieme [19]. However, although alternative biological matrices, such as hair or nail, are not forbidden in doping control, any negative analytical testing results obtained from them shall ne be used to counter adverse analytical findings or atypical findings from urine or blood as mentioned paragraph 5.3.6.4 of the International Standard for Laboratories [20]. Of course, nail testing for steroids is not limited to anti-doping control, but has numerous forensic applications, including evidence of drug abuse, documentation of psychiatric disorders with violent behavior, law enforcement, long-term poisoning, drug trafficking, or postmortem investigations. It is a complementary test that allows a retrospective investigation of continuous drug use and can be useful to identify in utero drug exposure [9] or new drug practices, such as in the context of Chemsex [21].

In this study, stanozolol, nandrolone, trenbolone, drostanolone, and testosterone enantate tested positive in the nail clippings. Results are presented Table 3. Obviously, concentrations are low, in the pg/mg range, which is also the range of steroids concentrations in hair [22–25]. In cases 3 and 5, the subjects were using simultaneously 2 anabolic drugs. Typical LC-MS/MS (Figure 1) and GC-MS/

TABLE 2 Individual validation parameters in nail

Parameters	Stanozolol	Nandrolone	Trenbolone	Drostanolone	T enantate
Linearity (pg/mg)	1–100 r^2 = 0.9995	1–100 r^2 = 0.9993	1–100 r^2 = 0.9996	1–100 r^2 = 0.9992	5–100 r^2 = 0.9991
Limit of detection (pg/mg)	0.2	0.4	0.2	0.5	2
Limit of quantitation (pg/mg)	1	1	1	1	5
Precision (20 pg/mg, n = 8), (%)	14.8	16.1	15.8	17.1	17.8

T enantate, testosterone enantate

MS (Figure 2) chromatograms are presented for case 2 and case 6. Given concentrations were always in the low pg/mg range, the use of tandem mass spectrometry appears as a prerequisite. It can be anticipated that the detection of "micro-dosing" of anabolic steroids will be very difficult to document. Indeed, the minimal detectable dose of steroid that can be detected in nail clippings is unknown. The toxicological significance of the measured concentrations is difficult to establish given the lack of suitable reference. It is therefore not possible to interpret the data in terms of dosage and frequency of use. In particular, nothing is known about the incorporation into the keratin matrix after intake and the correlation between dose and nail concentrations.

It is difficult to compare the concentrations in fingernail versus the concentration in toenail, given the limited number of specimens.

TABLE 3 Concentrations of anabolic steroids in various nail clipping specimens

Case	Steroid	Results
1	Stanozolol	Fingernail clippings: 7 pg/mg
2	Trenbolone	Fingernail clippings: 67 pg/mg Toenail clippings: 89 pg/mg
3	Trenbolone Nandrolone	Fingernail clippings: 26 pg/mg Fingernail clippings: 6 pg/mg
4	Testosterone enantate	Toenail clippings: 14 pg/mg
5	Trenbolone Stanozolol	Fingernail clippings: 81 pg/mg Fingernail clippings: 24 pg/mg
6	Drostanolone	Fingernail clippings: 8 pg/mg Toenail clippings: 11 pg/mg

However, in 2 cases toenail clipping concentrations were higher than the ones measured in fingernail clippings. In the unique publication reporting such a comparison for cocaine and opiates [26], concentrations of all the analytes were higher in fingernails than in toenails. However, this may be due to the higher probability of external contamination of fingernails with these drugs. Interestingly, the identification in nail of testosterone enantate, that is, an ester form, is of importance, as this latter compound cannot be endogenous and helps in documenting the abuse of a synthetic compound. This is also achieved in routine in hair [24].

The major interest of using nail when compared to head hair is the extension of the window of detection. Using head hair, the window of detection is typically linked to the length of the submitted specimen; that is, each cm represents the growth of 1 month (e.g., 3 cm = 3 months). It can be easily observed that the male population who uses anabolic steroids has generally short hair, or even very short hair. Therefore, the window of steroid detection using head hair is generally 1–3 months before hair collection. Using nail, standard windows of detection are generally up to 8 or even 12 months before collection, depending on what is used by the different laboratories. Nail testing provides some additional advantages over hair testing for drug(s). Firstly, when hair is not available in suitable amount (e.g., in case of alopecia, during chemotherapy, or during the first weeks or months after birth), nail analysis can be an important tool to gain retrospective information on drug exposure. Secondly, in contrast to hair, nail does not contain melanin. Since drug incorporation may be influenced by melanin concentrations, hair pigmentation can be an important source of bias when interpreting drug concentrations or to avoid ethnical discriminations. Thirdly, nail grows slower than hair,

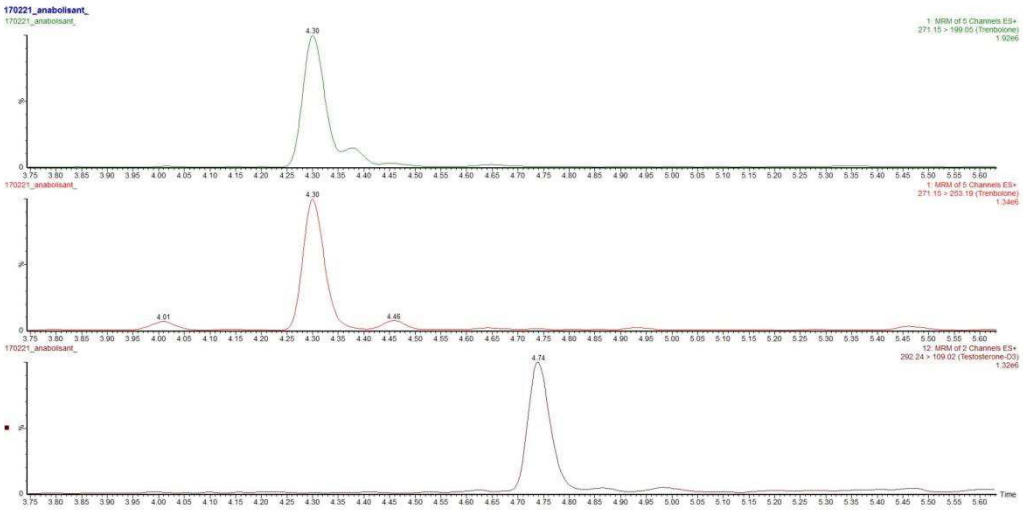


FIGURE 1 Chromatogram obtained after extraction of the fingernail clippings in case 3. Trenbolone concentration was 26 pg/mg. From top to bottom: 2 transitions for trenbolone (m/z 271.1 > 199.0 and 271.1 > 253.2) and 1 transition for testosterone-d_3 (IS, m/z 292.2 > 109.0) [Colour figure can be viewed at wileyonlinelibrary.com]

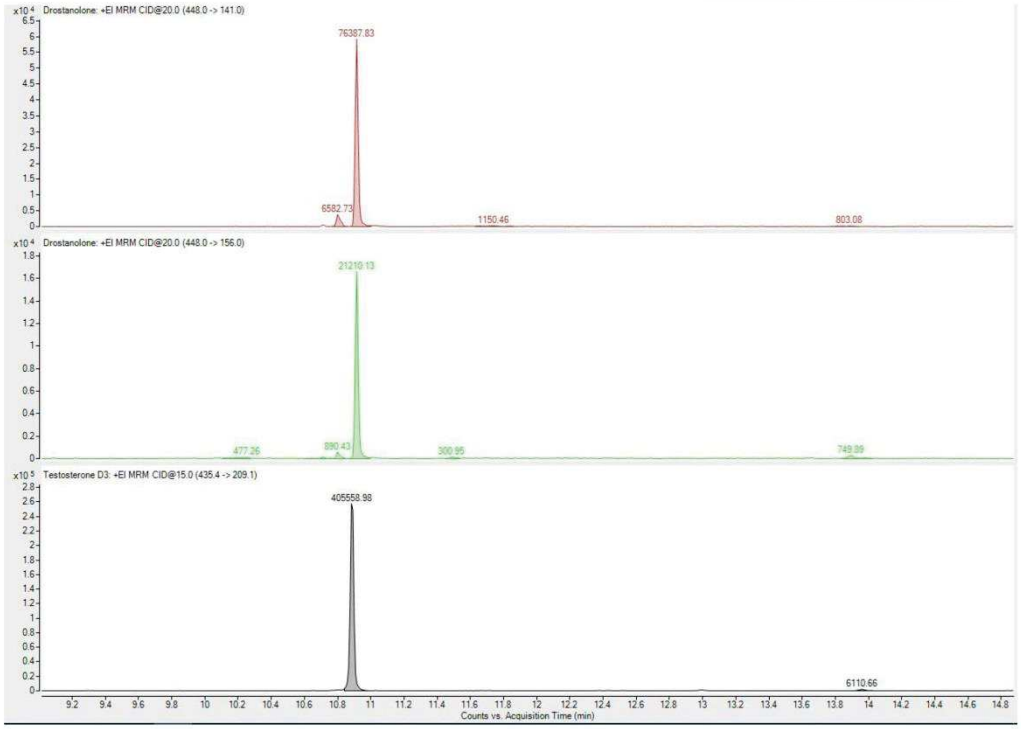


FIGURE 2 Chromatogram obtained after extraction of the fingernail clippings in case 6. Drostanolone concentration was 8 pg/mg. From top to bottom: 2 transitions for drostanolone (m/z 448.0 > 141.0 and 448.0 > 156.0) and 1 transition for testosterone-d_3 (IS, m/z 435.4 > 209.1) [Colour figure can be viewed at wileyonlinelibrary.com]

which provides the opportunity to detect smaller exposure levels and/or to investigate longer periods of time. This can be of paramount importance as this is a limitation of anabolic steroid investigations in human hair [24]. Fourthly, while hair is characterized by a cyclic growth rate with different stages, nails grow at a constant rate, which facilitates the interpretation of results. Finally, compared to hair sampling, nail clipping collection can be aesthetically more acceptable for some people, easier to collect, and less intrusive.

Taken together, these advantages underline the potential of nails as an interesting and useful matrix for the retrospective detection of steroids; pending criteria and data for interpretation will be available in the scientific literature. This is what was requested from the clients and has proven the interest of testing for synthetic steroids in human nail clippings, both with anti-doping and forensic applications.

In addition, according to a recent publication [27] the combination of nail and hair testing presents some forensic interests in extensively decomposed bodies, but the authors have indicated that it is challenging to determine the time, and even more, the dose of exposure(s). However, at this time, there is no publication dealing with the simultaneous identification of anabolic steroids in both hair and nail specimens.

4 | CONCLUSION

The detection of anabolic steroids in nail has been seldom described in the literature. Although there is no possibility to perform segmental analyses with nail clippings, testing for anabolic steroids in nail specimens has 2 major advantages: 1. Nail can replace hair when this specimen cannot be collected; and 2. the window of drug detection is generally much longer in nail clippings when compared to head hair, particularly in male athlete presenting short head hair. This study has provided evidence that nails have a relatively long window of detection and could potentially be used to retrieve information about long-term consumption of anabolic steroids, even if relatively low concentrations are detected in nails.

ORCID

Pascal Kintz https://orcid.org/0000-0002-4174-5362
Laurie Gheddar https://orcid.org/0000-0002-7804-3946

REFERENCES

1. Cadwallader AB, Lim CS, Rollins DE, Botrè F. The androgen receptor and its use in biological assays: looking toward effect-based

testing and its applications. J Anal Toxicol. 2011;35(9):594–607. https://doi.org/10.1093/anatox/35.9.594.
2. Muscular Development. Anabolic steroids – What is the right dose? https://www.musculardevelopment.com/news/the-mcgough-report/15139-anabolic-steroids-what-is-the-right-dose.html#.YECfky17RTY. Accessed 4 Mar 2021.
3. Van Amsterdam J, Opperhuizen A, Hartgens F. Adverse health effect of anabolic-androgenic steroids. Regul Toxicol Pharmacol. 2010;57(1):117–23. https://doi.org/10.1016/j.yrtph.2010.02.001.
4. Trenton AJ, Currier GW. Behavioural manifestations of anabolic steroid use. CNS Drugs. 2005;19(7):571–95. https://doi.org/10.2165/00023210-200519070-00002.
5. Pope HG, Kouri EM, Hudson JI. Effects of supraphysiologic doses of testosterone on mood and aggression in normal men: a randomized controlled trial. Arch Gen Psychiatry. 2000;57(2):133–40. https://doi.org/10.1001/archpsyc.57.2.133.
6. Khoodoruth MAS, Khan AA. Anabolic steroids-induced delirium. Medicine. 2020;99(33):e21639. https://doi.org/10.1097/MD.0000000000021639.
7. Lood Y, Eklund A, Garle M, Ahlner J. Anabolic androgenic steroids in police cases in Sweden 1999–2009. Forensic Sci Int. 2012;219(1–3):199–204. https://doi.org/10.1016/j.forsciint.2012.01.004.
8. Beaver KM, Vaughn MG, Delisi M, Wright JP. Anabolic-androgenic steroid use and involvement in violent behaviour in a nationally representative sample of young adult males in the United States. Am J Public Health. 2008;98(12):2185–7. https://doi.org/10.2105/AJPH.2008.137018.
9. Solimini R, Minutillo A, Kyriakou C, Pichini S, Pacifici R, Busardo FP. Nails in forensic toxicology: an update. Curr Pharm Des. 2017;23(36):5468–79. https://doi.org/10.2174/1381612823666170704123126.
10. Krumbiegel F, Hastedt M, Westendorf L, Niebel A, Methling M, Parr MK, et al. The use of nails as an alternative matrix for the long-term detection of previous drug intake: validation of sensitive UHPLC-MS/MS methods for the quantification of 76 substances and comparison of analytical results for drugs in nail and hair samples. Forensic Sci Med Pathol. 2016;12(4):416–34. https://doi.org/10.1007/s12024-016-9801-1.
11. Baumgartner MR. Nails: an adequate alternative matrix in forensic toxicology for drug analysis. Bioanalysis. 2014;6(17):2189–91. https://doi.org/10.4155/bio.14.165.
12. Khelil MB, Tegethoff M, Meinlschmidt G, Jamey C, Ludes B, Raul JS. Simultaneous measurement of endogenous cortisol, cortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulphate in nails by use of UPLC-MS/MS. Anal Bioanal Chem. 2011;401(4):1153–62. https://doi.org/10.1007/s00216-011-5172-3.
13. Voegel CD, Hofmann M, Kraemer T, Baumgartner MR, Binz TM. Endogenous steroid hormones in hair: investigations on different hair types, pigmentation effects and correlation to nails. Steroids. 2020;154:108547. https://doi.org/10.1016/j.steroids.2019.108547.
14. Voegel CD, la Marca-Ghaemmaghami P, Ehlert U, Baumgartner MR, Kraemer T, Binz TM. Steroid profiling in nails using liquid chromatography-tandem mass spectrometry. Steroids. 2018;140:144–50. https://doi.org/10.1016/j.steroids.2018.09.015.
15. Gugoasa LA, Stefan-van Staden RI. Advanced methods for the analysis of testosterone. Curr Med Chem. 2018;25(33):4037–49. https://doi.org/10.2174/0929867324666170724102602.
16. Kintz P, Gheddar L, Raul JS. Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis. Drug Test Anal. 2021;https://doi.org/10.1002/dta.3020.
17. Kintz P. Hair analysis in forensic toxicology: an update review with a special focus on pitfalls. Curr Pharm Des. 2017;23(36):5480–6. https://doi.org/10.2174/1381612823666170929155628.
18. Brown HG, Perrett D. Detection of doping in sports: detecting anabolic-androgenic steroids in human fingernail clippings. Med Leg J. 2011;79(2):67–9. https://doi.org/10.1258/mlj.2011.011013.
19. Thieme D. Potential and limitations of alternative specimens in doping control. Bioanalysis. 2012;4(13):1613–22. https://doi.org/10.4155/bio.12.150.
20. World Anti-doping Agency. World anti-doping code – International standard: Laboratories. https://www.wada-ama.org/sites/default/files/resources/files/isl_2021.pdf. Accessed 24 Jan 2021.
21. Busardò FP, Gottardi M, Pacifici R, Varì MR, Tini A, Volpe AR, et al. Nails analysis for drugs used in the context of Chemsex: a pilot study. J Anal Toxicol. 2020;44(1):69–74. https://doi.org/10.1093/jat/bkz009.
22. Deng XS, Kurosu A, Pounder DJ. Detection of anabolic steroids in head hair. J Forensic Sci. 1999;44(2):343–6.
23. Salomone A, Gerace E, Di Corcia D, Alladio E, Vincenti M, Kintz P. Hair analysis can provide additional information in doping and forensic cases involving clostebol. Drug Test Anal. 2019;11(1):95–101. https://doi.org/10.1002/dta.2469.
24. Kintz P, Gheddar L, Ameline A, Arbouche N, Raul JS. Hair testing for doping agents. What is known and what remains to do. Drug Test Anal. 2020;12(3):316–22. https://doi.org/10.1002/dta.2766.
25. Thieme D, Anielski P. Doping, applications of hair analysis. In: Kintz P, Salamone A, Vincenti M, editors. Hair analysis in clinical and forensic toxicology. London, U.K.: Academic Press; 2015. p. 275–99.
26. Engelhart DA, Jenkins AJ. Detection of cocaine analytes and opiates in nails from post-mortem cases. J Anal Toxicol. 2002;26(7):489–92. https://doi.org/10.1093/jat/26.7.489.
27. Wiart J-F, Hakim F, Andry A, Eiden C, Drevin G, Lelièvre B, et al. Pitfalls of toxicological investigations in hair, bones and nails in extensively decomposed bodies: illustration with two cases. Int J Leg Med. 2020;134(4):1339–44. https://doi.org/10.1007/s00414-020-02267-3.

How to cite this article: Kintz P, Gheddar L, Raul J-S. Testing for anabolic steroids in human nail clippings. *J Forensic Sci*. 2021;66:1577–1582. https://doi.org/10.1111/1556-4029.14729

Article 7 : In a case of death involving steroids, hair testing is more informative than blood or urine testing

Pascal Kintz, Laurie Gheddar, Adeline Blanchot, Alice Ameline, Jean-Sébastien Raul

Journal of Analytical Toxicology. 2021;45(8):829-834.

Journal of Analytical Toxicology, 2021;45:829–834
doi:https://doi.org/10.1093/jat/bkab048
Advance Access Publication Date: 15 May 2021
Special Issue

Special Issue

In a Case of Death Involving Steroids, Hair Testing is More Informative than Blood or Urine Testing

Pascal Kintz[1,2,*], Laurie Gheddar[2], Adeline Blanchot[2], Alice Ameline[2] and Jean-Sébastien Raul[2]

[1]X-Pertise Consulting, 42 rue principale, Mittelhausbergen 67206, France and [2]Institut de Médecine Légale, 11 rue Humann, Strasbourg 67000, France

*Author to whom correspondence should be addressed. Email: pascal.kintz@wanadoo.fr

Abstract

A 59-year-old male was found dead at home, with two empty vials of an oily preparation obtained from a manufacturer from East Europe. There was no label on the vial. The subject was a former weightlifter, also known as an anabolic steroids abuser. The local prosecutor ordered a body examination, which was unremarkable, and allowed collecting femoral blood, urine and scalp hair (6 cm, brown). He was treated for cardiac insufficiency with quinidine. Biological specimens were submitted not only to standard toxicological analyses including a screening with liquid chromatography (LC)–quadrupole time of flight, but also to a specific LC–tandem mass spectrometry method for anabolic steroids testing. Ethanol was not found in both blood and urine. Quinidine blood concentration (791 ng/mL) was therapeutic. No drug of abuse was identified. In blood, testosterone was less that 1 ng/mL and no other steroid was identified. In urine, testosterone/epitestosterone was 1.56 and boldenone was present at a concentration of 9 ng/mL. The hair test results, performed on the whole length, demonstrated repetitive steroids abuse, including not only testosterone (140 pg/mg), testosterone propionate (605 pg/mg) and testosterone decanoate (249 pg/mg), but also boldenone (160 pg/mg), trenbolone (143 pg/mg) and metandienone (60 pg/mg). Since forensic laboratories have limited access to steroid urinary metabolite reference material due to specific regulations (to avoid testing athletes before anti-doping verifications), hair analyses seem to be the best approach to document anabolic agents abuse. Indeed, in hair, the target drug is the parent compound; in addition, when compared to blood or urine, this matrix has a much larger window of detection. The pathologist concluded cardiac insufficiency in a context involving repetitive abuse of anabolic drugs. This case indicates that more attention should be paid to anabolic steroids, in a context of sudden cardiac death.

Introduction

Anabolic steroids are synthetic derivatives of the endogenous testosterone. These drugs are abused because it has been claimed that they increase lean body mass, increase strength, increase aggressiveness, increase endurance and lead to a shorter recovery time between workouts (1). When dealing with anabolic steroids, the immediate thought is to associate their use with an anti-doping violation. Indeed, in 2019, the World Anti-Doping Agency (WADA) reported in total 4,180 adverse analytical findings, including 1,825 for anabolic agents, corresponding to 44% of all the Anti-Doping Administration and Management System's reported findings (2). Most popular anabolic agents are stanozolol, drostanolone, 19-norandrosterone (nandrolone), boldenone and metandienone.

However, abuse of anabolic steroids has numerous health and forensic consequences. Today, anabolic steroids are mostly available on the Internet, at low costs, both in oral and injectable forms (generally as esters). Very few preparations are creams or gels. In most cases, these compounds are used in dosages much higher than what is recommended by the manufacturers. Therapeutic uses of anabolic steroids are very limited. Numerous side effects have been described after long-term anabolic steroids abuse, which include increase in blood pressure, alterations to the cardiovascular system, liver toxicity, acne, hirsutism, gynecomastia, atrophic testes and sexual dysfunction (3). When abuse is prolonged, subjects can develop psychiatric effects, which include antisocial lifestyle, disinhibition, frustration, psychosis, low self-confidence, childhood conduct disorders, delirium, depression and aggressiveness (4, 5). In the past years, it was demonstrated that the abuse of anabolic steroids constitutes an increased risk for violence and that there is a link between their use and criminality (6).

The prolonged abuse of anabolic steroids is associated with cardiovascular diseases, including cardiac hypertrophy, myocardial infarction and sudden cardiac death (7). Pathological changes in steroid abusers consist of various levels of interstitial and perivascular fibrosis, fibroadipous metaplasia and perineural fibrosis within the myocardium (8). In a series of 24 deaths involving anabolic–androgenic steroids, Darke et al. (9) noticed extensive cardiovascular disease, including 30.4% of left ventricular hypertrophy and 26.1% of moderate-to-severe narrowing of the coronary arteries.

Very few death cases after misuse of anabolic substances and subsequent toxicological analyses have been described in the literature (10–13). Because anabolic steroids are seldom tested in forensic laboratories and even less in hair specimens, the present observation, documented with toxicological analyses in blood, urine and hair, allows establishing a link between a cardiac death and addiction to this class of agents.

Case Report

A 59-year-old male (1.87 m tall, weighing 129 kg) was found dead at home in dorsal decubitus position, with two empty vials of an oily preparation obtained from a manufacturer from East Europe near the body, on a table. There was no label on the vial. Syringes and needles were present on the scene. The subject was a former weightlifter and was known as an anabolic steroids abuser. The exact regimen of the subject is unclear and the remaining steroids were not submitted to analyses to verify both the composition and the purity.

The local prosecutor ordered a body examination, which was unremarkable (except for dark liquid foam on the nose and the mouth). It allowed collecting femoral blood, urine and scalp hair (6 cm, brown). The subject was treated for cardiac insufficiency with quinidine. Given the circumstances of death and the lack of criminal evidence (confirmed by a whole-body scanner), the prosecutor did not ask the medico-legal team to perform an autopsy. As a consequence, no anatomic pathology test was possible. The prosecutor considered toxicological investigations suitable only to determine the cause of death.

All specimens but hair were stored at +4°C until analyses.

Materials and Methods

Chemicals and reference material

Ammonium formate, 99%, was provided by Alfa Aesar (Schiltigheim, France). Formic acid, 99–100%, AnalaR NORMAPUR was purchased from VWR Prolabo (Fontenay-sous-Bois, France). Acetonitrile for liquid chromatography (LC)–mass spectrometry (MS) grade, ethyl acetate, methanol, diethyl ether, hexane, isoamyl alcohol and dichloromethane were purchased from Merck (Molsheim, France). Steroids and the deuterated standards (testosterone-d_3, nandrolone-d_3 and stanozolol-d_3) were purchased from Cayman Chemical (Ann Arbor, MI, USA). ISOLUTE C_{18} columns were purchased from Interchim (Montlucon, France). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), 2-mercaptoethanol, ammonium iodide, β-glucuronidase and arylsulfatase were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France).

Blood and urine analysis

Blood and urine were subjected to the standard toxicological screening. Ethanol was tested in blood by headspace gas chromatography (GC)–flame ionization detector (FID) on a Perkin Elmer system (TurboMatrix 40 and Clarus 580) using a standard validated procedure. Carboxyhemoglobin (HbCO) was determined with a Radiometer ABL80. Cyanides were tested by headspace GC–MS on a Thermo system (Focus GC and DSQII) using a standard validated procedure. Enzyme-linked immunosorbent assay (ELISA) tests for pharmaceuticals and drugs of abuse were achieved using NeoGen kits using the recommendations of the manufacturer. A comprehensive screening for pharmaceuticals and drugs of abuse, including New Psychoactive Substances (NPS) (about 200 compounds), was performed using LC–tandem mass spectrometry (MS-MS) (Waters Xevo TQ-S micro) and LC–High Resolution Mass Spectrometry (HRMS) (Waters Xevo G2 QToF). Due to the nature of the case, anabolic steroids were specifically tested by a LC–MS-MS method, using a validated procedure (13). Briefly, steroids were extracted from 1 mL of blood or hydrolyzed urine (with β-glucuronidase and arylsulfatase) in the presence of 10 ng of testosterone-d_3 used as internal standard, with 1 mL of borate buffer at pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 30 µL of ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters BEH C18 column (100 × 2.1 mm × 1.7 µm) maintained at 50°C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in LC–MS-grade acetonitrile (mobile phase B) at flow rate of 0.4 mL/min. The initial gradient was 87% mobile phase A and the final gradient, at 15 min, was 5% mobile phase A. An injection volume of 2 µL was used in all cases. Ionization was achieved using electrospray in the positive ionization mode (ES+). MassLynx 4.1 software was used for quantification. Mass spectrometric parameters are presented in Table I.

Linearity was verified in blood and urine for concentrations ranging from 1 to 100 ng/mL. The limits of detection, with an signal to noise ratio of 3, varied from 0.1 to 0.5 ng/mL, depending on the drug. Quality control (QC) samples (5 ng/mL and 50 ng/mL), analyzed in duplicate in six independent experimental assays, were used for determining a coefficient of variation (CV) for precision. These CVs were lower than 20%. Urine was tested using the blood calibration.

Hair analysis

Because the nature of the anabolic steroids used by the victim was unknown, a screening procedure, already published (14), was used. Briefly, 29 mg of finely cut hair, previously decontaminated by two dichloromethane baths (5 mL, 2 min), was weighted. Internal standards (1 ng of testosterone-d_3, nandrolone-d_3 and stanozolol-d_3)

Table I. Mass Spectrometric Data for the Tested Compounds (A) with LC–MS-MS and (B) with GC–MS-MS

A

Drug	Transitions *m/z*	Collision energy eV	Cone voltage V
Boldenone	287.2 > 121.1	20	24
	287.2 > 135.1		
Metandienone	301.2 > 121.1	22	22
	301.2 > 149.1		
Trenbolone	271.2 > 199.1	20	46
	271.2 > 253.2		
Testosterone-d_3	292.2 > 109.0	22	30

B

Drug	Transitions *m/z*	Collision energy eV
Testosterone propionate	416.3 > 209.0	10
	416.3 > 401.01	
Testosterone decanoate	514.3 > 209.0	10
	514.3 > 499.01	
Testosterone-d_3	435.4 > 209.1	15

were added together with 1 mL of methanol for a 90-min ultrasonic bath at room temperature. After centrifugation, the organic phase was collected and evaporated and diluted with phosphate buffer at pH 7.0 (Part A). The remaining hair, at the bottom of the tube, was collected and hydrolyzed with 1 mL of 1 M NaOH (Part B). Liquid–liquid extractions were operated on Parts A and B with 2 mL of ethyl acetate. Extracts were combined and evaporated to dryness. The residue was dissolved in 1 mL of dichloromethane, which was submitted to solid-phase extraction, using ISOLUTE C_{18} columns. Columns were conditioned with 3 mL of dichloromethane and then the following sequence was used: 1 mL of the specimen in dichloromethane (Phase 1), 1 mL of dichloromethane (Phase 2) and finally 2 mL of dichloromethane/ethyl acetate (3/1) (Phase 3). All three phases (1, 2 and 3) were collected and evaporated using a SpeedVac Concentrator (Thermo Scientific SPD121P) and solubilized in 30 µL of methanol to be divided into two parts: for injection onto LC–MS-MS apparatus (Waters TQS micro) and onto GC–MS-MS apparatus (Agilent 7010B) after evaporation and trimethyl silyl (TMS) derivatization (MSTFA/NH_4I/2-mercaptoethanol, 1000:2:5) for 20 min at 60°C.

Androstenedione, testosterone, boldenone, tetrahydrogestrinone, metandienone, metenolone, methyltestosterone, nandrolone, chlorodehydromethyltestosterone, oxandrolone, stanozolol and trenbolone were tested by LC–MS-MS. Dehydroepiandrosterone, dihydrotestosterone, drostanolone, mesterolone and all esters (testosterone, nandrolone and drostanolone) were tested by GC–MS-MS.

Linearity was verified in hair for concentrations ranging from 5 to 200 pg/mg. The limits of detection, with an S/N ratio of 3, were in the range 0.5–5 pg/mg, depending on the drug. QC samples (10 pg/mg and 50 pg/mg), analyzed in duplicate in six independent experimental assays, were used for determination a CV for precision. These CVs were lower than 20%.

Table II. Toxicological Results after Screening for Anabolic Steroids in Various Biological Matrices

Compound	Blood	Urine	Hair (6 cm)
Dihydrotestosterone	<1 ng/mL	1.3 ng/mL	115 pg/mg
Dehydroepiandrosterone	<1 ng/mL	1.4 ng/mL	135 pg/mg
Testosterone	<1 ng/mL	1.4 ng/mL	140 pg/mg
Boldenone	ND	9 ng/mL	160 pg/mg
Metandienone	ND	ND	60 pg/mg
Trenbolone	ND	ND	143 pg/mg
Testosterone propionate	ND	ND	605 pg/mg
Testosterone decanoate	ND	ND	249 pg/mg

ND: Not detected.

Results and Discussion

Blood and urine tested negative for ethanol. HbCO was 1.2% and cyanides were at physiological concentrations (<80 ng/mL). ELISA screenings were negative for pharmaceuticals and drugs of abuse. The screening of the femoral blood revealed the presence of quinidine at a concentration of 791 ng/mL, consistent with the medical treatment of the subject. Quinidine is a Class 1a antiarrhythmic drug that was found at therapeutic concentration (15). No other pharmaceutical substance was identified. Specimens were free of drugs of abuse.

A summary of the findings for steroids is presented in Table II. In blood, only endogenous steroids were found, i.e., testosterone (<1 ng/mL), dihydrotestosterone (<1 ng/mL) and dehydroepiandrosterone (<1 ng/mL). These concentrations were physiological. No synthetic anabolic steroid was identified. In urine, after subsequent hydrolysis with β-glucuronidase and arylsulfatase, endogenous steroids were again detected: testosterone (1.4 ng/mL), epitestosterone (0.9 ng/mL), dihydrotestosterone (1.3 ng/mL) and dehydroepiandrosterone (1.4 ng/mL). The T/E ratio was 1.56—a value that is not considered by WADA as indicative of testosterone derivatives abuse, suggesting that the victim did not recently inject testosterone or testosterone esters. The screening for steroids indicated the presence of boldenone at a concentration of 9 ng/mL. This is a significant concentration, which demonstrates exposure during the previous 2–4 days (16). Although numerous metabolites of boldenone have been identified in human urine (16), including long-term metabolites (17), these compounds are not available for non-WADA-accredited laboratories as reference material. Indeed, there is an agreement between WADA and the manufacturers to not provide reference material to third parties that could favor survey of athletes before anti-doping tests. Only tedious and time-consuming paperwork and a delay of more than 9 months allow obtaining the standards of steroid metabolites, when available. This is the reason why only the parent compound was tested. Although traces of boldenone can be found in human urine as a consequence of natural secretion (15), the measured concentration in the decedent urine is too high to account for this situation. No other synthetic anabolic steroid was identified. Finally, the whole length of hair lock (6 cm) without segmentation was submitted for analysis. This was due to the limited amount of hair collected during the body examination, that is, less than 30 mg, which is the amount used during the validation process, allowing the performance of detection. Indeed, the whole sample of hair only weighed 29 mg, which prevented any

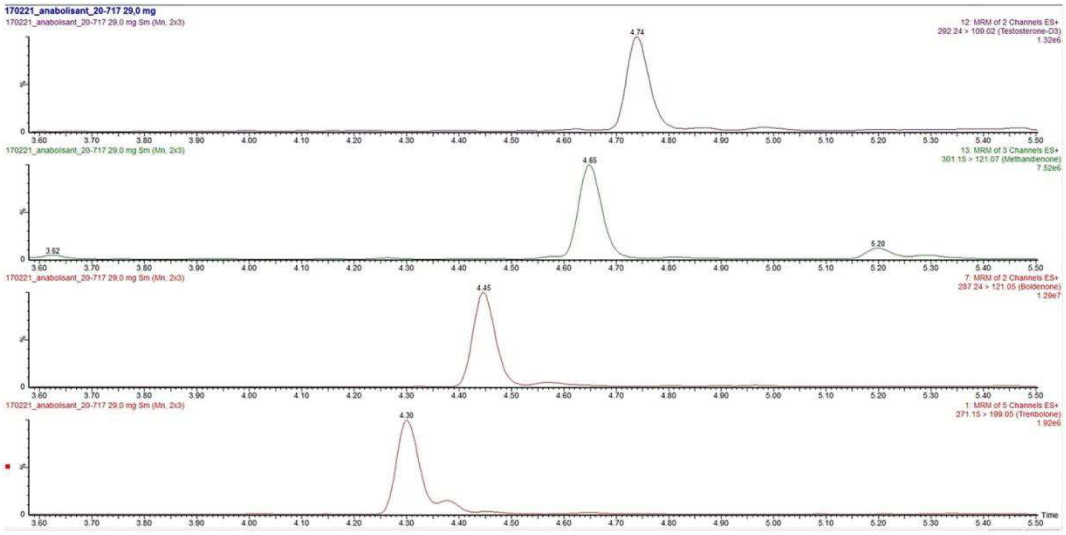


Figure 1. LC–MS-MS chromatogram obtained after extraction of the hair of the victim. From top to bottom: Quantitative transitions for testosterone-d_3, metandienone (60 pg/mg), boldenone (160 pg/mg) and trenbolone (143 pg/mg).

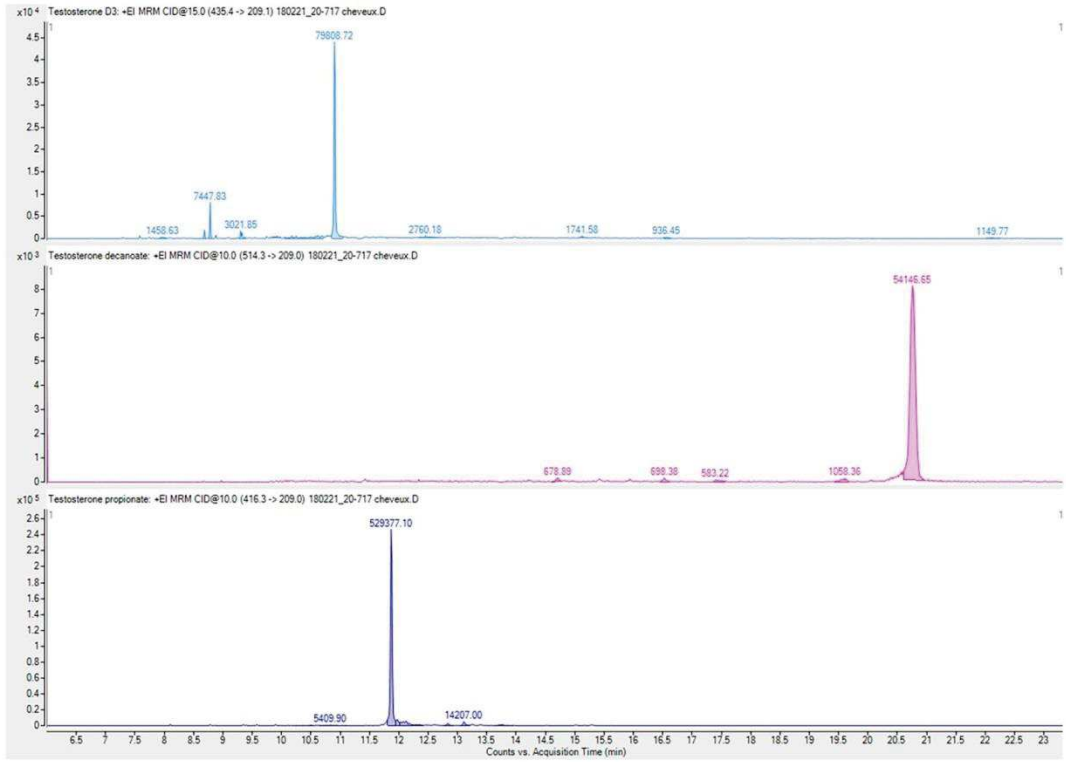


Figure 2. GC–MS-MS chromatogram obtained after extraction of the hair of the victim. From top to bottom: Quantitative transitions for testosterone-d_3, testosterone decanoate (249 pg/mg) and testosterone propionate (605 pg/mg).

segmentation, as there was a need to be very sensitive, because anabolic steroids are poorly incorporated into hair. The authors can accept that the definitive demonstration of repetitive and long-term abuse is based on the presence of a drug in several segments, but this was simply not possible to achieve in this case. On the other hand, it has been published in the past that a single dose of 50 mg of nandrolone undecanoate (18) or nandrolone decanoate (19) or of various testosterone esters, including testosterone enanthate, undecanoate and propionate is not detectable in hair (19). Given the concentrations measured in hair are significant, it is acceptable, on a scientific level, to consider that they are the consequence of substantial adminstration of steroids. Indeed, the hair test results are highly indicative of long-term steroids abuse, with the identification of several drugs, including not only testosterone (140 pg/mg), testosterone propionate (605 pg/mg) and testosterone decanoate (249 pg/mg), but also boldenone (160 pg/mg), trenbolone (143 pg/mg) and metandienone (60 pg/mg). Although most of the steroids were analyzed by LC–MS-MS (Figure 1), better results for testosterone esters were obtained using GC–MS-MS (Figure 2). This is probably due to weak ionization of the non-polar long chain of the esters.

Testosterone concentration in hair was higher than the physiological concentration range, generally lower than 10 pg/mg (20, 21). This is highly indicative of testosterone or testosterone esters abuse. Concentrations of dihydrotestosterone (115 pg/mg) and dehydroepiandrosterone (135 pg/mg) were also higher than the normal physiological range (22, 23). Concentrations of the other three synthetic steroids (boldenone, trenbolone and metandienone) can be considered important when compared to the existing literature (24–26). This suggests that the decedent was a regular anabolic steroids abuser. Despite a normal T/E ratio in urine, testosterone abuse by the subject can be unambiguously demonstrated by the presence of two testosterone esters, propionate and decanoate, in his hair, which cannot be considered to correspond to endogenous compounds. Testing for esters of testosterone is an extra attribute of hair when compared to urine. Indeed, it can be sometimes complicated to document exogenous testosterone administration, particularly at low concentrations. The discrimination of endogenous versus exogenous testosterone is achieved by isotope ratio MS (^{13}C vs. ^{12}C), a procedure often challenged during an anti-doping violation. On the contrary, the unique identification of an ester is a definitive proof of exogenous agent administration.

The presence of several different steroids can be explained by a special regimen, a pattern named cycling, which is use of steroids over a specific period of time followed by an abstinence period and again followed by a new period of use (27). In addition, as each anabolic steroid has its specific presentation (oral or injectable) and its specific pharmacology with more or less androgen effects and increase in muscle building, a mix between several agents is recommended to enhance the benefit of the administration. For example, metandienone (or methandrostenolone) is an orally effective anabolic steroid, first marketed by the Ciba Pharmaceutical Company in 1958. Production has decreased sharply in recent years due to its strong liver toxicity and the release of more effective testosterone esters in the market. However, these esters have to be injected intramuscularly but they are not toxic to the liver because they do not have a 17-alpha methyl group. As a consequence, testosterone propionate and testosterone decanoate do have therapeutic indications. Their main drawback is that they get inactive rapidly and therefore need to be administered frequently (for testosterone propionate) or must be taken in high doses to be effective (for testosterone decanoate). Trenbolone can be purchased as parent drug (pills) for oral administration or as trenbolone acetate, hexahydrobenzyl carbonate or enanthate for intramuscular injection. Its main use, in ester form, is in veterinary medicine in livestock to increase muscle growth and appetite. There is no medical use for boldenone in humans but it can be abused by athletes as it stimulates protein synthesis and erythropoietin release in the kidneys. Boldenone is only available for intramuscular injection as boldenone undecylenate. Its activity is mainly anabolic with a limited androgenic effect.

In addition to enabling the establishment of the pattern of abuse over months, hair has several other advantages as it can confirm past exposure in contrast to blood and urine. This is particularly useful when a drug becomes harmful when administered on a long-term basis. In the present case, the identification of several steroids in hair was of paramount importance to determine the cause of death. The negative findings in blood are adequately completed by the positive results in hair. More importantly, given steroids are mostly excreted in urine as metabolites, which are quite impossible to obtain from manufacturers as reference material (see supra), hair seems to be a suitable alternative in forensic toxicology, where the parent drug is the target analyte (and not its metabolites) (28). It is accepted by the scientific community that the parent drug is better incorporated in hair when compared to its metabolite(s), probably because the parent drug is less polar than the metabolite(s). In addition, reference material for parent drugs is always much more easier to purchase in comparison to its metabolite(s).

In male subjects, long hair can be missing, and it is frequent that only some millimeters of head hair are available for investigations. This is a limitation of the potential for documenting long-term drug administrations. Alternatively, it is recommended, in such a situation, to collect body hair or nail clippings—two matrices that were recently suggested to be suitable for anabolic steroids identification—allowing a dramatic increase in the window of detection (14, 29).

In the absence of traumatic cause of death, and given the toxicological investigations, the pathologist concluded cardiac insufficiency in a context involving repetitive abuse of anabolic drugs.

Conclusion

Toxicologists and forensic pathologists should be aware of possible abuse of steroids proposed as image- and performance-enhancing drugs to promote body changes and nice appearance (30). Steroid agents can also be used to lose weight or to enhance athletic performance, even in subjects older than 50 years of age. The possibility of cardiac insufficiency in a steroid-abusing subject is a forensic situation that has to be documented by a comprehensive toxicological screening, targeting all the performance-enhancing drugs in the market. Monitoring all the drugs with a cardiac tropism, even indirectly, is of importance when death is unexpected. This is particularly necessary as only very few postmortem cases dealing with steroids are available in the literature, although these drugs are abused by millions of subjects.

References

1. Cadwallader, A.B., Lim, C.S., Rollins, D.E., Botrè, F. (2011) The androgen receptor and its use in biological assays: looking toward effect-based testing and its applications. *Journal of Analytical Toxicology*, **35**, 594–607.
2. World Anti-Doping Agency. https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf (Accessed Mar 2, 2021).

3. Van Amsterdam, J., Opperhuizen, A., Hartgens, F. (2010) Adverse health effect of anabolic-androgenic steroids. *Regulatory Toxicology Pharmacology*, **57**, 117–123.
4. Trenton, A.J., Currier, G.W. (2005) Behavioural manifestations of anabolic steroid use. *CNS Drugs*, **19**, 571–595.
5. Khoodoruth, M.A.S., Khan, A.A. (2020) Anabolic steroids-induced delirium. *Medicine*, **99**, e21639.
6. Beaver, K.M., Vaughn, M.G., Delisi, M., Wright, J.P. (2008) Anabolic-androgenic steroid use and involvement in violent behaviour in a nationally representative sample of young adult males in the United States. *American Journal of Public Health*, **98**, 2185–2187.
7. Frati, P., Busardo, F.P., Cipolloni, L., De Dominicis, E., Fineschi, V. (2015) Anabolic androgenic steroids (AAS) related deaths: autoptic, histopathological and toxicological findings. *Current Neuropharmacology*, **13**, 146–159.
8. Lusetti, M., Licata, M., Silingardi, E., Bonetti, L.R., Palmiere, C. (2015) Pathological changes in anabolic androgenic steroid users. *Journal of Forensic and Legal Medicine*, **33**, 101–104.
9. Darke, S., Torok, M., Duflou, J. (2014) Sudden or natural deaths involving anabolic-androgenic steroids. *Journal of Forensic Sciences*, **59**, 1025–1028.
10. Fineschi, V., Riezzo, I., Centini, F., Silingardi, E., Licata, M., Beduschi, G., et al. (2007) Sudden cardiac death during anabolic steroid abuse: morphologic and toxicologic findings in two fatal cases of bodybuilders. *International Journal of Legal Medicine*, **121**, 48–53.
11. Lehmann, S., Thomas, A., Schiwy-Bochat, K.-H., Geyer, H., Thevis, M., Glenewinkel, F., et al. (2019) Death after misuse of anabolic substances (clenbuterol, stanozolol and metandienone). *Forensic Science International*, **303**, 109925.
12. Park, M., Sim, J., Jeon, Y., Yeon, S., Lee, J., In, S. (2019) Determination of boldenone in post-mortem specimens including blood and urine samples using LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, **169**, 111–115.
13. Kintz, P., Gheddar, L., Ameline, A., Dumestre-Toulet, V., Verschoore, M., Comte, J., et al. (2019) Complete post-mortem investigations in a death involving clenbuterol after long-term abuse. *Journal of Analytical Toxicology*, **43**, 660–665.
14. Kintz, P., Gheddar, L., Raul, J.-S. (2021) Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis. *Drug Testing and Analysis*.
15. Batra, K.K., Omand, J., Baselt, R.C. (1981) Serum quinidine concentrations as measured by direct fluorometry, double-extraction fluorometry, and enzyme immunoassay. *Clinical Chemistry*, **27**, 780–781.
16. Wu, X., Gao, F., Zhang, W., Ni, J. (2015) Metabolism study of boldenone in human urine by gas chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, **115**, 570–575.
17. Gomez, C., Pozo, O.J., Geyer, H., Marcos, J., Thevis, M., Schänzer, W., et al. (2012) New potential markers for the detection of boldenone misuse. *The Journal of Steroid Biochemistry and Molecular Biology*, **132**, 239–246.
18. Kintz, P., Cirimele, V., Ludes, B. (2000) Pharmacological criteria that can affect the detection of doping agents in hair. *Forensic Science International*, **107**, 325–334.
19. Segura, J., Pichini, S., Peng, S.H., De la Torre, X. (2000) Hair analysis and detectability of single dose administration of androgenic steroid esters. *Forensic Science International*, **107**, 347–359.
20. Kintz, P., Cirimele, V., Jeanneau, T., Ludes, B. (1999) Identification of testosterone and testosterone esters in human hair. *Journal of Analytical Toxicology*, **23**, 352–356.
21. Voegel, C.D., Baumgartner, M.R., Kraemer, T., Wüst, S., Binz, T.M. (2021) Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS – Insights into EC baseline values and correlation to steroid concentrations. *Talanta*, **222**, 121499.
22. Choi, M.H., Chung, B.C. (1999) GC-MS determination of steroids related to androgen biosynthesis in human hair with pentafluorophenyldimethylsilyl-trimethylsilyl derivatization. *Analyst*, **124**, 1297–1300.
23. Kintz, P., Cirimele, V., Ludes, B. (1999) Physiological concentrations of DHEA in human hair. *Journal of Analytical Toxicology*, **23**, 424–428.
24. Fabresse, N., Grassin-Delyle, S., Etting, I., Alvarez, J.-C. (2017) Detection and quantification of 12 anabolic steroids and analogs in human whole blood and 20 in hair using LC-HRMS: application to real cases. *International Journal of Legal Medicine*, **131**, 989–999.
25. Kintz, P. (2017) A new series of hair test results involving anabolic steroids. *Toxicologie Analytique Et Clinique*, **29**, 320–324.
26. Thieme, D., Anielski, P. Doping, application of hair analysis. In: Kintz P., Salamone A., Vincenti M. (eds). *Hair Analysis in Clinical and Forensic Toxicology*. Academic Press: London, 2015; pp 275–299.
27. American Addiction Centers. https://www.drugabuse.gov/publications/research-reports/steroids-other-appearance-performance-enhancing-drugs-apeds/how-are-anabolic-steroids-used (Accessed Mar 2, 2021).
28. Kintz, P. (2017) Hair analysis in forensic toxicology: an update review with a special focus on pitfalls. *Current Pharmaceutical Design*, **23**, 5480–5486.
29. Kintz, P., Gheddar, L., Raul, J.S. (2021) Testing for anabolic steroids in human nail clippings. *Journal of Forensic Sciences*.
30. Lusetti, M., Licata, M., Silingardi, E., Bonsignore, A., Palmiere, C. (2018) Appearance/image- and performance-enhancing drug users: a forensic approach. *American Journal of Forensic Medicine and Pathology*, **39**, 325–329.

Article 8 : Testing for trenbolone, an anabolic steroid, in biological fluids and head hair in a postmortem case

Laurie Gheddar, Anne-Laure Pélissier, Jacques Desfeux, Fabrice Niort, Jean-Sébastien Raul, Pascal Kintz

Journal of Analytical Toxicology. 2021. 19:bkab091. DOI:10.1093/jat/bkab091.

Journal of Analytical Toxicology, 2021, **00**, 1–4
DOI: https://doi.org/10.1093/jat/bkab091
Advance Access Publication Date: 19 August 2021
Case Report

Testing for Trenbolone, an Anabolic Steroid, in Biological Fluids and Head Hair in a Postmortem Case

Laurie Gheddar[1,*], Anne-Laure Pélissier[2], Jacques Desfeux[2], Fabrice Niort[2], Jean-Sébastien Raul[1] and Pascal Kintz[1]

[1]Toxicologie, Institut de Médecine Légale, 11 rue Humann, Strasbourg, Alsace 67000, France
[2]Service de Médecine Légale, Aix-Marseille Université, 27 boulevard Jean Moulin, Marseille, Provence-Alpes-Côte d'Azur 13385, France
*Author to whom correspondence should be addressed. Email: laurie.gheddar@etu.unistra.fr

Abstract

A 60-year-old man was found dead at home by his wife. The man was known to be a regular trainer in a fitness center and was described as a long-term user of anabolic steroids. The autopsy revealed heart failure with cardiomegaly, visceral congestion, and pulmonary edema. The local prosecutor ordered toxicological analyses but no anatomic pathology test. Initial analyses by the liquid chromatography coupled to quadrupole time-of-flight mass spectrometry system (UPLC-q-TOF-MS) system indicated the presence in femoral blood of trenbolone, an anabolic steroid. Trenbolone was quantified with a liquid chromatography system coupled with a tandem mass spectrometer in various autopsy specimens, including hair. Concentrations of trenbolone were 3.9, 3.2, 231, and lower than 0.5 ng/mL in femoral blood, cardiac blood, bile, and vitreous humor, respectively. Moreover, in head hair, the concentration was 9 pg/mg. The presence of trenbolone in hair can be interpreted as repetitive exposures when compared to the limited literature data. Repetitive misuse of trenbolone can explain advanced heart failure. This paper highlights the importance of testing biological specimens for anabolic steroids in case of heart failure.

Introduction

Trenbolone (17β-trenbolone) is a synthetic anabolic-androgenic steroid (AAS). It was first used in animals as a growth promoter in cattle and was administered as pelletized implants for subcutaneous administration at 120–200 mg (1).

Due to its anabolic properties, humans can misuse trenbolone, especially athletes for doping purposes, in order to improve muscle mass, enhance endurance, or reduce the recovery time. Trenbolone is available on the Internet as a tablet (25 mg) for oral administration and as an oily solution for intramuscular injection (75 mg/mL), mostly as esters forms (1). Because of its misuse in sport, the World Anti-Doping Agency (WADA) has prohibited trenbolone in and out of competition and classified it as S1.1. AAS class. According to the statistics of the WADA, the drug is commonly used by athletes. Indeed, it represented 5% of the adverse analytical findings in the S1.1 class in 2019 (top 10) (2).

Anabolic steroids are misused not only by top athletes but also by amateurs for aesthetic purposes, for example, to improve their physical appearance. However, long-term abuse of trenbolone can be a health problem because AAS are often used at high dosages (10–100 times more than recommended doses), and their abuse can lead to severe side effects, including increase in blood pressure, alterations of the cardiovascular system, liver toxicity, acne, hirsutism, sexual dysfunction, and a large series of psychiatric effects (antisocial lifestyle, disinhibition, frustration, psychosis, low self-confidence, depression, and aggressiveness) (3). It has been published in the scientific literature that daily consumption of anabolic agents can lead to cardiac death. Lehman et al. described a death after the misuse of anabolic steroids mix, namely clenbuterol, stanozolol, and metandienone (4). Kintz et al. reported in another paper that the misuse of clenbuterol and stanozolol could lead to a cardiovascular failure (5). Fabresse et al. (6) also reported two cardiac deaths due to the misuse of boldenone, stanozolol, nandrolone, and trenbolone.

In this paper, the authors report a postmortem case that describes cardiomegaly and ischemic heart disease involving regular consumption of trenbolone and present concentrations in different biological fluids and head hair.

Case Report

A 60-year-old man was found dead at home, in the bathroom, by his wife in the evening. He did not present any medical pathology history. However, he was known to be a regular trainer in a fitness center. His wife reported that he was a regular user of anabolic steroids, with no indication of the frequency, dose, and nature of these steroids.

The man measured 1.82 m and weighed 93 kg. The external body revealed needle marks in the thigh. An autopsy was requested and was performed 1 day after death. The autopsy revealed heart failure with cardiomegaly (heart weighting 579 g, whereas normal values are in the range 250–350 g for an adult male (5)), visceral congestion, and pulmonary edema. Athermanous plaque was observed in the coronaries with lumen reduction to 50–80%. In the left atrium, an aneurysm of 3 cm in diameter was identified. Moreover, the autopsy revealed general organ congestion and acute

asphyxiated syndrome in the lungs. During the autopsy, toxicological specimens were collected, including cardiac and femoral blood, vitreous humor, bile, and head hair (1–1.5 cm in length, white in color). Urine specimen, that is, the matrix used by antidoping laboratories for testing AAS, was not available during the autopsy. Unfortunately, no anatomic pathology test was requested by the local prosecutor, who only ordered a comprehensive toxicological analysis.

Biological specimens were stored at 4°C until analysis, except for hair, which was stored at ambient temperature.

Experienced pathologists concluded that the cause of death was ischemic heart disease linked to the lack of perfusion of the organs.

Initial Toxicological Screening

The following standard analyses were carried out in femoral blood: immunochemical screening for pharmaceuticals and drugs of abuse by ELISA (NeoGen), assay of ethanol in blood by headspace gas chromatography with flame-ionization detection (HS-GC-FID), cyanides and volatile substances by headspace gas chromatography with mass spectrometry (HS-GC-MS) in femoral blood, and level of carboxyhemoglobin by a CO-oximeter in cardiac blood. Two other screenings were carried out by liquid chromatography method to test for drugs, narcotics, and new psychoactive substances (about 200 compounds) in femoral blood. The last screening was achieved on UPLC–q-TOF-MS (Waters Corporation, Milford, MA, USA) system in femoral blood. As the initial screening by UPLC–q-TOF-MS identified trenbolone, a specific method on a liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system was used to quantify the drug in the autopsy specimens.

Trenbolone Analysis

Material and methods

Trenbolone and testosterone-D_3 were obtained from LGC (Teddington, United Kingdom) and from Sigma Aldrich (Steinheim, Germany), respectively. The standard solutions, prepared in methanol (MeOH), were stored at –20°C.

Phosphate, ammonium acetate for HPLC, dichloromethane, heptane, and isoamylic alcohol were purchased from Merck (Darmstadt, Germany) and MeOH and acetonitrile (ACN) for HPLC Isocratic Grade from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Disodium tetraborate decahydrate, diethyl ether, and hexane were purchased from VWR Chemicals Prolabo (Fontenay-sous-Bois, France).

For method validation, blank hair specimens from laboratory staff were used after checking the absence of response at the retention times of trenbolone and the internal standard.

Preparation of samples

For the analysis in fluids (blood, bile, and vitreous humor), trenbolone was extracted from 1 mL of matrix in the presence of 10 ng of testosterone-D_3, used as internal standard (IS), with 1 mL of borate buffer pH 9.5 and 5 mL of a mix of ether/dichloromethane/hexane/isoamylic alcohol (50/30/20/0.5). After agitation for 15 min and centrifugation (15 min, 3,000 rpm), the organic phase was collected and evaporated to dryness under a nitrogen flow. The dry residue was dissolved in 30 µL of MeOH, and 2 µL were injected into the UHPLC system.

Hair was decontaminated twice with 5 mL of dichloromethane for 1 min at room temperature and dried. No segmentation was achieved due to the lack of material. Thirty milligrams of decontaminated hair were cut into very short segments (<1 mm) and were incubated in 1-mL phosphate buffer (pH 5.0) for 1 hour in ultrasonic bath in the presence of 1 ng of IS. After cooling, 5 mL of heptane were added. After agitation for 15 min and centrifugation (15 min, 3,000 rpm), the organic phase was collected and evaporated to dryness under a nitrogen flow. The dry residue was dissolved in 50 µL of MeOH, and 10 µL were injected into the UHPLC system.

Liquid chromatography/mass spectrometer (UHPLC–MS-MS) analysis

Chromatographic separations were performed with a liquid chromatography Waters Acquity UPLC™ system (Waters Corporation, Milford, MA, USA) using an Acquity UPLC BEH C18 column (100 × 2.1 mm, i.d. 1.7 µm particle size). The column temperature was set to 50°C, and the flow rate at 0.4 mL/min. A gradient elution was performed using 1.0 mM acetate buffer (mobile phase A) and 0.1% formic acid in acetonitrile LC-MS (mobile phase B). Analysis time was 11.0 min, and the following gradient pattern (eluent A) was used for the separation of trenbolone and testosterone-D3: 0 min, 80%; 1.00 min, 80%; 6.00 min, 5%; 7.50 min, 5%; 7.60 min, 80%; 11.00 min, 80%. Trenbolone and testosterone-D_3 were eluted in 4.40 and 4.80 min, respectively. Detection was performed using a triple quadrupole (XEVO™ TQ-S micro, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionization source (ESI) and used in the positive mode (ES+). Nitrogen was used as drying gas as well as nebulizing gas. The desolvation gas flow was set to 800 L/h, and the desolvation temperature was 500°C. The source temperature was 150°C. The capillary voltage was set at 3.0 kV. Cone voltage and collision energy were adjusted to optimize the signal of the two most abundant product ions of trenbolone: m/z 271.2 > 199.1 and 271.2 > 253.2 and for testosterone-D_3: m/z 292.2 > 109.0. MassLynx 4.1 software was used for quantification.

Results and Discussion

Preliminary toxicology analyses did not detect any drugs, narcotics, or alcohol in femoral blood. Nevertheless, the screening by UPLC–q-TOF-MS in femoral blood identified trenbolone, an anabolic steroid. Therefore, trenbolone was

Table I. Postmortem Concentration of Trenbolone

Specimen	Trenbolone
Femoral blood	3.9 ng/mL
Cardiac blood	3.2 ng/mL
Bile	231 ng/mL
Humor vitreous	Less than 1 ng/mL (LOQ)
Hair	9 pg/mg

LOQ, limit of quantification.

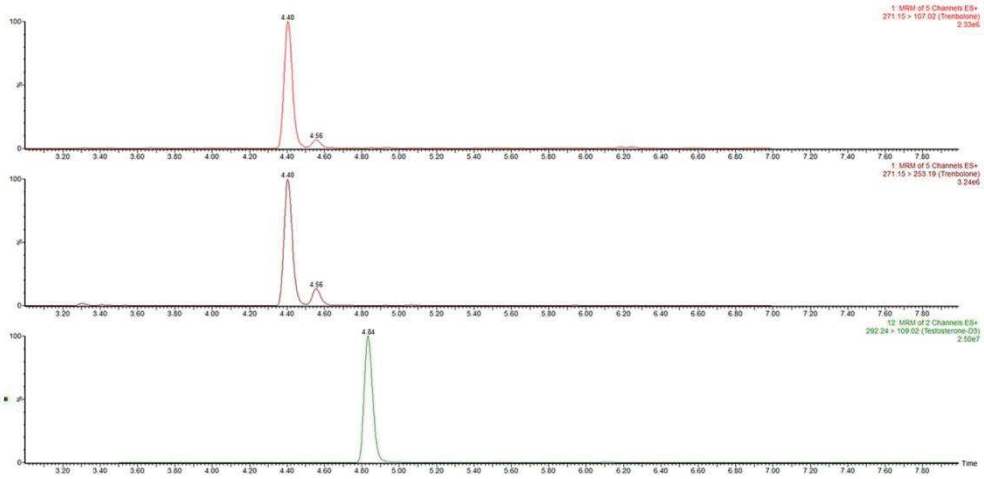


Figure 1. Chromatogram of cardiac blood (3.2 ng/mL) of deceased: from top to bottom: two transitions of trenbolone, one transition for IS testosterone-D_3.

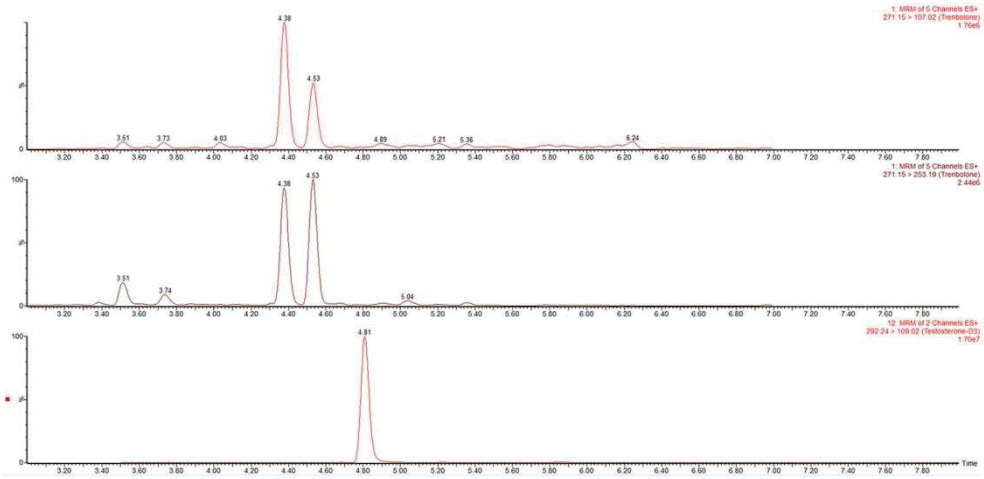


Figure 2. Chromatogram of bile (231 ng/mL) of deceased: from top to bottom: two transitions of trenbolone, one transition for IS testosterone-D_3.

tested in all fluids and head hair by a specific UPLC–MS-MS method.

The analytical approaches were validated in blood and hair. Linearity was observed in blood from 1 to 500 ng/mL, with a correlation coefficient of 0.9996. The limit of detection was evaluated at 0.5 ng/mL with an S/N ratio of 3. The limit of quantification was the first point of the calibration (1 ng/mL). QC samples (10 ng/mL and 100 ng/mL) were analyzed in duplicate in six independent experimental assays and were used to determine the CVs for precision and accuracy, which were found to be lower than 20%. Matrix effect was evaluated with 10 blank blood specimens and was found lower than 15%. Other liquid matrices were tested using blood calibration. As described by Putz et al., the LC–MS-MS technique is favored for the analysis of trenbolone and its metabolites and allows avoiding the derivatization step required for GC–MS/MS analysis (7). According to WADA Technical Document TD2019MRPL, antidoping laboratories should be able to analyze exogenous AAS with a minimum required performance level of 5 ng/mL (8). The method used and described in this article allows the identification of trenbolone up to 0.5 ng/mL

in liquid matrices, which is comparable with WADA's methods.

Linearity was also observed from 1 to 200 pg/mg in hair, with a correlation coefficient of 0.9994. The limit of detection was evaluated at 0.4 pg/mg with an S/N ratio of 3. The limit of quantification was the first point of the calibration (1 pg/mg). QC samples (10 pg/mg and 100 pg/mg) were analyzed in duplicate in six independent experimental assays and were used to determine the CVs for precision and accuracy, which were found to be lower than 20%. Matrix effect was evaluated with 10 blank hair specimens and was found to be lower than 15%.

Concentrations of trenbolone are summarized in Table I. Concentrations in femoral and cardiac blood are very close, which seems to indicate that there was no postmortem redistribution. The chromatograms obtained for bile and cardiac blood (Figures 1 and 2), showed the presence of the trenbolone (4.40 min) and its major metabolite, epitrenbolone, (17α-trenbolone) at 4.53 min. According to the chromatograms of bile and blood, the response of epitrenbolone seems to represent less than 30% of the response of trenbolone. Epitrenbolone was not detected in head hair and vitreous humor. Its absence can be explained by the very low concentrations observed in these two specimens. The lack of information in the scientific literature does not allow interpreting the concentrations in the various biological fluids. Indeed, this is the first time that trenbolone concentrations in body fluids are reported, except the one of Fabresse et al. (6), who reported a blood concentration of 163 ng/mL in a cardiac death case. The presence of trenbolone in blood, bile, and vitreous humor can be interpreted as a marker of a recent consumption.

In addition, hair of the deceased was tested to document possible repetitive misuse. Hair tested positive for trenbolone at 9 pg/mg. In literature, several authors have reported trenbolone hair concentration after repetitive consumption. Thieme and Anielski reported hair concentrations ranging from 2 to 330 pg/mg (9). In a recent paper, Kintz et al. identified trenbolone with concentrations at 14 pg/mg and 121 pg/mg in head hair specimens (10). Finally, Fabresse et al. (6) detected trenbolone at 23 pg/mg. The concentration at 9 pg/mg of the deceased can be interpreted as corresponding to repetitive consumption.

Trenbolone (Parabolan ™) is an anabolic steroid presented to be three times more powerful than testosterone and is a derivative of nandrolone (19-nortestosterone). It can be bought as parent drug in tablets or as esters such as acetate, enantate, cyclohexylmethylcarbonate, undecylate, or undecanoate in injectable forms. Cardiomegaly and ischemic heart disease observed during the autopsy can be the consequences of the regular administrations of trenbolone.

It has been demonstrated that the misuse of AAS at high dosages can lead to severe adverse cardiovascular effects, including hypertrophy, arrhythmia, and hypertension (11, 12) but also myocardial infarction as reported for a young man after repetitive exposure of trenbolone acetate (13). Some authors have also reported that AAS misuse could lead to atherosclerosis that increases the risk of myocardial ischemia (11).

Conclusion

The authors report in this paper the observation of an acute asphyxia syndrome caused by an advanced cardiomegaly and ischemic heart disease. The pathologists concluded that the cause of death was not directly linked to the consumption of trenbolone but that this misuse could explain the advanced heart disease.

Funding

The authors declare that no funding received for this work.

References

1. Baselt, R.C. Trenbolone. In: Baselt (ed), *Disposition of Toxic Drugs and Chemicals in Man*. 12th edition. Biomedical Publications: Seal Beach, CA, 2020; p. 2135.
2. WADA. (2019) *Anti-Doping Testing Figures*. https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf (accessed Mar 01, 2021).
3. Trenton, A.J., Currier, G.W. (2005) Behavioural manifestations of anabolic steroid use. *CNS Drugs*, **19**, 571–595.
4. Lehmen, S., Andreas, T., Schiwy-Bochat, K.H., Geyer, H., Thevis, M., Glenewinkel, F., et al. (2019) Death after misuse of anabolic substances (clenbuterol, stanozolol and metandienone). *Forensic Science International*, **303**, 109925.
5. Kintz, P., Gheddar, L., Ameline, A., Dumestre-Toulet, V., Verschoore, M., Comte, J., et al. (2019) Complete postmortem investigation in a death involving clenbuterol after long-term abuse. *Journal of Analytical Toxicology*, **43**, 660–665.
6. Fabresse, N., Grassin-Delyle, S., Etting, I., Alvarez, J.C. (2017) Detection and quantification of 12 anabolic steroids anad analogs in human whole blood and 20 in hair using LC-HRMS/MS: application to real cases. *International Journal of Legal Medicine*, **131**, 989–999.
7. Putz, M., Piper, T., Thevis, M. (2020) Identification of trenbolone metabolites using hydrogen isotope ratio mass spectrometry and liquid chromatography/high accuracy/ high resolution mass spectrometry for doping control analysis. *Frontiers in Chemistry*, **8**, 435.
8. WADA. (2019) *WADA Technical Document – TD2019MRPL*. https://www.wada-ama.org/en/resources/science-medicine/td2019mrpl (accessed Aug 03, 2021).
9. Thieme, D., Anielski, P. Doping, applications of hair analysis. In: Kintz P., Salomone A., Vincenti M. (eds.). *Hair Analysis in Clinical and Forensic Toxicology*. Chapter 10. Academic Press, Elsevier: London, UK, 2015; pp 275–299.
10. Kintz, P., Gheddar, L., Raul, J.S. (2021) Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared to the standard head hair analysis. *Drug Testing and Analysis*, **13**, 1445–1451.
11. Seara, F.A.C., Olivares, E.L., Nascimento, J.H.M. (2020) Anabolic steroid excess and myocardial infarction: from ischemia to reperfusion injury. *Steroids*, **161**, 108660.
12. Sullivan, M.L., Martinez, C.M., Gennis, P., Gallagher, E.J. (1998) The cardiac toxicity of anabolic steroids. *Progress in Cardiovascular Diseases*, **41**, 1–15.
13. Shahsavari Nia, K., Rahmani, F., Ebrahimi Bakhtavar, H., Hashemi Agdam, Y., Balafar, M. (2014) A young man with myocardial infarction due to trenbolone acetate; a case report. *Emergency*, **2**, 43–45.

Article 9 : Anabolic steroids and extreme violence : a case of murder after chronic intake and under acute influence of metandienone and trenbolone

Frédéric Aknouche, Laurie Gheddar, Angéline Kernalléguen, Christophe Maruejouls, Pascal Kintz

International Journal of Legal Medicine. 2021;135(4):1449-1453.

International Journal of Legal Medicine
https://doi.org/10.1007/s00414-021-02587-y

CASE REPORT

Anabolic steroids and extreme violence: a case of murder after chronic intake and under acute influence of metandienone and trenbolone

Frédéric Aknouche[1] · Laurie Gheddar[2] · Angeline Kernalléguen[1] · Christophe Maruejouls[1] · Pascal Kintz[2,3]

Received: 25 January 2021 / Accepted: 25 March 2021

Abstract
A 32-year-old male went to the police to claim he just killed his girlfriend by inflicting several stabs with a kitchen knife. He was very nervous and particularly aggressive. About 90 min after the assault, a blood specimen was collected with natrium fluoride as preservative. The blood was free of alcohol, pharmaceuticals and drugs of abuse, but tested positive by LC–MS/MS for metandienone (32 ng/mL) and trenbolone (9 ng/mL). The perpetrator admitted regular consumption of anabolic steroids to enhance his muscular mass, as he was a professional security agent. To document long-term steroid abuse, a hair specimen was collected 3 weeks after the assault, which tested positive for both drugs. Segmental analyses revealed in the proximal 1.5 cm segment, corresponding to the period of the assault, the simultaneous presence of metandienone (11 pg/mg) and trenbolone (14 pg/mg), while only metandienone (3 pg/mg) was identified in the distal 1.5 cm segment. As aggressiveness and violence can be associated with abuse of anabolic steroids, the aetiology of this domestic crime was listed to be due impulsive behaviour in a context of antisocial lifestyle.

Keywords Murder · LC–MS/MS · Metandienone · Trenbolone · Blood · Hair

Introduction

Athletes, body builders, students, fitness practitioners and security-involved agents can use anabolic steroids because it has been claimed that these drugs increase lean body mass, increase strength, increase aggressiveness, increase endurance and lead to a shorter recovery time between workouts. These properties are due to their effects on human androgen receptors [1]. Anabolic steroids are mostly available on the Internet, at low costs, both as oral (methyltestosterone, stanozolol, metandienone, chlorodehydromethyltestosterone, tetrahydrogestrinone, stanozolol …) or injectable (methenolone esters, drostanolone esters, nandrolone esters, testosterone esters, trenbolone esters …) forms. In most cases, these compounds are used in dosages much higher than what is recommended by the manufacturers. Therapeutic uses are very limited, and, for example, in France, only testosterone enantate (Androtardyl™) is available after medical prescription.

Numerous side effects after long-term anabolic steroids misuse have been described, and include increase in blood pressure, alterations of the cardiovascular system, liver toxicity, acne, hirsutism, gynecomastia, atrophic testes and sexual dysfunction [2]. When misuse is prolonged, subjects can develop psychiatric effects, which include antisocial lifestyle, disinhibition, frustration, psychosis, low self-confidence, childhood conduct disorders, delirium, depression and aggressiveness [3–5].

Since the demonstration by Kindlundh et al. in 2003 [6] that 2 weeks of treatment with nandrolone decanoate cause a significant alteration in the density of serotoninergic 5HT1B and 5HT2 receptors in the male rat brain, a testosterone-serotonin link [7] was established, which could explain changes in emotional states and behaviour. It seems that anabolic steroids can modulate serotonin receptors activity, which will affect aggressiveness, as it is associated to decreased serotonin neurotransmission [8].

Epidemiological studies, both in Europe [9–11] and in the USA [12] have demonstrated that the misuse of anabolic

✉ Pascal Kintz
pascal.kintz@wanadoo.fr

1 Laboratoire Barla, 6 rue Barla, 06000 Nice, France

2 Institut de Médecine Légale, 11 rue Humann, F-67000 Strasbourg, France

3 X-Pertise Consulting, 42 rue principale, F-67206 Mittelhausbergen, France

Published online: 04 April 2021

steroids constitutes an increased risk for violence and that there is an association between their use and criminality. There is an increased risk of developing an antisocial lifestyle [13] and the frequency of crimes of violence and weapons offences is increased once anabolic steroids are consumed for a long period [14]. These data mainly come from case reports rather than from complete epidemiological studies.

Because anabolic steroids are seldom tested in forensic laboratories, and even less in hair specimens, the present observation, documented with toxicological analyses in both blood and hair, allows establishing a link between the aggressive behaviour of a murderer and his addiction to this class of compounds.

Case history

A 32-year-old man went to the police station some minutes after murdering his girlfriend by inflicting several stabs with a kitchen knife. He reported a dispute that started at home because she arrived late from work. She gave him a slap in the face for bad wording during the initial phase, but very rapidly the man went nervous, excited and took a knife to injure the neck, the face, the arms and the back of his girlfriend.

The man was a security agent and was not known to the police. There was no reported family history of mental illness. Relatives and witnesses did not report signs of violence before the homicide. Quite daily, he was practicing muscular improvement in a fitness centre and confessed using anabolic steroids to enhance his athletic figure and to promote muscle growth. Last exposure to oral steroids was about 90 min before the assault. He reported using metandienone (Dianabol™) and trenbolone. Later at home, the police also discovered tablets of clenbuterol and ampoules of Androtardyl™ (testosterone enantate). The exact regimen of the subject is unclear and the steroids were not submitted to analyses to verify both the composition and the purity.

At the police station, 2 vials of blood (Vacutainer® with grey top, Becton, Dickinson and Company, Le Pont de Claix, France) containing sodium fluoride as preservative were collected about 3.5 h after the assault (i.e. corresponding to about 5 h after the oral administration of the steroids). Three weeks later, an experienced physician collected 3 strands of hair as close as possible from the scalp in the vertex region (black in colour, 3 cm in length, as the subject was short-haired).

Toxicological analyses

Blood analysis

First, the blood was subjected to the standard toxicological screening. Ethanol was tested in blood by head-space GC/FID on a Perkin Elmer system (TurboMatrix 40 & Clarus 580, Every, France) using a standard validated procedure. ELISA tests for benzodiazepines and drugs of abuse (opiates, cannabis, cocaine, amphetamines, methamphetamines and methadone) were achieved using Neogen kits (Lexington, KY, USA) using the recommendations of the manufacturer. GHB was tested by GC/MS on an Agilent 5971 (Santa Clara, CA, USA). A comprehensive screening for pharmaceuticals and drugs of abuse, including NPS (about 200 compounds), was performed on blood using LC/MS–MS (Waters Xevo TQD, Milford, MA, USA). Due to the nature of the case, anabolic steroids were specifically tested by a MRM method using LC/MS–MS, using a validated procedure [15]. Briefly, steroids were extracted from 1 mL of blood in presence of 10 ng of testosterone-d_3 (Sigma-Aldrich, Saint Louis, MI, USA) used as internal standard, with 1 mL borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5, vol%). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 30 µL of ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters BEH C18 column (100 × 2.1 mm × 1.7 µm) maintained at 50 °C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in LC/MS-grade acetonitrile (mobile phase B) at flow rate of 0.4 mL/min. The initial composition was 87% mobile phase A and the final composition, at 15 min, was 5% mobile phase A. An injection volume of 2 µL was used in all cases. Ionization was achieved using electrospray in the positive ionization mode (ESI+). MassLynx 4.1 software was used for quantification. Mass spectrometric parameters are presented in Table 1.

Hair analysis

Because there was a request to test for several anabolic steroids, a screening procedure, already published [16], was used. Briefly, 30 mg of finely cut hair, previously decontaminated by 2 dichloromethane bathes (5 mL, 2 min) and segmented (2 × 1.5 cm) were weighted. The hair was mixed with 1 ng of testosterone-d_3, used as internal standard and 1 mL of methanol and submitted to an ultrasonic bath for a period of 90 min. After centrifugation, the organic phase was collected and evaporated and diluted with phosphate buffer pH 7.0 (part A).

Table 1 Mass spectrometric data for the tested compounds

Drug	Transitions *m/z*	Collision energy eV	Cone voltage V
Metandienone	301.2 > 121.1 301.2 > 149.1	22	22
Trenbolone	271.2 > 199.1 271.2 > 253.2	20	46
Testosterone-d3	292.2 > 109.0 292.2 > 97.0	22	30

The remaining hair, at the bottom of the tube, was collected and hydrolyzed with 1 mL NaOH 1 M (part B). Liquid–liquid extractions were operated on parts A and B with 2 mL ethyl acetate. Extracts were combined and evaporated to dryness. The residue was dissolved in 1 mL dichloromethane, which was submitted to solid-phase extraction, using Isolute C18 columns. The final elution phase was evaporated and solubilized in 30 µL methanol to be divided in two parts: for injection onto LC–MS/MS apparatus (Waters TQS micro, Milford, MA, USA) and onto GC–MS/MS apparatus (Agilent 7010B, Santa Clara, CA, USA) after evaporation and TMS derivatization (MSTFA/NH_4I/2-mercaptoethanol, 1000:2:5, vol%).

Testosterone, metandienone and trenbolone were tested by LC–MS/MS.

The major validation of the analytical procedures for these 2 compounds, both in blood and hair, are presented in Tables 2 and 3.

Results and discussion

Ethanol tested negative in blood. GHB was within the physiological range (1.1 mg/l). ELISA screenings were negative for pharmaceuticals and drugs of abuse. The screening of the blood by LC/MS–MS was negative, except for metandienone and trenbolone.

Metandienone and trenbolone tested positive in blood at 32 and 9 ng/mL, respectively. These concentrations are highly indicative that the murderer was under the influence of anabolic steroids at the time of the assault. Results are presented Table 4. Very few data are available in the literature for steroid blood concentrations, and particularly for metandienone or trenbolone. In a death after misuse of anabolic substances, Lehmann et al. [17] identified metandienone in post mortem femoral blood at 8 ng/mL. It seems that the pharmacokinetic parameters of trenbolone has not been established in humans, while the elimination half-live of metandienone is about 3 to 6 h. Blood testosterone concentration was 0.9 ng/mL, which can be interpreted as a physiological concentration. No clenbuterol was identified (limit of detection is 0.1 ng/mL).

In the recent years, hair specimens have been proposed to document drug exposure in any situation in which a history of past rather than recent drug use is requested. In addition, keratinous matrices, such as nail or hair, irrespective of the anatomical location of collection are the most commonly used matrices allowing documenting long-term exposure to drugs. Finally, testing head hair by segmentation allows establishing a retrospective calendar of an individual's drug use and the distinction between single and repetitive use [18–21]. Segmental analyses of the hair of the murderer went back positive and allowed interpreting the results as an increase in steroid consumption in the more recent period. Concentrations of metandienone and trenbolone were in the low pg/mg range, i.e. 11 and 3 pg/mg and 14 pg/mg and not detected in the 2×1.5 cm segments, respectively. Although concentrations of anabolic steroids are low in hair when compared to drugs of abuse, such as cocaine, opiates or amphetamines identified in the ng/mg range, a suitable chromatographic signal was obtained in hair (Fig. 1). No clenbuterol and testosterone enantate were identified in any of the 2 segments. Testosterone concentrations were less than 1 pg/mg in the 2 hair segments. Again, anabolic steroids have been seldom reported in hair. In most cases, bodybuilders have been tested. Bresson et al. [22] reported metandienone concentrations in the range of 7 to 108 pg/mg after segmental analysis of the scalp hair collected from a football player. In 2 series of results [23, 24], Kintz et al. identified several metandienone abusers, with concentrations in the range 7 to 2800 pg/mg. Thieme and Anielski [25] in their compendium presented concentrations varying from 4 to 250 pg/mg. For trenbolone, data are also limited. Fabresse et al. [26] found 23 pg/mg in a suicide case and Thieme and Anielski [25] reported concentrations in the range 2 to 290 pg/mg.

Metandienone or methandrostenolone (Dianabol™) is an orally effective anabolic steroid. The drug was once one of the most widely produced steroids by the Ciba Pharmaceutical Company. Production and use have decreased sharply in the more recent years due to liver toxicity and the release

Table 2 Validation parameters in blood

Parameters	Metandienone	Trenbolone
Linearity	1 to 100 ng/mL, $r^2=0.9998$	1 to 100 ng/mL, $r^2=0.9997$
Limit of detection	0.3 ng/mL	0.5 ng/mL
Limit of quantitation	1 ng/mL	1 ng/mL
Precision (10 ng/mL, $n=8$)	14.7%	15.8%
Precision (50 ng/mL, $n=8$)	13.8%	13.6%
Matrix effect (10 ng/mL, $n=15$)	<20%	<20%

Table 3 Validation parameters in hair

Parameters	Metandienone	Trenbolone
Linearity	1 to 200 pg/mg, $r^2=0.9995$	1 to 200 pg/mg, $r^2=0.9993$
Limit of detection	0.1 pg/mg	0.4 pg/mg
Limit of quantitation	1 pg/mg	1 pg/mg
Precision (10 pg/mg, $n=8$)	16.6%	17.8%
Precision (100 pg/mg, $n=8$)	14.9%	15.8%
Matrix effect (10 pg/mg, $n=15$)	<20%	<20%

Table 4 Distribution of metandienone and trenbolone in blood and hair

Specimen	Metandienone	Trenbolone
Blood	32 ng/mL	9 ng/mL
Hair (0–1.5 cm)	11 pg/mg	14 pg/mg
Hair (1.5–3 cm)	3 pg/mg	Not detected

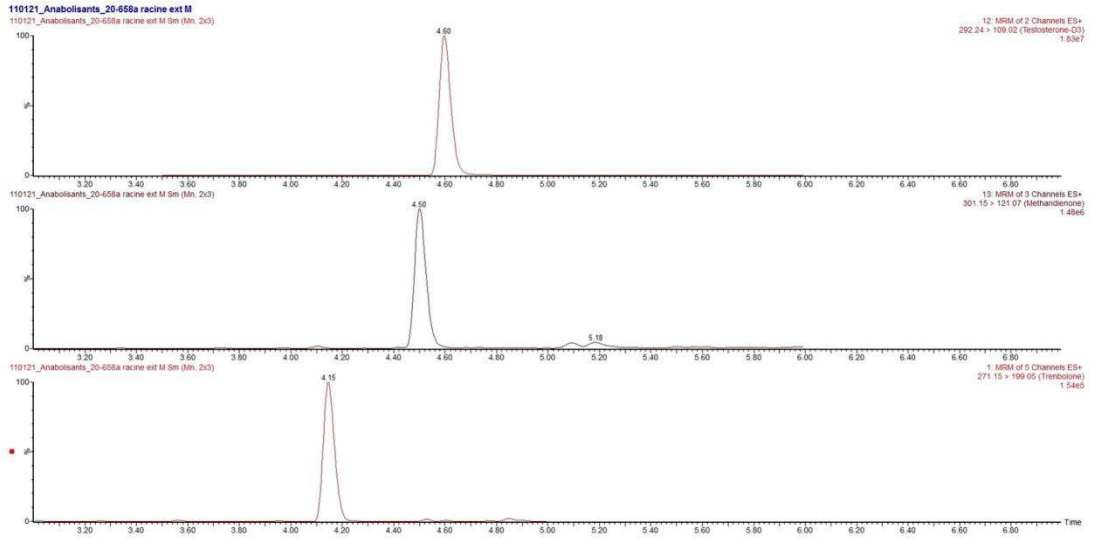


Fig. 1 Chromatogram obtained after extraction of the proximal hair segment of the suspect. Metandienone and trenbolone concentrations were 11 and 14 pg/mg, respectively. From top to bottom: quantification transitions for testosterone-d_3 (IS), metandienone and trenbolone

of safer testosterone esters. Trenbolone (Parabolan™) is an anabolic steroid presented to be 3 times more powerful than testosterone, and is a derivative of nandrolone (19-nortestosterone). It can be bought as parent drug in tablets or as esters such as acetate, enantate, cyclohexylmethylcarbonate, undecylate or undecanoate in injectable forms. Elimination half-life is much shorter when trenbolone is not used as ester forms (i.e. 2–3 days versus 3–12 days).

None of these publications was dealing with a situation of violence. Obviously, the murderer was under the influence of 2 steroids at the time of the assault. The test in an alternative matrix, i.e. scalp hair, allowed confirming long-term abuse. This seems of major importance to establish a clear picture of drug involvement.

It is not surprizing that 2 steroids were identified at the same time, as people who misuse steroids can take two or more different drugs and mix injectable and/or oral preparations [27]. It has been indicated in the literature [27] that steroids can be taken in a cycle of six to twelve weeks, with increasing doses, followed by a decrease at the end of the cycle. In the same review, authors have pointed that anabolic steroids increase both irritability and aggression and that personality traits are overrepresented in steroid abusers.

Conclusion

Regarding these results, and current known effects of long-term anabolic steroids, which appear to be nevertheless variable across individuals, the present case suggests an association between misuse of anabolic steroids and irritability/aggression. Abusers of steroids are more prone to be involved in criminal acts, as demonstrated by epidemiological studies. However, in all these reports, none was supported by toxicological analyses, which seem to have a major importance in forensic science. The authors have presented analytical results on biological specimens collected from a subject under the influence of both metandienone and trenbolone and recommend analyses of blood and hair to prove an acute impairment and the negative impact of a possible misuse. In this case, metandienone and trenbolone were simultaneously detected in blood and hair. The combination of these 2 specimens allowed establishing the involvement of steroids at the time of the assault and the confirmation that the perpetrator was a long-term steroids misuser.

Declarations

Ethics approval and consent to participate This study does not need ethical approval.

Informed consent not required

Conflict of interest The authors declare no competing interests.

References

1. Cadwallader AB, Lim CS, Rollins DE, Botrè F (2011) The androgen receptor and its use in biological assays: looking toward effect-based testing and its applications. J Anal Toxicol 35:594–607
2. Van Amsterdam J, Opperhuizen A, Hartgens F (2010) Adverse health effect of anabolic-androgenic steroids. Regul Toxicol Pharmacol 57:117–123
3. Trenton AJ, Currier GW (2005) Behavioural manifestations of anabolic steroid use. CNS Drugs 19:571–595
4. Pope HG, Kouri EM, Hudson JI (2000) Effects of supraphysiologic doses of testosterone on mood and aggression in normal men: a randomized controlled trial. Arch Gen Psychiatry 57:133–140
5. Khoodoruth MAS, Khan AA (2020) Anabolic steroids-induced delirium. Medicine. https://doi.org/10.1097/MD.0000000000021639
6. Kindlundh AMS, Lindblom J, Bergström L, Nyberg F (2003) The anabolic-androgenic steroid nandrolone induces alterations in the density of serotoninergic HHT1B and 5HT2 receptors in the male rat brain. Neuroscience 119:113–120
7. Birger M, Swartz M, Cohen D, Alesh Y, Grishpan C, Kooteir M (2003) Aggression: the testosterone-serotonin link. Isr Med Assoc J 5:653–658
8. Anderson PD, Bokor G (2012) Forensic aspects of drug-induced violence. J Pharm Pract 25:41–49
9. Klötz F, Garle M, Granath F, Thiblin I (2006) Criminality among individuals testing positive for the presence of anabolic androgenic steroids. Arch Gen Psychiatry 63:1274–1279
10. Lundholm L, Käll K, Wallin S, Thiblin I (2010) Use of anabolic androgenic steroids in substance abusers arrested for crime. Drug Alcohol Depend 111:222–226
11. Lood Y, Eklund A, Garle M, Ahlner J (2012) Anabolic androgenic steroids in police cases in Sweden 1999–2009. Forensic Sci Int 219:199–204
12. Beaver KM, Vaughn MG, Delisi M, Wright JP (2008) Anabolic-androgenic steroid use and involvement in violent behaviour in a nationally representative sample of young adult males in the United States. Am J Public Health 98:2185–2187
13. Thiblin I, Pärlklo T (2002) Anabolic androgenic steroids and violence. Acta Psychiatr Scand Suppl 412:125–128
14. Skärberg K, Nyberg F, Engström I (2010) Is there an association between the use of anabolic-androgenic steroids and criminality? Eur Addict Res 16:213–219
15. Kintz P, Gheddar L, Ameline A, Dumestre-Toulet V, Verschoore M, Comte J, Raul JS (2019) Complete post-mortem investigations in a death involving clenbuterol after long-term abuse. J Anal Toxicol 43:660–665
16. Kintz P, Gheddar L, Raul JS (2021) Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis. Drug Test Anal. https://doi.org/10.1002/dta.3020
17. Lehmann S, Thomas A, Schiwy-Bochat KH, Geyer H, Thevis M, Glenewinkel F, Rothschild MA, Andresen-Streichert H, Juebner M (2019) Death after misuse of anabolic substances (clenbuterol, stanozolol and metandienone). Forensic Sci Int 303:109925. https://doi.org/10.1016/j.forsciint.2019.109925
18. Kintz P (2017) Hair analysis in forensic toxicology: an updated review with special focus on pitfalls. Curr Pharm Des 23:5480–5486
19. Pragst F, Balikova M (2006) State of the art in hair analysis for detection of drug and alcohol abuse. Clin Chim Acta 370:17–49
20. Musshoff F, Schwartz G, Sachs H, Skopp G, Franz T (2020) Concentration distribution of more than 100 drugs and metabolites in forensic hair samples. Int J Legal Med 134:989–995
21. Salomone A, Gerace E, Di Corcia D, Allardio E, Vincenti M, Kintz P (2019) Hair analysis can provide additional information in doping and forensic cases involving clostebol. Drug Test Anal 11:95–101
22. Bresson M, Cirimele V, Villain M, Kintz P (2006) Doping control for metandienone using hair analysed by gas chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 836:124–128
23. Kintz P, Vaysette F, Deveaux M (2014) Compendium of results from hair tested for anabolics. Toxicol Anal Clin 26:197–200
24. Kintz P (2017) A new series of hair test results involving anabolic steroids. Toxicol Anal Clin 29:320–324
25. Thieme D, Anielski P (2015) Doping, application of hair analysis. In: Kintz P, Salamone A, Vincenti M (eds) Hair analysis in clinical and forensic toxicology. Academic Press, London, pp 275–299
26. Fabresse N, Grassin-Delyle S, Etting I, Alvarez JC (2017) Detection and quantification of 12 anabolic steroids and analogs in human whole blood and 20 in hair using LC-HRMS: application to real cases. Int J Leg Med 131:989–999
27. NIDA (2020). Why are anabolic steroids misused? Retrieved from https://www.drugabuse.gov/publications/research-reports/steroids-other-appearance-performance-enhancing-drugs-apeds/why-are-anabolic-steroids-misused. Accessed 23 Mar 2021

Article 10 : Evidence of use of drostanolone, an anabolic steroid, at the time the subject committed a murder: place of hair analysis

Pascal Kintz, Laurie Gheddar

Toxicologie Analytique et Clinique. 2021 ; 33 : 22-225.

Toxicologie Analytique & Clinique 33 (2021) 222–225

Disponible en ligne sur
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com

CASE REPORT

Evidence of use of drostanolone, an anabolic steroid, at the time the subject committed a murder: Place of hair analysis

Pascal Kintz[a,b,*], **Laurie Gheddar**[b]

[a] *X-Pertise Consulting, 42, rue principale, 67206 Mittelhausbergen, France*
[b] *Institut de médecine légale, 11, rue Humann, 67000 Strasbourg, France*

Received 26 January 2021; received in revised form 18 May 2021; accepted 18 May 2021
Available online 1 June 2021

KEYWORDS
Violence;
Anabolic steroids;
Drostanolone;
Hair

Summary A 21-year old male was arrested after killing a rival during a dramatic episode of violence, due to jealousy. The perpetrator was sent to prison, where he revealed, several months later, that he was using anabolic steroids at the time of the assault. The steroids were used to enhance his muscular mass. To document possible steroids abuse, a hair specimen (9 cm in length, black in colour) was collected 7 months after the event and segmented in 3 × 3 cm segments. A specific steroids screening procedure, involving both LC-MS/MS and GC-MS/MS, was used, which demonstrated the presence of drostanolone and its ester form, drostanolone enantate in the distal segment (i.e. the one corresponding to the period of the assault). Concentrations were 3 and 14 pg/mg for drostanolone and drostanolone enantate, respectively. The etiology of this murder was finally considered to be due to an aggressive behaviour, which can have been enhanced by side effects due to anabolic steroids abuse.

Introduction

Athletes, but also amateurs, can use anabolic steroids because it has been claimed that these drugs increase lean body mass, strength, aggressiveness, endurance and lead to a shorter recovery time between workouts [1]. Today, anabolic steroids are mostly available on the Internet, at low costs, both as oral or injectable forms (generally as esters). In most cases, these compounds are used in dosages much higher than what is recommended by the manufacturers. Therapeutic uses of anabolic steroids are very limited, and, for example, in France, only testosterone enantate (Androtardyl™) is available after medical prescription for intramuscular injection into the buttock.

Numerous side-effects after long-term anabolic steroids abuse have been described, and include increase

* Corresponding author at: X-Pertise Consulting, 42, rue principale, Mittelhausbergen 67206, France.
E-mail addresses: pascal.kintz@wanadoo.fr, pkintz@x-pertise.com (P. Kintz).

https://doi.org/10.1016/j.toxac.2021.05.002
2352-0078/

in blood pressure, alterations of the cardiovascular system, liver toxicity, acne, hirsutism, gynecomastia, atrophic testes and sexual dysfunction [2]. When abuse is prolonged, subjects can develop psychiatric effects, which include antisocial lifestyle, disinhibition, frustration, psychosis, low self-confidence, childhood conduct disorders, delirium, depression and aggressiveness [3—5].

Epidemiological studies, both in Europe [6] and in the United States of America [7] have demonstrated that the abuse of anabolic steroids constitutes an increased risk for violence and that there is an association between their use and criminality. There is an increased risk of developing an antisocial lifestyle but the frequency of crimes of violence and weapons offences is increased once anabolic steroids are consumed for a long period [8].

Because anabolic steroids are seldom tested in forensic laboratories, and even less in hair specimens, the present observation allows establishing a link between the aggressive behaviour of a murderer and his addiction to this class of compounds.

Case history

A 21-year old male was arrested after killing a rival during a dramatic episode of violence, due to jealousy. He was the new boyfriend of a girl, who was previously in couple with the victim. The perpetrator was sent to prison, where he revealed, several months later, that he was using anabolic steroids at the time of the assault. The exact regimen of the subject is unclear and the steroids were not identified. He refused to indicate which compounds were used and how they were purchased.

A medical doctor was requested to collect a hair specimen about 7 months after the assault. The perpetrator only accepted to give one strand of hair, which was 9 cm in length and black in colour.

Toxicological analyses

Because the identity of the involved steroid(s) was not known, a screening procedure, already published [9] was used. Briefly, 30 mg of finely cut hair, previously decontaminated by 2 dichloromethane bathes (5 mL, 2 min) and segmented (3 × 3 cm) were weighted. The hair was mixed with 1 ng of testosterone-d3, used as internal standard and 1 mL of methanol and submitted to an ultrasonic bath for a 90 min. Then, in one side, the organic phase was evaporated and diluted with phosphate buffer pH 7.0 (part A), and in another side, the hair remain was incubated with 1 mL NaOH 1 M (part B). Liquid-liquid extractions were operated on parts A and B with 2 mL ethyl acetate. Extracts were combined and evaporated to dryness. The residue was dissolved in 1 mL dichloromethane, which was submitted to solid phase extraction, using Isolute C18 columns. The final elution phase was evaporated and TMS derivatization (MSTFA/NH_4I/2-mercaptoethanol, 1000:2:5) was achieved. Drostanolone and drostanolone enantate were tested by GC-MS/MS (Agilent 7010B) (Table 1).

Results and discussion

The validation of the analytical procedures for drostanolone and drostanolone enantate in hair is presented in Table 2.

In the recent years, hair specimens have been proposed to document drug exposure in any situation in which a history of past rather than recent drug use is requested. In addition, keratinous matrices are the unique matrices allowing documenting long-term exposure to drugs. Finally, testing head hair by segmentation allows establishing a retrospective calendar of an individual's drug use and the distinction between single and repetitive use [10]. Segmental analyses of the hair of the murderer went back positive for the distal end and allowed interpreting the results as steroid consumption at the period corresponding to the assault, assuming a normal hair growth rate at 1 cm per month. Concentrations of drostanolone and drostanolone enantate were in the low pg/mg range, i.e. 3 and 14 pg/mg, respectively. Although concentrations were low, a suitable chromatographic signal was obtained in the distal hair segment (Fig. 1). The proximal segment and the segment of the middle were both negative for drostanolone and drostanolone enantate, roughly corresponding to the period the subject was in jail. Testosterone concentrations were less than 1 pg/mg in the 3

Table 1 GC-MS/MS mass spectrometric data for the tested compounds.

Drug	Transitions m/z	Collision energy eV		Retention time min
Drostanolone	448.0 > 141.0448.0 > 156.0	20	20	10.94
Drostanolone enantate	488.4 > 141.1488.4 > 156.1	25	15	15.42
Testosterone-d3	435.4 > 209.1	15		10.89

Table 2 GC-MS/MS validation parameters in hair.

Parameters	Drostanolone	Drostanolone enantate
Linearity	1 to 100 pg/mg, $r^2 = 0.9998$	5 to 100 pg/mg, $r^2 = 0.9992$
Limit of detection	0.5 pg/mg	2 pg/mg
Limit of quantitation	1 pg/mg	5 pg/mg
Precision (20 pg/mg, $n = 8$)	16.2%	17.9%
Matrix effect (20 pg/mg, $n = 15$)	< 20%	< 20%

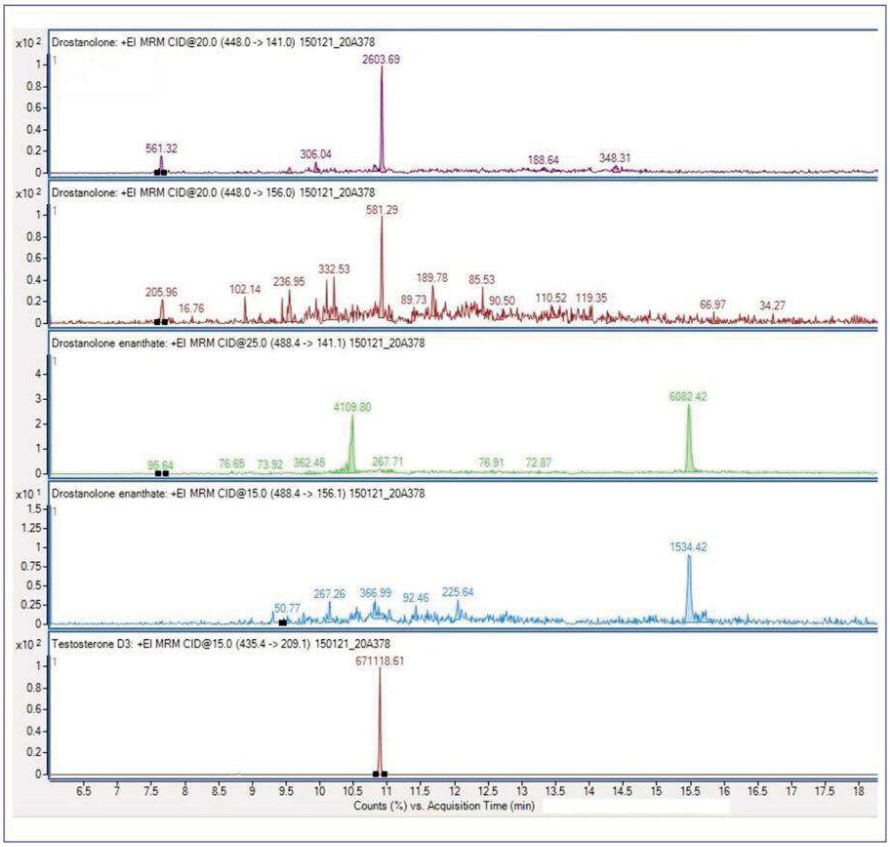


Figure 1. GC-MS/MS chromatogram obtained after extraction of the distal hair segment of the suspect. Drostanolone and drostanolone enantate concentrations were 3 and 14 pg/mg, respectively. From top to bottom: 2 transitions for drostanolone (11.03 min, m/z 448.0 > 141.0 and 448.0 > 156.0), 2 transitions for drostanolone enantate (15.51 min, m/z 488.4 > 141 and 488.4 > 156.1) and 1 transition for testosterone-d3 (10.92 min, IS, m/z 435.4 > 209.1).

segments, which is the limit of quantitation of the method. Anabolic steroids have been seldom reported in hair and, in most cases, bodybuilders have been tested. Only Thieme and Anielski [11], in their compendium presented concentrations for drostanolone and drostanolone enantate, which were 4 and 30 pg/mg in one case and 2 and 2 pg/mg in a second one.

Drostanolone, or dromostanolone, is a potent synthetic androgenic anabolic steroid. Currently, there is no human indication for drostanolone, either in the United States or in European countries. In the past, it has been indicated in postmenopausal women with recurrent breast cancer, in a combined hormone therapy.

As drostanolone has the potential to be misused for performance enhancement in sport, it can attract athletes looking for power and strength improvement. As a consequence, drostanolone has been prohibited by the World Anti-Doping Agency (WADA) at-all-times in the category of ''anabolic agents'' under section S1 of the WADA List. On the WADA 2019 testing figures report [12], there were 4180 adverse analytical findings (AAF) and among them, 163 were for drostanolone. This indicates that drostanolone is easy to obtain and is largely abused.

According to witnesses, the murderer was aggressive at the time of the assault. The test in an alternative matrix, i.e. scalp hair, allowed confirming long-term drostanolone abuse. This seems of major importance to establish a clear picture of drug involvement. However, the authors can accept that some parameters about the detection of drostanolone in hair have not been addressed: dose necessary to give a positive result, repeatability, robustness, contamination, carry-over, interferences, variable incorporation into hair, external factors that may have an impact, etc. The minimum dose that had to be ingested by a subject in order to get a ''positive'' detection in one of the hair sections is unknown.

Conclusion

Since many years, numerous publications have indicated that there is an association between the abuse of anabolic steroids and violence. Abusers of steroids are more prone to be involved in criminal acts, as demonstrated by epidemiological studies. However, in all these reports, none are supported by toxicological analyses, which seem to be the basis in forensic science. The authors have presented toxicological investigations in a murderer who was abusing drostanolone at the time of the assault. This situation appears to be highly suitable for the trial hearing by demonstrating that a prohibited and harmful substance was involved.

Disclosure of interest

The authors declare that they have no competing interest.

References

[1] Cadwallader AB, Lim CS, Rollins DE, Botrè F. The androgen receptor and its use in biological assays: looking toward effect-based testing and its applications. J Anal Toxicol 2011;35:594—607.
[2] Van Amsterdam J, Opperhuizen A, Hartgens F. Adverse health effect of anabolic-androgenic steroids. Regul Toxicol Pharmacol 2010;57:117—23.
[3] Trenton AJ, Currier GW. Behavioural manifestations of anabolic steroid use. CNS Drugs 2005;19:571—95.
[4] Pope HG, Kouri EM, Hudson JI. Effects of supraphysiologic doses of testosterone on mood and aggression in normal men: a randomized controlled trial. Arch Gen Psychiatry 2000;57:133—40.
[5] Khoodoruth MAS, Khan AA. Anabolic steroids-induced delirium. Medicine 2020, http://dx.doi.org/10.1097/MD.0000000000021639.
[6] Lood Y, Eklund A, Garle M, Ahlner J. Anabolic androgenic steroids in police cases in Sweden 1999-2009. Forensic Sci Int 2012;219:199—204.
[7] Beaver KM, Vaughn MG, Delisi M, Wright JP. Anabolic-androgenic steroid use and involvement in violent behaviour in a nationally representative sample of young adult males in the United States. Am J Public Health 2008;98:2185—7.
[8] Skärberg K, Nyberg F, Engström I. Is there an association between the use of anabolic-androgenic steroids and criminality? Eur Addict Res 2010;16:213—9.
[9] Kintz P, Gheddar L, Raul J-S. Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis. Drug Test Anal 2021, http://dx.doi.org/10.1002/dta.3020.
[10] Kintz P. Hair analysis in forensic toxicology: an updated review with special focus on pitfalls. Curr Pharm Des 2017;23:5480—6.
[11] Thieme D, Anielski P. Doping, application of hair analysis. In: Kintz P, Salamone A, Vincenti M, editors. Hair analysis in clinical and forensic toxicology. London: Academic Press; 2015. p. 275—99, https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf (Accessed 24 January 2021).

Article 11 : Forensic investigations in a case of aggressive behavior of three dogs : identification of dietary supplements contamination by metandienone and confirmation by hair tests

Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz

Forensic Science International: Animals and Environments. 2021 ; doi:10.1016/j.fsiae.2021.100022.

Forensic Science International: Animals and Environments 1 (2021) 100022

Contents lists available at ScienceDirect

Forensic Science International: Animals and Environments

journal homepage: www.sciencedirect.com/journal/forensic-science-international-animals-and-environments

Case Report

Forensic investigations in a case of aggressive behavior of three dogs: Identification of dietary supplements contamination by metandienone and confirmation by hair tests

Laurie Gheddar [a,*], Jean-Sébastien Raul [a], Pascal Kintz [a,b]

[a] Institut de Médecine Légale, 11 rue Humann, 67000, Strasbourg, France
[b] X-Pertise Consulting, 42 rue Principale, 670206, Mittelhausbergen, France

ARTICLE INFO

Keywords:
Anabolic steroid
Aggressive behavior
Hair
Dog
Dietary supplement contamination

ABSTRACT

After observing a modification in behavior of his three dogs, an owner consulted a laboratory to determine if a drug could be involved. Hair strands of each animal were collected from the neck area and sent to the laboratory for anabolic steroid screening. Initial screening by liquid chromatography coupled to high-resolution mass spectrometry identified the presence of metandienone, an anabolic androgenic steroid, in all three samples. In order to quantify metandienone in hair, a specific method was developed using a liquid chromatography to tandem mass spectrometry system. In addition, sixteen animal pharmaceutical products and food supplements that the dogs had received were tested for a possible source of contamination.

The hair specimens of the dogs tested positive for metandienone at 42, 13 and 32 pg/mg. Final interpretation of these concentrations was difficult as there is no data about metandienone detection in hair collected from dogs. Analysis of the animal pharmaceutical and food supplements demonstrated the presence of metandienone in two supplements, with amounts at 25 and 452 ng/g.

Simultaneous analyses of the hair of the dogs and their supplements enabled to make an association with the observed aggressive behavior in the dogs. Testing for anabolic steroids in the hair of the dog in case of aggressiveness can be useful to help to understand an unexpected behavior in dogs.

1. Introduction

Doping agents can be used in human as well as animal sports. As in human competitions, the use of performance-enhancing drugs in animal sports is prohibited in competition and even out-of-competition for some products [1]. In these sports, including horse or camel racing, greyhound dog racing or pigeon racing, doping agents are used to illicitly improve an animal's physical performance (i.e. increase of muscle mass, decrease of fat) and to modify behavior (increase courage and endurance) [2]. For example, corticosteroids are some of the more popular doping agents used in animal sports, especially for horse racing. Numerous doping cases involving horses, pigeons or camels have been reported in literature [1,3,4].

In animal sports, anti-doping analyses are carried out mainly on urine because this matrix is non-invasive [1]. Nevertheless, using urine has two major disadvantages: 1) the drug is excreted in free form or as conjugated metabolites; 2) the parent drug is generally present in small amounts [1]. Therefore some researchers have preferred to use keratinous specimens such as hair, nail, hoof and even feathers because it is a non-invasive matrix which allows testing for the parent drug. For example, analysis in pigeon feathers was developed for doping purposes to analyze for corticosteroids [3]. In other cases hair specimens of horses, camels or even dogs were used to test for glucocorticoids and anabolic steroids [1,4].

Anabolic androgenic steroids (AAS) are largely misused in animal sports. Some authors have described the analysis of AAS in greyhound dog [5] or in racing pigeons [3]. Beside the doping issues, the misuse of these drugs can be a forensic problem, i.e. lead to physical and psychological health problems. Physical side effects of misuse of AAS are well described in humans including high blood pressure, edema, and cardiac diseases [6,7]. Psychological side effects such as delirium, violence (homicide, suicide), mania and depression have also been reported after a repetitive use of AAS [7–9]. For example, aggressive behaviors have been described for human in the scientific literature after

* Corresponding author.
E-mail address: l.gheddar@gmail.com (L. Gheddar).

https://doi.org/10.1016/j.fsiae.2021.100022
Received 15 December 2020; Received in revised form 18 May 2021; Accepted 2 July 2021
Available online 13 July 2021

Table 1
Supplementary analysis on veterinary products and dietary supplements (ND: not detected, with a limit of positivity at 1 ng/g).

Products	Nature	Metandienone (quantity)
1	**Dietary supplement (protein)**	**452 ng/g**
2	**Dietary supplement (vitamin)**	**25 ng/g**
3	Intestinal hygiene	ND
4	Anti-Flatulence tablets	ND
5	Digestive supplement	ND
6	Antibacterial and anti-inflammatory gel	ND
7	Non-steroidal anti-inflammatory	ND
8	Non-steroidal anti-inflammatory	ND
9	Vitamins	ND
10	Bone Builder (complementary feed)	ND
11	Dietary supplement	ND
12	Dietary supplement	ND
13	Dietary supplement	ND
14	Dietary supplement	ND
15	Vermifuge	ND
16	Vermifuge	ND

using a testosterone esters mixture [9]. This can be extended to animals. A study has highlighted that AAS interact indirectly with the serotonergic and dopaminergic systems to modulate aggressive behavior in Syrian hamsters [10]. Another case of aggressiveness was published for lizards. In this paper, the authors administered testosterone to lizards, studied their behavior and concluded that testosterone administration had modified their behavior and increased their aggressiveness [11]. The misuse of AAS has also been described for fighting dogs. People administer AAS to their dogs to improve their aggressiveness during fighting which enhances the value of animals [12].

In a laboratory of forensic toxicology, it could be of interest to test for AAS in hair specimens when a modification of behavior is suspected or when an unusual aggressive behavior is observed. In this paper, the authors report a case of aggressive behavior in association with the administration of supplements contaminated with metandienone, an anabolic steroid, in dogs.

2. Material and method

2.1. Case report

Three healthy adult dogs became suddenly aggressive towards each other and towards their owner. Observing these unusual behaviors, the owner consulted with a laboratory of toxicology if a drug could be involved to explain this aggressive behavior. The laboratory proposed the collection of one hair strand for each animal. The three samples were cut as close as possible from the skin, in the neck region, at home and received at the laboratory in three separate plastic bags, which were stored at ambient temperature until analysis. Hair strands were brown, blond and dark in color and were 1 to 2 cm in length. In agreement with the request, a screening of AAS was carried out on liquid chromatography coupled to high-resolution mass spectrometry system, which showed the possible presence of metandienone, also known as methandrostenolone. This drug is sold under the name Danabol® or Dianabol® on the internet and available as tablets (2.5–50 mg) for human oral administration [13].

Therefore, the authors developed a specific quantitative method for metandienone in hair using a liquid chromatography to tandem mass spectrometry system (UHPLC–MS/MS).

2.2. Chemicals and reagents

Metandienone and testosterone-D_3 were obtained from Cerilliant – Sigma Aldrich (Steinheim, Germany) and from Sigma Aldrich (Steinheim, Germany), respectively. Ammonium acetate for HPLC, dichloromethane and heptane were purchased from Merck (Darmstadt, Germany) and methanol (MeOH) and acetonitrile (ACN) for HPLC Isocratic Grade from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Sodium hydroxide (NaOH) was purchased from Merck (Darmstadt, Germany). The standard solutions, prepared in MeOH, were stored at − 20 °C.

2.3. Hair testing

Hair strands were first decontaminated twice with 5 mL of dichloromethane for 2 min at room temperature and dried. The two solutions of decontamination bath were combined and stored at 4 °C until their analyses. Then, hair strands were cut into very short segments (< 1 mm). 30 mg of decontaminated hair were incubated in 1 mL NaOH for 10 min at 95 °C, in the presence of 10 ng of testosterone-D_3, used as internal standard. Then, after cooling, 5 mL of heptane were added. After agitation for 15 min and centrifugation (15 min, 3000 rpm), the organic phase was collected and evaporated to dryness under a nitrogen flow. The dry residue was dissolved in 30 µL of MeOH, and 2 µL was injected into the UHPLC-system.

For method validation, blank hair from laboratory staff was used after checking the absence of response at the retention times of metandienone and the internal standard.

2.4. Supplements analysis

In order to explain the presence of metandienone in the three hair samples, the owner requested, in agreement with the toxicologist, additional analyses of animal pharmaceutical and supplements, regularly administered with standard food. These supplements had been fed to dogs for approximately 6 months. In total, sixteen products (Table 1) were ground and/or diluted in MeOH (according to the type of samples), mixed, vigorously agitated, centrifuged and then diluted in MeOH to obtain a concentration at 1 g/L. After a second dilution in MeOH (10 mg/L), the solutions were injected into the UPLC-MS/MS system and compared with a reference solution of metandienone at 10 mg/L in MeOH.

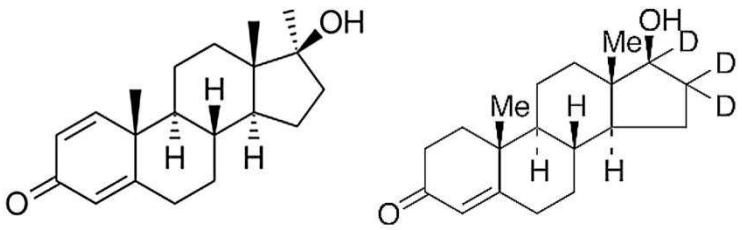


Fig. 1. From left to right: metandienone (300.4 g/moL) – testosterone-D_3 (291.4 g/moL).

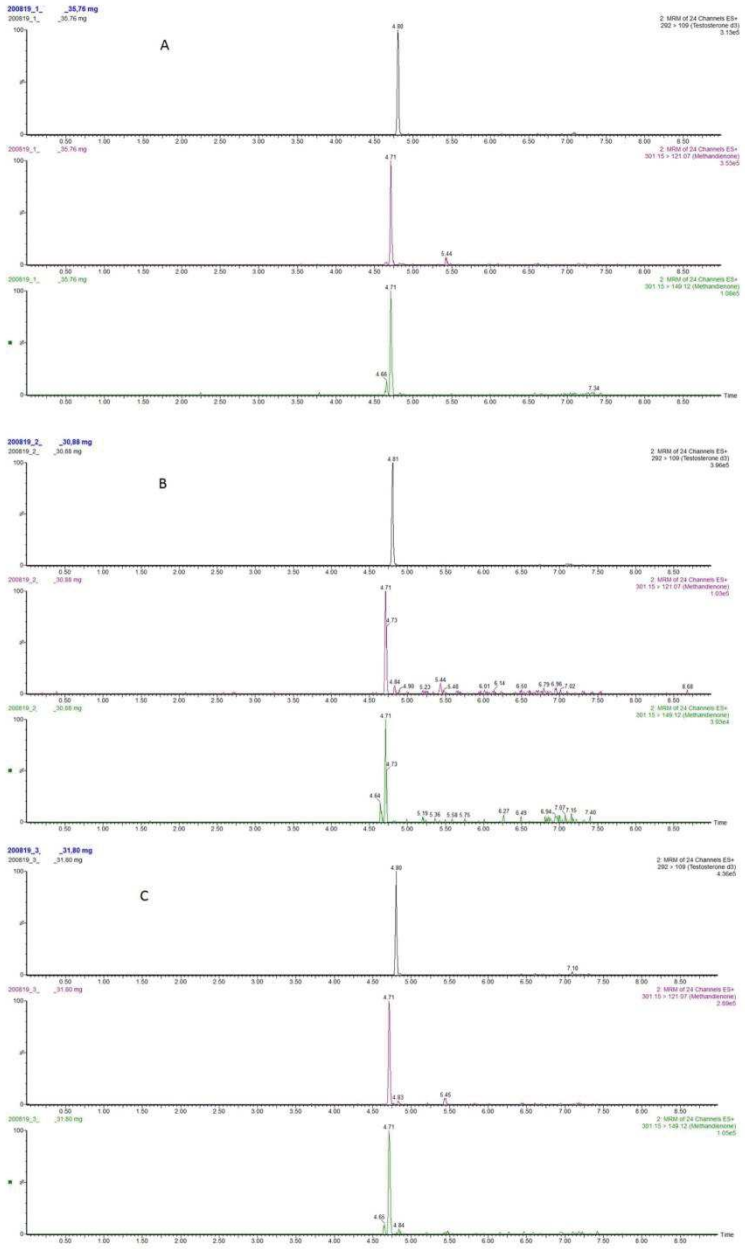


Fig. 2. Chromatograms of the hair strands of 3 dogs (from top to bottom: 1 transition of testosterone-D_3, 2 transitions of metandienone). Metandienone concentrations were: A – 42 pg/mg; B – 13 pg/mg; C – 36 pg/mg.

2.5. Toxicological analysis

Chromatographic separations were performed with a liquid chromatography Waters Acquity UPLC™ system (Waters corporation, Milford, MA, USA) using an Acquity UPLC HSS C18 column (150 mm x 2.1 mm, i.d. 1.8 µm particle size). The column temperature was set to 50 °C, and the flow rate at 0.4 mL/min. The mobile phase consisted of 1.0 mM ammonium acetate in water (eluent A) and ACN with 0.1 % formic acid (eluent B). Analysis time was 9.0 min, and the following gradient pattern (eluent A) was used for the separation of metandienone and testosterone-D3: 0 min, 90 %; 1.00 min, 90 %; 6.00 min, 0 %; 6.50 min, 0%; 4.60 min, 90 %; 9.00 min, 90 %. Metandienone and testosterone-D_3 were eluted in 4.71 and 4.80 min respectively. Detection was performed using a triple quadrupole (XEVO™ TQD, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionization source (ESI) and used in the positive mode (ES+). Nitrogen was used as drying gas as well as nebulizing gas. The desolvation gas flow was set to 800 L/h and the desolvation temperature was 400 °C. The source temperature was 150 °C. The capillary voltage was set at 1.50 kV. Cone voltage and collision energy were adjusted to optimize the signal of the 2 most abundant product ions of metandienone: m/z 301.2 > 121.1 and 301.2 > 149.1 and for testosterone-D_3: m/z 292 > 109. MassLynx 4.1 software was used for quantification.

3. Results and discussion

3.1. Hair analysis

Linearity for metandienone in hair was observed for concentrations from 5 to 500 pg/mg with a correlation coefficient of 0.9989. Testosterone-D_3 was used as internal standard because its chemical structure is similar to the one of metandienone (Fig. 1). A weighted linear regression of 1/x was applied. The limit of detection was evaluated at 3 pg/mg with a S/N ratio of 3. The limit of quantification was the first point of the calibration (5 pg/mg). Quality control samples (5 pg/mg and 100 pg/mg) were analyzed in duplicate in 6 independent experimental assays and were used to determine the coefficients of variation for precision and accuracy, which were found to be lower than 20 %. Matrix effect was evaluated with 10 blank hair specimens and was found lower than 15 %.

The hair of the three dogs tested positive for metandienone at 42, 13 and 36 pg/mg. Chromatograms obtained after the extraction of the three hair specimens are presented in Fig. 2. Decontamination baths were analyzed and were found negative for metandienone.

Although frequently observed in cases of anti-doping rules violation, metandienone has been seldom reported in hair. For human, in the literature, metandienone was identified at 7–108 pg/mg in the hair of a top athlete and was considered representative of a doping practice [14]. Two other authors have published a series of hair test results, where metandienone was identified in human hair in the range from 4 to 2800 pg/mg [15,16]. Unfortunately, results for animal are more limited and only two authors have described hair concentrations for metandienone in animals. One author reported a concentration at 15.2 pg/mg in a horse hair specimen [17] and a second author found metandienone at about 3000 pg/mg in the hair of a guinea pig four days after one single injection [18].

Nevertheless, there is no data in the literature dealing with the identification of metandienone in hair collected from a dog. According to a recent study, the growth rate of the hair of the dog is around 0.065 mm/day corresponding approximately to a growth period of 5 months. In our cases, dogs have been exposure 5–10 months according to the length of hair specimens. However, the growth rate depends on the breed of dog [19]. Given the lack of literature, one cannot be more accurate in the interpretation of the results in dog hair. Therefore, in this case, concentrations of metandienone are difficult to interpret in terms of dosage and frequency of exposures. However, it is now possible to confirm that metandienone is detectable in hair collected from dogs.

3.2. Supplement analysis

With respect to the analysis of animal pharmaceutical products and dietary supplements, results are summarized in Table 1. Two products from sixteen were positive for metandienone. These products were advertised for containing proteins and vitamins. Analysis of a standard reference at 10 mg/L enabled to estimate the dosages and metandienone was quantified at 25 and 452 ng/g in each supplement. In the literature, cases of contamination of dietary supplements by metandienone have been reported. For example, Odoardi et al. have found metandienone in three dietary supplements for human, whereas this AAS was not labelled on the list of ingredients. Metandienone tested positive in three fat burners and energy enhancers at 130–8850 ng/g [20]. Another study had showed that metandienone was present at about 50 ng/g in several products such as vitamin C [21]. According to Geyer et al., several products can be processed on the same production line [21]. Therefore, the presence of metandienone in these two supplements may be due to a cross-contamination between the production of supplements and metandienone.

In view of the results of these dietary supplements, regularly consumed by the dogs in addition to their food, it is possible to link the presence of metandienone in their hair of the three dogs and to speculate about their aggressive behavior [11].

In further support of the association between the supplements and the dogs' behavior, the owner reported that the dogs were back to their normal behavior four months after withdrawal of the contaminated supplements.

4. Conclusion

After noticing a marked change in behavior of the three dogs, further investigations were carried out, including hair and nutritional supplements analysis. A return to normal behavior was observed after the supplements were stopped. These different investigations and observations allowed us to identify the cause of this unexpected situation. As has been observed on many occasions for humans with potential anti-doping rules violations the use of supplements can be an issue when not properly prepared and controlled. Indeed, dietary supplements can contain drugs that are not labelled on the list of ingredients and can be the source of various disorders in behavior.

Declaration of Competing Interest

The authors report no declarations of interest.

References

[1] J. Segura, R. Ventura, M. Gonzalez, C. Jimenez, Doping substances in human and animal sport, in: Handbook of Analytical Separations, Chapter 15, Vol. 2, Elsevier, London UK, 2000, pp. 531–566.
[2] L. Thippeswamy, A. Pathak, C. Patil, R. Saika, S. Choudhury, A. Shukla, Doping in animals: a concise outlook, Int. J. Sci. Environ. Technol. 7 (2018) 1427–1430.
[3] F.X. Moreira, H. Carmo, A. Melo, R. e Silva, Z. Azevedo, M.L. Bastos, P.G. de Pinho, The use if feathers from racing pigeons for doping control purposes, J. Anal. Toxicol. 43 (2019) 307–315, https://doi.org/10.1093/jat/bky088.
[4] S.S. Ashraf, W. El-Gabany, A. Alraeesi, L. Al-Hajj, A. Al-Maidalli, I. Shah, Analysis of illicit glucocorticoid levels in camel hair using competitive ELISA – comparison with LC-MS/MS, Drug Test. Anal. 12 (2020) 458–464, https://doi.org/10.1002/dta.2750.
[5] B. Gray, L. Tuckley, C. Cutler, S. Biddle, S. Hudson, S. Gower, L. Vanhaecke, Investigations into the analysis of intact drug conjugates in animal sport doping control – development and assessment of a rapid and economical approach for screening greyhound urine, Drug Test. Anal. 12 (2020) 731–742, https://doi.org/10.1002/dta.2779.
[6] P. Kintz, L. Gheddar, A. Ameline, V. Dumestre-Toulet, M. Verschoore, J. Comte, J. S. Raul, Complete post-mortem investigation in a death involving clenbuterol after long-term abuse, J. Anal. Toxicol. 43 (2019) 660–665, https://doi.org/10.1093/jat/bkz058.

[7] Q. Zhang, S. Shan Khine, A. Raza, N. Manda, T. Nace, A rare case and literature review of anabolic-androgenic steroids (AAS)-induced acute myocardial infarction, Cureus 12 (2020), e8332, https://doi.org/10.7759/cureus.8332.
[8] K.T. Ganson, T.J. Cadet, Exploring anabolic-androgenic steroid use and teen dating violence among adolescent males, Subst. Use Misuse 54 (2019), https://doi.org/10.1080/10826084.2018.1536723.
[9] M.A.S. Khoodoruth, A.A. Khan, Anabolic steroids-induced delirium, Medicine 99 (2020), e21639, https://doi.org/10.1097/MD.0000000000021639.
[10] R.J. Harrison, D.F. Connor, C. Nowak, K. Nash, R.H. Melloni Jr., Chronic anabolic-androgenic steroid treatment during adolescence increases anterior hypothalamic vasopressin and aggression in intact hamsters, Psychoneuroendocrinology 25 (2000) 317–338, https://doi.org/10.1016/s0306-4530(99)00057-8.
[11] B.S. Done, H. Heatwole, Effetcs of hormones on the aggressive behaviour and social organization of the scincid lizard, Sphenomorphus kosciuskoi, Z. Tierpsychol. 44 (1977) 1–12, https://doi.org/10.1111/j.1439-0310.1977.tb00981.x.
[12] H. Lawson, Dangerous Dogs "Injected with Steroids to Increase their Value for Fighting and Intimidation, Daily mail., 2013. https://www.dailymail.co.uk/news/article-2304296/Dangerous-dogs-injected-steroids-increase-value-fighting-intimidation.html.
[13] R.C. Baselt, Disposition of Toxic Drugs and Chemicals in Man, 12th edition, Biomedical publications, Seal Beach, CA, 2020, p. 1281.
[14] M. Bresson, V. Cirimele, M. Villain, P. Kintz, Doping control for metandienone using hair analysed by gas chromatography-tandem mass spectrometry, J. Chromatogr. B 836 (2006) 124–128, https://doi.org/10.1016/j.jchromb.2006.03.040.
[15] D. Thieme, P. Anielski, Doping, applications of hair analysis. Hair Analysis in Clinical and Forensic Toxicology, Chapter 10, Academic Press, Elsevier, London UK, 2015, pp. 275–299.
[16] P. Kintz, A new series of hair test results involving anabolic steroids, Toxicologie Analytique et Clinique 29 (2017) 320–324, https://doi.org/10.1016/j.toxac.2017.05.003.
[17] Y. Gaillard, F. Vayssette, A. Balland, G. Pepin, Gas chromatographic-tandem mass spectrometric determination of anabolic steroids and their esters in hair. Application in doping control and meat quality control, J. Chromatogr. B 735 (1999) 189–205, https://doi.org/10.1016/s0378-4347(99)00416-8.
[18] M. Shen, P. Xiang, H. Yan, B. Shen, M. Wang, Analysis of anabolic steroids in hair: time courses in guinea pigs, Steroids 74 (2009) 773–778, https://doi.org/10.1016/j.steroids.2009.04.008.
[19] M.D. Schuldenfrei, J.B. Pieper, Evaluation of hair follicle parameters using TrichoScale Pro in healthy dogs: a pilot study, Vet. Dermatol. 31 (2020) 181–e137, https://doi.org/10.1111/vde.12836.
[20] S. Odoardi, E. Castrignano, S. Martello, M. Chiarotti, S. Strano-Rossi, Determination of anabolic agents in dietary supplements by liquid chromatography-high-resolution mass spectrometry, Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 32 (2015) 635–647, https://doi.org/10.1080/19440049.2015.1014868.
[21] H. Geyer, M.K. Parr, K. Koehler, U. Mareck, W. Schänzer, M. Thevis, Nutritional supplements cross-contaminated and faked with doping substances, J. Mass Spectrom. 48 (2008) 892–902, https://doi.org/10.1002/jms.1452.

Troisième partie – Les autres agents anabolisants

3.1. Présentation

La classe S1.2 comme définie par l'AMA, « autres agents anabolisant », regroupe le clenbuterol, la tibolone, le zéranol, le zilpatérol et les SARMs. Cette liste n'est pas exhaustive *[Liste d'interdiction AMA, 2021]*.

Au vu des cas rencontrés et des analyses demandées, mes travaux m'ont permis de travailler plus particulièrement sur le clenbutérol et sur les SARMs.

3.1.1. Le Clenbutérol

Le clenbutérol est un β-agoniste développé initialement à des fins vétérinaires. Il est régulièrement retrouvé dans la viande. L'AMA a ainsi annoncé et prévenu que la viande consommée en Chine ou au Mexique pouvait mener à une contamination par du clenbuterol *[WADA, 2019]*. Il est utilisé en complément des SAA comme brûleur de graisse par les sportifs amateurs et de haut niveau. Dans la littérature, plusieurs effets secondaires sur la santé sont décrits comme une hypokaliémie ou des décès par vasospame coronarien *[Dufayet, 2020]*. Généralement utilisé en combinaison avec les SAA, le clenbutérol a été intégré à la méthode de criblage des stéroïdes anabolisants (comme décrit en partie 2).

Le cas décrit ci-dessous est un cas de décès associant l'usage du clenbutérol et du stanozolol, un stéroïde anabolisant :

Un homme de 61 ans est retrouvé décédé chez lui autour de minuit en position allongée par sa femme. Cet homme est connu pour s'entraîner régulièrement en salle de musculation et est connu pour utiliser de façon régulière des SAA. La police a retrouvé chez lui deux produits : le Clenox® (clenbutérol) et du Stanox-10® (stanozolol). L'analyse des produits a montré que les quantités retrouvées n'étaient pas celles indiquées sur les étiquettes (7,3 µg/g pour le clenbutérol et 11,5 mg/g pour le stanozolol au lieu de 40 µg/g et 10,0 mg/g). A l'autopsie, une cardiomégalie ainsi qu'une congestion multi viscérale sont observés. L'analyse toxicologique a mis en évidence uniquement du clenbutérol et du stanozolol. Le clenbutérol a été identifié dans le sang (1,1 ng/mL), les urines (7,2 ng/mL), la bile (2,4 ng/mL), le contenu gastrique (3,2 ng/mL) et les cheveux (23 pg/mg). Quant au stanozolol, il a été identifié uniquement dans les cheveux (11 pg/mg). Les experts ont conclu à une insuffisance cardiaque dans un contexte d'abus de SAA.

Ce cas est un exemple de l'intérêt de travailler en collaboration avec les forces de l'ordre, les médecins légistes mais également avec les anatomopathologistes lorsque cela est possible ***[Article 12]***.

Article 12 : Complete post-mortem investigations in a death involving clenbuterol after long-term abuse

Pascal Kintz, Laurie Gheddar, Alice Ameline, Véronique Dumestre-Toulet, Marion Verschoore, Julien Comte, Jean-Sébastien Raul

Journal of Analytical Toxicology. 2019 ;43(8) :660-665.

Journal of Analytical Toxicology, 2019;1–6
doi: 10.1093/jat/bkz058
Case report

Case report

Complete Post-mortem Investigations in a Death Involving Clenbuterol After Long-term Abuse

Pascal Kintz[1,2,*], Laurie Gheddar[2], Alice Ameline[2], Véronique Dumestre-Toulet[3], Marion Verschoore[4], Julien Comte[4] and Jean-Sébastien Raul[2]

[1]X-Pertise Consulting, 42 Rue Principale, F-67206 Mittelhausbergen, France [2]Institut de Médecine Légale, 11 Rue Humann, F-67000 Strasbourg, France [3]Laboratoire ToxGen, 11 Rue du Commandant Cousteau, F-33100 Bordeaux, France [4]Unité de Medicine Légale, Place Amélie Raba-Léon, F-33000 Bordeaux, France

*Author to whom correspondence should be addressed. Email: pascal.kintz@wanadoo.fr

Abstract

The body of a 61-year-old man was found at his home by his wife, lying on the floor, near the bathroom, around midnight. He was known to be training for bodybuilding, using anabolic steroids. Police investigations revealed the presence of two types of tablets at home, one supposed to contain clenbuterol (0.040 mg) and the other stanozolol (10 mg). Testing the tablets revealed different dosages from what was expected, i.e., 0.073 and 11.5 mg/tablet, for clenbuterol and stanozolol, respectively. External body examination and autopsy, which was performed the next day, revealed generalized organ congestion and lack of any traumatic injury (confirmed by radiology). Cardiomegaly, with a heart weighing 692 g, was obvious. Anatomic pathology tests did not reveal evidence of malformations, but atheromatous plaque was identified in the coronaries during complete histology investigations. Femoral blood, urine, bile, gastric contents and two strands of hair (6 cm) were collected for toxicology. These specimens were submitted to standard analyses, but also to a specific LC–MS-MS method for clenbuterol and stanozolol testing. Clenbuterol was identified in all the tissues, including femoral blood (1.1 ng/mL), urine (7.2 ng/mL), bile (2.4 ng/mL), gastric content (3.2 ng/mL) and hair (23 pg/mg). Stanozolol only tested positive in hair (11 pg/mg). All other analyses were negative, including blood alcohol and drugs of abuse. The pathologists concluded to cardiac insufficiency with support of cardiomegaly, in a context involving repetitive abuse of anabolic drugs. This case indicates that more attention should be paid to clenbuterol, a drug widely used as a stimulant by people who want to lose weight, athletes and bodybuilding practitioners.

Introduction

Clenbuterol is a long-acting, oral β2-adrenergic partial agonist that stimulates the receptors to relax bronchial smooth muscle, inhibits the release of inflammatory mediators, decreases mucus production and may increase the rate of mucociliary transport in the airways. In addition, clenbuterol decreases body fat and increases muscle mass, which has prompted its abuse in the athletes and, particularly, in the bodybuilders community. Abuse has also been observed in subjects who want to lose weight (1). Clenbuterol is often presented as a substitute for anabolic steroids (2).

Currently, there is no human indication for clenbuterol, either in the USA or in most European countries. Some countries can sell it as a prescription drug for the treatment of acute asthma exacerbations. Several case reports, involving users of clenbuterol-adulterated heroin (3), subjects eating contaminated livestock (4), and mostly bodybuilders (5–7) to gain mass or to lose weight, have been described in the scientific international literature.

Proposed for the treatment of reactive airway disease, clenbuterol has a similar chemical structure of salbutamol, but is 100 times more potent. Its anabolic properties, and therefore its use as a performance-enhancing drug have resulted in numerous adverse analytical findings (AAF) during doping control. For example, in 2017, the World Anti-Doping Agency (WADA) reported 294 AAFs (7.2%) for clenbuterol over a total of 4076 AAFs (8).

Athletes can abuse clenbuterol using a tablet dosed at 40 µg, once or twice daily, but the drug is also available as syrup, injectable solutions and aerosols. In case of overdose, clenbuterol toxicity is first observed with headache, tachycardia, agitation, palpitations and tremor. At increased dosages, myocardial ischemia, as well as hypo-kalemia, lactic acidosis and hyper-glycemia can be observed. Other symptoms can be seizures, vomiting, anxiety or psychosis, myocardial infarction, ventricular arrhythmias or rhabdomyolysis (5–7, 9, 10). Even at low dosage (20 µg), clenbuterol can produce sinus tachycardia and electrolyte derangement (11).

In most of the intoxication cases, clenbuterol was not tested in biological fluids, thus a true 'therapeutic' or 'toxic' range of concentrations is difficult to obtain. Analytically confirmed reports of clenbuterol use are relatively rare, probably due to the very low concentrations found in blood or other biological specimens. Clenbuterol concentrations (n = 12) ranged from not detectable to 76 ng/mL in blood collected from contaminated heroin abusers (3). In a more recent series (12), clenbuterol serum concentrations (n = 7) ranged from 3 to 38 ng/mL caused by an adulterated heroin. Clenbuterol post-mortem concentrations in human specimens other than blood have never been reported.

The authors present here a fatal case involving clenbuterol, abused by a 61-year-old man for bodybuilding purposes.

Case report

The body of an athletic 61-year-old man (1.8 m tall, weighing 110 kg) was found at home by his wife around midnight, lying on the floor near the bathroom. She reported that her husband had experienced nausea and vomiting the evening before death. He was known to be training as a bodybuilder at a fitness center, using anabolic steroids at various intervals for at least 15 years. The day prior to his death, he had reportedly consumed several tablets (impossible to be more precise). Police investigations at home revealed the presence of two types of tablets (Figure 1A and B): one containing clenbuterol (Clenox, Malay Tiger, 0.04 mg) and the other containing stanozolol (Stanox, Malay Tiger, 10 mg). One tablet of each drug was tested at the laboratory by LC–MS-MS after weighing, crushing and appropriate dilution with methanol in presence of their deuterated analogs, and the dosages did not match the labels. The laboratory found 0.073 and 11.5 mg/tablet for clenbuterol and stanozolol, respectively. Other batches of these tablets were tested by the French national laboratory. Clenbuterol was identified at 0.22 mg/tablet, much more than the supposed dosage, while stanozolol was identified at 0.6 mg, much less than the supposed dosage. This prompted the French Agency of Pharmaceutical Security (ANSM) to release a press warning, alerting the public to the health dangers of using both products (13).

External body examination and autopsy, performed the next day, revealed generalized organ congestion and the lack of any traumatic injury (confirmed by radiology). Cardiomegaly, with a heart weighing 692 g, was obvious (normal weight for a male adult is in the range 250–350 g). Anatomic pathology tests did not evidence malformations, but atheromatous plaque was identified in the coronaries. They were associated with metastatic calcification. Pulmonary oedema

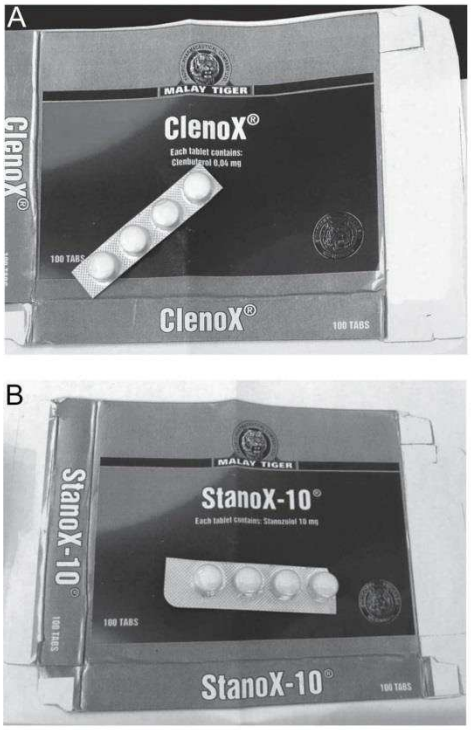


Figure 1. Pictures of confiscated tablets found at the home: (A) clenbuterol (Clenox) and (B) stanozolol (Stanox).

was also observed during the histology investigations. Non-specific congestion of liver and kidney was also noticed.

Femoral blood, urine, bile, gastric contents and two strands of hair (6 cm, dark) were collected for toxicology. All specimens but hair were stored at +4°C until analyses.

Material and methods

Toxicological initial screening

The case specimens were subjected to the laboratory's normal post-mortem toxicological screening battery. Ethanol was tested in blood and urine by head space GC/FID on a Perkin Elmer system (TurboMatrix 40 & Clarus 580) using a standard validated procedure. Carboxyhemoglobin was determined with a Radiometer ABL80. Volatiles and cyanides were tested by head space GC/MS on a Thermo system (Focus GC & DSQII) using standard validated procedures. ELISA tests for pharmaceuticals and drugs of abuse were achieved on blood and urine using NeoGen kits using the recommendations of the manufacturer. GHB was tested by GC/MS on an Aglilent 5971. Ethyl glucuronide was tested by LC–MS-MS on a Waters Quattro Micro. A comprehensive screening for pharmaceuticals and drugs of abuse, including New Psychoactive Substances (NPS) was performed on blood using LC/MS-MS (Waters Xevo TQD system). Due to the nature of the case, clenbuterol

and stanozolol were specifically tested by an Multtiple Reaction Monitoring (MRM) method using LC/MS-MS.

Clenbuterol analysis

Clenbuterol was extracted from 1 mL of fluid (blood, urine, bile, gastric content) in presence of 10 ng of clenbuterol-d_9 used as internal standard, with 1 mL borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 30 µL of ammonium formate buffer adjusted at pH 3.

Hair analysis of clenbuterol was performed after decontamination with dichloromethane. Twenty milligrams of cut hair were incubated overnight at 50°C in 1 mL borate buffer pH 9.5, in presence of 200 pg of clenbuterol-d_9. After incubation, the mixture was extracted as blood.

Chromatography was achieved using a Waters HSS C18 column (150 × 2.1 mm × 1.8 µm) maintained at 50°C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in LC/MS-grade acetonitrile (mobile phase B) at flow rate of 0.4 mL/min. The initial gradient was 87% mobile phase A and the final gradient, at 15 min, was 5% mobile phase A. An injection volume of 2 µL was used in all cases. A Xevo triple quadrupole mass spectrometer was used for the detection of the drugs. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of clenbuterol and the internal standard: capillary voltage at 1.5 kV, source block temperature at 150°C, desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple-reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision-induced dissociation of both species was performed. Collision energy was adjusted to optimize the signal for the 2 most abundant product ions of clenbuterol: *m/z* 279.1 > 204.9 (Collision Voltage (CV): 30 V, Collision Energy (CE): 16 eV) and 279.1 > 131.9 (CV: 30 V, CE: 16 eV) and the most abundant product ion of internal standard *m/z* 286.1 > 203.9 (CV: 26 V, CE: 16 eV). Transition *m/z* 279.1 > 204.9 was used for quantification of clenbuterol. Although the molecular weight of clenbuterol is 277.1 g/mol, the method development report (IntelliStart, Waters) indicated, after drug infusion, a parent *m/z* at 279.1. MassLynx 4.1 software was used for quantification.

Linearity was observed in blood for clenbuterol concentrations ranging from 1 to 100 ng/mL, with a correlation coefficient of 0.9999. The limit of detection was estimated to be 0.1 ng/mL, with an S/N ratio of 3. Quality Controls (QC) samples (5 ng/mL and 20 ng/mL), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision. These CVs were lower than 20%. Other liquid matrices were tested using the blood calibration.

Linearity was observed in hair for clenbuterol concentrations ranging from 1 to 100 pg/mg, with a correlation coefficient of 0.9989. The limit of detection was estimated to be 0.1 pg/mg, with an S/N ratio of 3. QC samples (5 pg/mg and 20 pg/mg), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision. These CVs were lower than 20%.

Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood or in hair. Recoveries (after spiking the specimens) of clenbuterol for both blood (at 10 ng/mL) and hair (at 10 pg/mg) were ~75%, which was found suitable for the purposes of the post-mortem tests. Once in the auto-sampler, analytes were found stable for at least 48 hours.

Stanozolol analysis

The method for stanozolol was modified from our previous paper (14). Briefly, stanozolol was extracted from 1 mL of fluid (blood, hydrolysed urine with ß-glucuronidase, bile, gastric content) in presence of 20 ng of stanozolol-d_3 used as internal standard, with 1 mL 1 M natrium hydroxide solution and 5 mL of ether. After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 30 µL of ammonium formate buffer adjusted at pH 3.

Hair analysis of stanozolol was performed after decontamination with dichloromethane. Thirty milligrams were incubated for 15 min at 95°C in 1 mL 1 M natrium hydroxide solution, in presence of 300 pg of stanozolol-d_3 and then, the mixture was extracted as blood.

Collision energy was adjusted to optimize the signal for the two most abundant product ions of stanozolol: *m/z* 329.3 > 81.1 (CV: 86 V, CE: 44 eV) and 329.3 > 121.1 (CV: 86 V, CE: 44 eV) and the most abundant product ion of internal standard *m/z* 332.3 > 81.1 (CV: 80 V, CE: 52 eV). Transition *m/z* 329.3 > 81.1 was used for quantification of stanozolol.

Linearity was observed in blood for stanozolol concentrations ranging from 1 to 50 ng/mL, with a correlation coefficient of 0.9956. The limit of detection was estimated to be 0.1 ng/mL, with a S/N ratio of 3. QC samples (5 ng/mL and 20 ng/mL), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision. These CVs were lower than 20%. Other liquid matrices were tested using the blood calibration.

Linearity was observed in hair for stanozolol concentrations ranging from 5 to 200 pg/mg, with a correlation coefficient of 0.99897. The limit of detection was estimated to be 0.5 pg/mg, with an S/N ratio of 3. QC samples (10 pg/mg and 50 pg/mg), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision. These CVs were lower than 20%.

Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood or in hair. Recoveries of stanozolol for both blood (at 10 ng/mL) and hair (at 10 pg/mg) were ~75%, which was found suitable for the purposes of the post mortem tests. Once in the auto-sampler, analytes were found stable for at least 48 hours.

Results and discussion

Ethanol tested negative in blood and urine. Ethyl glucuronide tested negative in urine. GHB was normal (4.1 mg/l) in blood. ELISA screenings were negative for pharmaceuticals and drugs of abuse. HbCO was 1.1%, and cyanides were at physiological concentrations (<80 ng/mL). The screening of blood by LC/MS-MS was negative. However, when testing the blood for clenbuterol and stanozolol by MRM, clenbuterol was identified at 1.1 ng/mL. Given this low concentration, it is not surprising that a screening method will not detect the drug. After a single administration of a 40 µg oral dose, the plasma level of clenbuterol reached maximum values of 0.2 ng/mL within 2.5 hours (15). After repeated administration of 40 µg oral doses twice daily, an average steady-state plasma level of 0.5–0.6 ng/mL has been observed in three healthy adults (15). In the

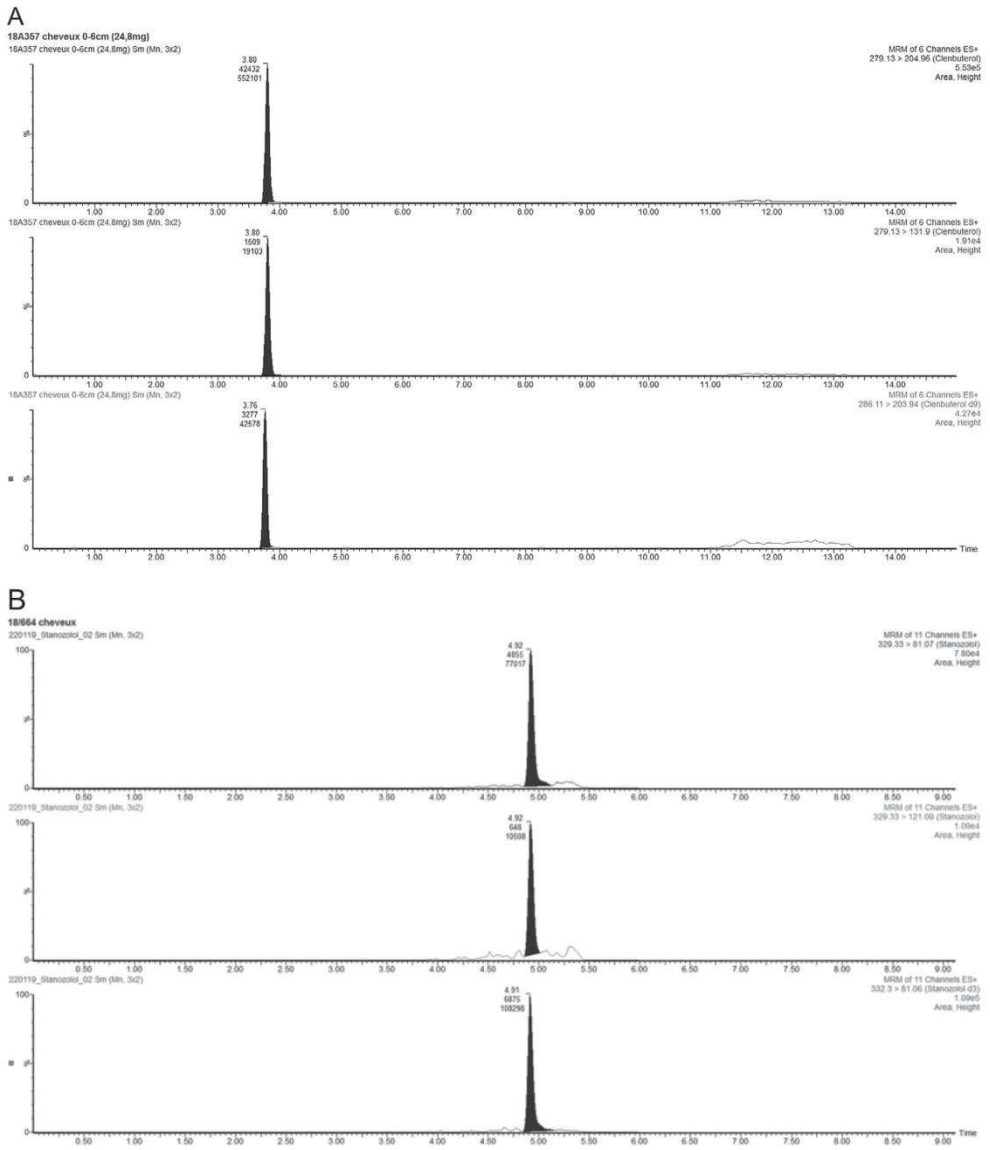


Figure 2. Chromatograms obtained after extraction of the hair of the victim. (A) Clenbuterol concentration was 23 pg/mg. From top to bottom: two transitions for clenbuterol and one transition for clenbuterol-d_9. (B) Stanozolol concentration was 11 pg/mg. From top to bottom: two transitions for stanozolol and one transition for stanozolol-d_3.

later study, the half-life of clenbuterol in plasma was estimated to be 35 hours. A 17-year-old adolescent who took 4000 µg with the goal of losing weight and was hospitalized for sinus tachycardia had a serum clenbuterol concentration of 5.9 ng/mL 7 hours post-ingestion. On the second day, her serum clenbuterol concentration was 2.6 ng/mL (16). In our case, low clenbuterol concentrations were also measured in urine (7.2 ng/ml), bile (2.4 ng/ml) and gastric content (3.2 ng/ml). It was not possible to back-calculate the total amount of clenbuterol, as the volume of the gastric content was not measured. All the data are presented in Table 1. The concentration found in the urine is

Table 1. Post-mortem Distribution of Clenbuterol and Stanozolol

Specimen	Clenbuterol	Stanozolol
Femoral blood	1.1 ng/mL	Not detected
Urine	7.2 ng/mL	Not detected
Bile	2.4 ng/mL	Not detected
Gastric content	3.2 ng/mL	Not detected
Hair	23 pg/mg	11 pg/mg

not surprising, as the cumulative urinary excretion of unchanged compound corresponded to ~20% of the administered dose as measured at 72 hours following a single oral administration (15). This is the first time that clenbuterol distribution is presented in a post-mortem bile specimen. A 2-fold concentration increase between blood and bile was observed. Bile sequestration of clenbuterol was already published in Holstein calves (17). No stanozolol was detected in blood, nor in any other bio-fluid, which should be consistent with a lack of exposure within the last days of life. There was no attempt to test the urine for 3-OH-stanozolol nor for the long-term metabolites of stanozolol, such as stanozolol glucuronide or stanozolol O- and N-sulfate metabolites as these drugs are not available as reference material for non-WADA laboratories.

The hair specimen (6 cm, dark) tested positive for both clenbuterol at 23 pg/mg and stanozolol at 11 pg/mg. Hair chromatograms are presented Figure 2A and B. A length of 6 cm can be representative of the past 6 months, assuming a growth rate of 1 cm per month. Clenbuterol was identified in the hair of contaminated subjects (Mexican soccer players) at low concentrations, between 0.02 and 1.90 pg/mg (18). In contrast, clenbuterol tested positive in the hair of six bodybuilders, at concentrations ranging from 15 to 122 pg/mg (19). Therefore, one can consider that the result of the hair test of the victim is in accordance with performance-enhancing practices on a long-term basis. The measured stanozolol concentration in the hair of the victim is in the range of previously published concentrations: 2–84 pg/mg [n = 4 (19)], 135–156 pg/mg [n = 2 (20)] and 17–1992 pg/mg [n = 9 (21)]. Stanozolol, 17a-methyl-17b-hydroxy-5a-andros-tano-(3,2-C)-pyrazole, has been one of the most abused anabolic steroids and is the unique drug of this class with a nitrogen atom in its chemical structure. For example, stanozolol ranked first in the S1.1 anabolic agents of the 2017 AAFs list of WADA (8), i.e., 284 cases from 4076, corresponding to 7% of the total AAFs. Athletes use stanozolol because it has been claimed that it increases lean body mass, increases strength, increases aggressiveness and leads to a shorter recovery time between workouts. The drug is available in oral and injectable forms, under the trade name Winstrol. Stanozolol has a particular chemical structure versus the other anabolic androgenic steroids, as it contains two nitrogen atoms. The structure of stanozolol differs from endogenous steroid hormones and from commercially available anabolic steroids as there is a pyrazole ring fused to the androstane ring system. As a consequence, stanozolol is less lipophilic than many other steroids (particularly those in the injectable form and/or in the ester forms) and therefore less prone to lipid deposition.

The combination of clenbuterol and stanozolol can be considered as a synergetic preparation. In this case, it was of paramount importance to test for both drugs in hair in order to document the case and the possible side effects responsible for enhancing the fatal process. Only clenbuterol was detectable in blood, at low concentration, and stanozolol was not identified. The use of hair allowed to have a parallel between heart disease with long-term anabolic drug abuse.

Although abused for increasing body mass, this mixture is likely to have played a major role in the final fatal outcome. It has been published that this combination can be the cause of myocardial infarction, even with normal coronary arteriograms due to coronary spasm (22). The misuse of these drugs is associated with serious adverse effects to different organs, and it has been reported that rapidly progressive dilated cardiomyopathy and acute hepatic injury can occur in a very short period of time, i.e., within a few days after starting abuse (23).

In the absence of traumatic cause of death and given the toxicological and histological investigations, the pathologists concluded to cardiac insufficiency with support of cardiomegaly, in a context involving repetitive abuse of anabolic drugs.

Conclusion

Toxicologists and forensic pathologists should be aware of possible abuse of clenbuterol as a product proposed to lose weight or to enhance athletic performance, even in subjects older than 60 years. The possibility of cardiac insufficiency in a subject suffering from silent cardiomegaly in the presence of occlusive coronary artery disease must be documented by complete autopsy and anatomic pathology tests. A comprehensive toxicological screening, targeting all the performance-enhancing drugs is of paramount importance in these types of cases, as clenbuterol and anabolic steroids are seldom tested and blood concentrations are generally very low. Even abused alone, clenbuterol (16) or stanozolol (24) will increase the risk of cardiovascular disease. Therefore, monitoring all the drugs with a cardiac tropism, even indirectly, is of importance when the death is unexpected, particularly in the bodybuilders' population or subjects who want to lose weight.

This fatal case demonstrates the absolute need of collaboration between the active investigators, including police, forensic pathologists (autopsy + anatomic pathology tests) and toxicologists. This is particularly necessary as only very few case reports dealing with the combination clenbuterol and steroids are available in the literature, although these drugs are abused by millions of subjects.

References

1. Milano, G., Chiappini, S., Mattioli, F., Martelli, A., Schifano, F. (2018) ß-2 agonists as misusing drugs? Assessment of both clenbuterol- and salbutamol-related European medicines agency pharmacovigilance database reports. *Basic & Clinical Pharmacology & Toxicology*, 123, 182–187.
2. Prather, E.D., Brown, D.E. (1995) Clenbuterol: a substitute for anabolic steroids. *Medicine and Science in Sports and Exercice*, 27, 1118–1121.
3. Wingert, W.E., Mundy, L.A., Nelson, L., Wong, S.C., Curtis, J. (2008) Detection of clenbuterol in heroin users in twelve post-mortem cases at the Philadelphia medical examiner's office. *Journal of Analytical Toxicology*, 32, 522–528.
4. Barbosa, J., Cruz, C., Martins, J., Silva, J.M., Neves, F., Noronha da Silveira, M.I. (2005) Food poisoning by clenbuterol in Portugal. *Food Additives & Contaminants*, 22, 563–566.
5. Huckins, D.S., Lemons, M.F. (2013) Myocrdial ischemia associated with clenbuterol abuse: report of two cases. *The Journal of Emergency Medicine*, 44, 444–449.
6. Grimmer, N.M., Gimbar, R.P., Bursua, A., Patel, M. (2016) Rhabdomyolisis secondary to clenbuterol use and exercice. *The Journal of Emergency Medicine*, 50, e71–e74.

7. Daubert, G.P., Mabasa, V.H., Leung, V.W.Y., Aaron, C. (2007) Acute clenbuterol overdose resulting in supraventricular tachycardia and atrial fibrillation. *Journal of Medical Toxicology*, 3, 56–60.
8. World Anti-Doping Agency. 2017 Anti-Doping Testing Figures. https://www.wada-ama.org/sites/default/files/resources/files/2017_anti-doping_testing_figures_en_0.pdf (accessed Feb 11, 2019.
9. Barry, A.R., Graham, M.M. (2013) Case report and review of clenbuterol cardiac toxicity. *Journal of Cardiology Cases*, 8, 131–133.
10. Demoulin, R., Poyet, R., Capilla, E., Tortat, A.V., Pons, F., Brocq, F.X. *et al.* (2018) Cardiovascular complications of doping products. *Annales de Cardiologie et d'Angéiologie*, 67, 365–369.
11. Waight, M., McGuinness, W. (2016) Case of low dose clenbuterol toxicity. *Britisch Medical Journal Case Reports*, 2016, bcr2016215157 doi: 10.1136/bcr-2016-215157. https://www.ncbi.nlm.nih.gov/pubmed/?term=Waight+McGuinness+2016%22BMJ%20Case%20Rep.%202016%20Apr%2015;2016:10.1136/bcr-2016-215157..
12. Hieger, M.A., Emswiler, M.P., Maskell, K.F., Sentz, J.T., Miller, K.B., Wolf, C.E. *et al.* (2016) A case series of clenbuterol toxicity caused by adulterated heroin. *The Journal of Emergency Medicine*, 51, 259–261.
13. ANSM. L'ANSM alerte sur les risques pour la santé des produits à visée anabolisante ou amaigrissante CLENOX® et STANOX-10® de Pacific Pharmaceutical Company LTD. Malay Tiger, vendus sur internet-Point d'information. https://www.ansm.sante.fr/S-informer/Points-d-information-Points-d-information/L-ANSM-alerte-sur-les-risques-pour-la-sante-des-produits-a-visee-anabolisante-ou-amaigrissante-CLENOX-R-et-STANOX-10-R-de-Pacific-Pharmaceutical-Company-LTD.-Malay-Tiger-vendus-sur-internet-Point-d-information (accessed Feb 19 2019).
14. Cirimele, V., Kintz, P., Ludes, B. (2000) Testing of the anabolic stanozolol in human hair by gas chromatography-negative ion chemical ionization mass spectrometry. *Journal of Chromatography B Biomedical Science Applications*, 740, 265–271.
15. Yamamoto, I., Iwata, K., Nakashima, M. (1985) Pharmacokinetics of plasma and urine clenbuterol in man, rat, and rabbit. *Journal of Pharmacobiodynamy*, 8, 385–391.
16. Quinley, K.E., Chen, H.Y., Yang, H.S., Lynch, K.L., Olson, K.R. (2016) Clenbuterol causing non-ST-segment elevation myocarial infarction in a teenage female desinring to lose weight: case and brief literature review. *The American Journal of Emergency Medicine*, 34, 1739.e5–1739.e7.
17. Smith, D.J., Paulson, G.D. (1997) Distribution, elimination, and residues of [14C] clenbuterol HCl in Holstein calves. *Journal of Animal Science*, 75, 454–461.
18. Krumbholtz, A., Anielski, P., Gfrerer, L., Graw, M., Geyer, H., Schänzer, W. *et al.* (2014) Statistical difference of hair analysis of clenbuterol to discriminate therapeutic use from contamination. *Drug testing and Analysis*, 6, 1108–1116.
19. Dumestre-Toulet, V., Cirimele, V., Ludes, B., Gromb, S., Kintz, P. (2002) Hair analysis of seven bodybuilders for anabolic steroids, ephedrine and clenbuterol. *Journal of Forensic Science*, 47, 211–214.
20. Kintz, P., Cirimele, V., Sachs, H., Jeanneau, T., Ludes, B. (1999) Testing for anabolic steroids in hair from two bodybuilders. *Forensic Science International*, 101, 209–216.
21. Kintz, P. (2017) A new series of hair test results involving anabolic steroids. *Toxicologie Analytique et Clinique*, 29, 320–324.
22. Goldstein, D.R., Dobbs, T., Krull, B., Plumb, V.J. (1998) Clenbuterol and anabolic steroids: a previous unreported cause of myocardial infarction with normal coronary arteriograms. *South Medical Journal*, 91, 780–784.
23. Li, C., Adhikari, B.K., Gao, L., Zhang, S., Liu, Q., Wang, Y. *et al.* (2018) Performance-enhancing drugs abuse caused cardiomyopathy and acute hepatic injury in a young bodybuilder. *American Journal of Men's Health*, 12, 1700–1704.
24. Mewis, C., Spyridopoulos, I., Kühlkamp, V., Seipel, L. (1996) Manifestation of severe coronary heart disease after anabolic drug abuse. *Clinical Cardiology*, 19, 153–155.

3.1.2. Les SARMs

Au cours des deux dernières décennies une nouvelle famille de produits dopants, les SARMs, a émergé. Les SARMs sont des ligands pouvant exercer sélectivement des effets agonistes et antagonistes sur les récepteurs des androgènes.

La classe de médicaments des SARMs comprend une grande variété de structures chimiques et peut être classée en composés stéroïdiens (YK-11, MK-0773) et non stéroïdiens (ostarine, andarine, LGD-4033, ACP-105) *[Thevis, 2018]*.

Ils agissent de manière sélective sur les récepteurs des androgènes des muscles et des os. Contrairement aux SAA, ils ne lient pas sur les récepteurs des androgènes de la prostate et évitent donc les effets secondaires associés, comme la virilisation.

Le premier SARM à avoir été développé est le bicalutamide, un anti-androgène, et a été développé dans les années 1990 pour traiter le cancer de la prostate *[Dalton, 1998]*.

D'après les forums de consommateurs, les SARMs stéroïdiens, sont moins appréciés du fait de leur ressemblance avec les SAA au niveau de la structure chimique (figure 3) et donc de leurs potentiels effets secondaires similaires.

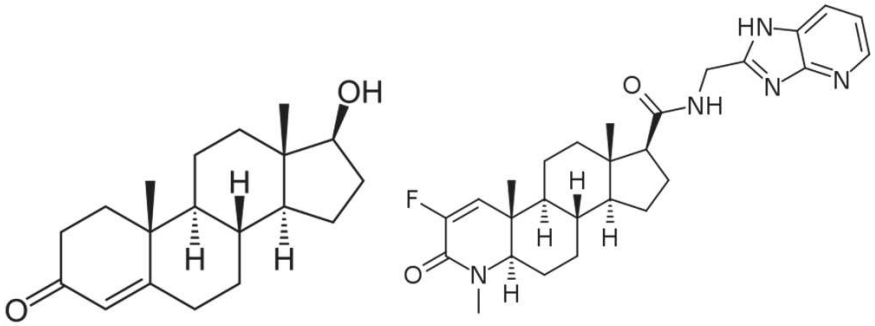


Figure 3 : Testostérone (gauche), MK-0773 (à droite)

Néanmoins aucun SARM n'a d'AMM à ce jour. Ils sont tous en essais cliniques à des stades plus ou moins avancés dans le but de traiter des maladies musculaires (cachexie) ou osseuses (ostéropose) ; pour certains, les essais cliniques ont même été arrêtés (figure 4) *[Zhang, 2013 ; Christiansen, 2020].*

Chemotype	SARM	Current status	Company
Aryl propionamide	Andarine	Phase I	GTx&Janssen
Aryl propionamide	Osterine™	Phase III (cancer cachexia)	GTx
Steroid	MK-0773	Phase II (sarcopenia)	Merck
Quinolinone	LGD-2226	Phase I (dis.)	Ligand
Quinolinone	LGD-2941	Phase I (frailty & osteoporosis)	Ligand&TAP
Quinolinone	LGD-3303	Preparing Phase I	Ligand
quinolinone	LGD-4033	Preparing Phase II	Ligand
Bicyclic hydantoin	BMS-564929	Phase I (age-related functional decline)	BMS&Pharmacopia
Unknown	GSK-971086	Phase I (safety and tolerability)	GSK
Aniline	ACP-105	Preparing trial	Acadia
Aniline	RAD140	Preparing trial	Radius

Figure 4: État des phases d'essais cliniques pour les SARMs en 2012 [Zhang, 2013]

Les SARMs non stéroïdiens sont très appréciés du grand public, dont la population féminine, car les effets virilisants sont évités avec ces molécules.

Ils présentent une bonne biodisponibilité orale due à leur structure chimique. Ils sont consommés par voie orale, ce qui présente un avantage par rapport aux esters de SAA. Il en existe sous forme de gélule, de comprimé et même de liquide.

Néanmoins très peu de données existent à leur sujet et les effets indésirables sur le long terme sont très mal connus. Leur achat ainsi que leur vente sont difficilement contrôlés.

3.2. Développement d'une méthode analytique

Du fait de l'émergence de ces molécules, il m'a semblé important de développer une méthode d'analyse spécifique et sensible dans les cheveux pour identifier les SARMs en circulation sur Internet. De plus, il n'y avait aucune donnée dans la littérature sur l'identification des SARMs dans des phanères authentiques lorsque j'ai commencé ma thèse en 2018.

Quelques données dans la littérature concernant l'identification des métabolites existent mais les métabolites ne sont pas commercialisés.

J'ai donc développé une méthode permettant d'identifier 11 SARMs (réels et vendus comme tels) dans les cheveux pour identifier les molécules « parents » dont : AC262536, ACP-105, andarine ou S-4, LGD-4033 ou ligandrol, MK-0773, MK 677, ostarine ou S22 ou MK 2866, RAD 140 ou testolone, S23, GW501516 ou cardarine et SR-9009.

La cardarine et le SR-9009 sont deux modulateurs métaboliques vendus sur Internet comme étant des SARMs. C'est pour cette raison qu'ils ont été ajoutés à la méthode spécifique de ces molécules. En effet, il n'est pas rare pour un consommateur d'associer un réel SARM à la cardarine.

Le bicalutamide-D_4 a été choisi en tant que standard interne du fait de la structure similaire avec les SARMs non stéroïdiens (figure 4).

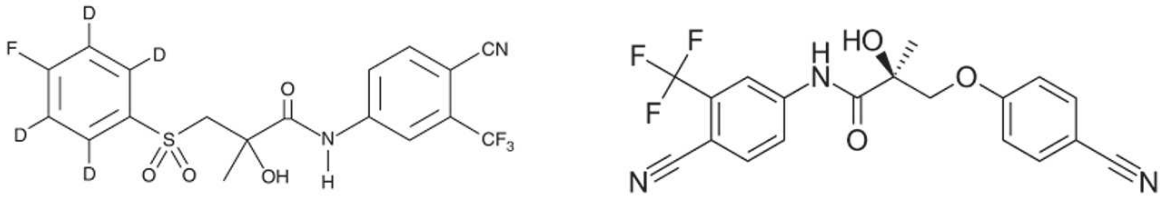


Figure 5 : Bicalutamide-D_4 (gauche) - Ostarine (droite)

Les échantillons sont analysés par LC-MS/MS en 6 min. La méthode a été optimisée, développée et validée. Les paramètres de la méthode sont décrits dans l'article ci-dessous. Cette méthode a été soumise à publication et est en cours de relecture à ce jour :

Afin de répondre à une demande croissante de la part des sportifs de haut niveau pour prouver leur bonne foi après un RAA de SARMs dans les urines, j'ai donc développée, optimisé et validé une méthode de criblage ciblé et de quantification dans les cheveux. Cette méthode permet d'identifier 9 SARMs et 2 modulateurs métaboliques largement associés aux SARMs et vendus sur certains sites internet comme étant des SARMs. Cette méthode a été appliquée à un cas de dopage, décrit dans cet article, pour lequel de l'ostarine, de l'andarine et du S23 ont été identifiés avec des profils de concentrations similaires. Il s'agit de la première identification du S23 dans les cheveux. Ce cas a mis en évidence une pratique dopante ainsi qu'une probable contamination d'un supplément d'andarine par de l'ostarine et du S23 ***[Article 13]***.

Article 13 : Development and validation of SARMs and metabolic modulators screening in hair using UHPLC-MS/MS : application to a doping case and first identification of S23 in authentic human hair

Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz

Soumis le 16 juillet 2021 dans Journal of Chromatography B

Journal of Chromatography B

Development and validation of SARMs and metabolic modulators screening in hair using UHPLC-MS/MS: application to a doping case and first identification of S23 in authentic human hair

--Manuscript Draft--

Manuscript Number:	CHROMB-D-21-00768R1
Article Type:	Full Length Article
Keywords:	SARMs; modulator metabolic; Hair; UHPLC-MS/MS; HRMS
Corresponding Author:	Laurie Gheddar Institut de Médecine Légale: Institut de Medecine Legale Strasbourg, FRANCE
First Author:	Laurie Gheddar
Order of Authors:	Laurie Gheddar
	Jean-Sébastien Raul
	Pascal Kintz
Abstract:	SARMs are a new class of doping drugs that emerged in sport since 2008. Easy access on the Internet also leads to their misuse by amateurs. It seems important for a laboratory of toxicology to develop a targeted screening of SARMs, given their health risks. A method has been developed and validated for the analysis in hair of 9 SARMs (AC262536, ACP-105, andarine, LGD-4033, MK-0773, MK 677, ostarine, RAD 410 and S23) and 2 other metabolic modulators (GW501516, SR9009), using liquid chromatography coupled to tandem mass spectrometry. After addition of bicalutamide-D 4 used as internal standard and incubation in phosphate buffer pH = 9.5, 20 mg of hair samples were extracted with liquid/liquid extraction. Linearity was verified for all compounds between 0.1-20 and 2000 pg/mg. LOD and LOQ were determined between 0.1-20 and 0.5-50 pg/mg respectively, according to the various analytes. Intra- and inter-day precision (CV < 20 %), matrix effects and recovery were evaluated for all compounds with CVs < 20 %. The application and the interest of SARMs screening was demonstrated in a doping case. Three SARMs were detected namely andarine (120-1644 pg/mg), ostarine (1-9 pg/mg) and S23 (0.6-16 pg/mg) in 6x1 cm segments of the subject.

Development and validation of SARMs and metabolic modulators screening in hair using UHPLC-MS/MS: application to a doping case and first identification of S23 in authentic human hair

Laurie Gheddar*[1], Jean-Sébastien Raul[1], Pascal Kintz[1, 2]

1: Institut de médecine légale, 11 rue Humann, 67000 Strasbourg, France

2: X-Pertise Consulting, Mittelhausbergen, France

*Author for correspondence, l.gheddar@gmail.com

Highlights

- Development and validation of a rapid method for SARMs in hair using UHPLC-MS/MS
- Simultaneous analysis of 9 SARMs and 2 metabolic modulators in hair
- First identification of S23 in authentic human hair in a doping case

Abstract

SARMs are a new class of doping drugs that emerged in sport since 2008. Easy access on the Internet also leads to their misuse by amateurs. It seems important for a laboratory of toxicology to develop a targeted screening of SARMs, given their health risks.

A method has been developed and validated for the analysis in hair of 9 SARMs (AC262536, ACP-105, andarine, LGD-4033, MK-0773, MK 677, ostarine, RAD 410 and S23) and 2 other metabolic modulators (GW501516, SR9009), using liquid chromatography coupled to tandem mass spectrometry. After addition of bicalutamide-D_4 used as internal standard and incubation in phosphate buffer pH = 9.5, 20 mg of hair samples were extracted with liquid/liquid extraction. Linearity was verified for all compounds between 0.1-20 and 2000 pg/mg. LOD and LOQ were determined between 0.1-20 and 0.5-50 pg/mg respectively, according to the various analytes. Intra- and inter-day precision (CV < 20 %), matrix effects and recovery were evaluated for all compounds with CVs < 20 %. The application and the interest of SARMs screening was demonstrated in a doping case. Three SARMs were detected namely andarine (120-1644 pg/mg), ostarine (1-9 pg/mg) and S23 (0.6-16 pg/mg) in 6x1 cm segments of the subject.

Keywords: SARMs, modulator metabolic, hair, UHPLC-MS/MS, HRMS

1. Introduction

In recent years, a new family of doping agents, named selective androgen receptor modulators (SARMs), has emerged. The class of SARMs includes a wide variety of chemical structures and can be classified into steroidal (example: MK-0773) and non-steroidal (example: ostarine, andarine) compounds [1].

The first SARMs were developed in the 1990s based on a non-steroidal anti-androgen, named bicalutamide, to fight diseases manifested by muscle loss (e.g., cachexia, sarcopenia) or osteoporosis [2]. They have similar properties to androgenic anabolic steroids (AAS) but have less marked androgenic properties. They activate the androgen receptors in muscles and bones selectively and avoid the appearance of side-effects (e.g., virilization). On the opposite of AAS, they also have good oral bioavailability. It should be noted that today, all SARMS are still in clinical trials without any therapeutic indication [3].

Since 2008, these products have been banned in-and-out of competition by the World Anti-Doping Agency (WADA) and classified as other anabolic agents (class S1.2). They are increasingly used by top athletes, as evidenced by the WADA statistics. In 2015, they accounted for only 32 from 3809 of adverse analytical findings (AAF), compared to 142 from 4180 in 2019 [4].

Like AAS, the misuse of SARMs could extend to the general public as they are easily available on the Internet. Therefore, it seems important for a toxicology laboratory to develop a screening method to identify SARMs. Contrary to urine, in which it is necessary to test for metabolites that are not generally marketed, hair analysis seems to be the gold choice because the target analyte in hair is often the parent molecule. Furthermore, hair specimens allow increasing the window of detection, to document the consumption pattern when segmentation is possible and to trace back a possible source of contamination.

In this study, the authors focused on SARMs available on the Internet. Among these SARMs, two are classified as metabolic modulators (GW501416, SR9009) and categorized as SARMs by users and sellers. Therefore, the authors decided to include them in the screening. GW501516 or cardarine is a peroxisome proliferator-activated receptor delta (PPARδ) agonist. It acts on the receptor to increase fat-burning capacity and muscle production. SR9009, also known as stenabolic, is REV-ERB agonist. It increases fat-burning, muscle production and endurance. These two products have been prohibited by the WADA and are classified as S4. hormone and metabolic modulators. In 2019, GW501516 and SR9009 represented 32 cases and 2 cases on 362 of AAF, respectively [4]. Moreover, Leaney *et al.* [5] have detected these two compounds in SARMs tablets purchased on the Internet. Indeed, some products are associated with GW501516 and/or SR9009; this is the case for capsules that include andarine, GW501516 and SR9009. In addition, in this previous study, GW501516 was found in one

tablet for which it was not declared on the product label. This capsule was supposed to contain RAD-140, LGD-4033 and ostarine [5].

In this paper, the authors describe the development and the validation of a screening method for 9 SARMs and 2 metabolic modulators (categorized as SARMs on the Internet) in hair and apply it to a real doping case.

2. Material and method

2.1. Chemicals and reagents

Andarine (S-4), ACP-105, GW501516 (GW1516), Ostarine (S-22 or enobosarm), S23, RAD 140 (testolone), MK 677 (ibutamoren), SR 9009 and bicalutamide-D_4 were obtained from Toronto Research Chemicals (Toronto, Canada). Ligandrol (LGD-4033) and AC262536 were obtained from Cayman Chemical (Ann Arbour, USA) and Sigma-Aldrich (Saint-Louis, USA) respectively. MK-0773 (Dynamic Performance) was obtained on the Internet. Ammonium formate 99 % was provided by Alfa Aesar (Schiltigheim, France). Dichloromethane was purchased from Merck (Darmstadt, Germany). Methanol for HPLC LC-MS Grade (MeOH), acetonitrile for HPLC LC-MS Grade (ACN) and formic acid were purchased from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Diethyl ether was purchased from Honeywell Riedel-de Haën (Seelze, Germany). HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany). Water for the formate buffer (eluent A for chromatographic separation) was purified using a Milli-Q system (Millipore) (Molsheim, France).

The standard solutions, prepared in MeOH, were stored at -20 °C or at + 4°C according to the supplier recommendations.

2.2. Instrumentation

2.2.1. UHPLC-MS/MS analysis

Chromatographic separation was performed with a liquid chromatography Waters Acquity UPLC™ system (Waters corporation, Milford, MA, USA) using an Acquity UPLC HSS C18 column (150 mm x 2.1 mm, i.d. 1.8 µm particle size). The column temperature was set to 50°C, and the flow rate at 0.4 mL/min. A gradient elution was performed using formate buffer adjusted to pH 3 (eluent A) and 0.1% formic acid in acetonitrile LC-MS (eluent B). Analysis time was 6.0 min and the following gradient pattern (eluent A) was used for the separation of SARMs and bicalutamide-D_4, used as internal standard (IS): 0 min, 60%; 0.5 min, 60%; 1.50 min, 5 %; 3.0 min, 5%; 4.00 min, 60%; 6.00 min, 60%. Retention times are presented in table 1.

Detection was performed using a triple quadrupole (XEVO™ TQS micro, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionization source (ESI) and

used in the positive and negative mode (ES+/ES-). Nitrogen was used as drying gas as well as nebulizing gas. Parameters were optimized during the development period. The desolvation gas flow was set to 800 L/h and the desolvation temperature was 550°C. The source temperature was 150°C. The capillary voltage was tested and set at 1.50 kV (ES-) and 3.0 kV (ES+). Cone voltage and collision energy were automatically adjusted to optimize the signal of the 2 most abundant product ions of SARMs and bicalutamide-D_4 using Intellistart software. Transitions, cone voltage and collision energy are resumed in table 1. MassLynx 4.1 software was used for identification and quantification.

2.2.2. HRMS analysis

HRMS system enables to confirm the presence of SARMs in samples targeting the exact mass. Chromatographic separations were performed with identical equipment than UHPLC-MS/MS. The applied parameters (column temperature, mobile phase, gradient pattern) were the same compared to UHPLC-MS/MS system.

For the analysis of the doping case described in 5.2 section, the gradient pattern was different as follow: 0 min, 50%; 0.5 min, 50%; 1.50 min, 5 %; 2.5 min, 5%; 3.00 min, 50%; 4.00 min, 50% to analyse only ostarine (t_R=1.91 min), andarine (t_R=1.67 min) and S23 (t_R=2.24 min).

Detection was performed using a high-resolution (XEVO^{TM} G2XS Q-TOF, Waters Corporation, Milford, MA, USA) mass spectrometer operating in positive-and negative-ion mode and in sensitivity mode. The desolvation gas flow was set to 600 L/h at a temperature of 400 °C with the cone gas set to 20 L/h and the source temperature set to 150 °C. The capillary voltage and the cone voltage were set to 2000 (ESI +) / 800 (ESI -) and 20 V, respectively. Leucine-enkephalin was used as the lock mass (*m/z* 556.2766 - *m/z* 554.2620) for accurate mass calibration and introduced using the lockspray interface at 10 µL/min and a concentration of 200 pg/µL in 50% aqueous ACN, 0.1 % formic acid. In MS scanning, data were acquired from 50 to 1000 *m/z*. As for MS/MS fragmentation of target ions, collision energy ranging from 10 to 40 V was applied. Unifi software was used for data, chromatograms and spectra acquisition.

2.3. Sample treatment

The hair strands were decontaminated twice with 5 mL of dichloromethane for 1 min at room temperature and dried. For the validation, hair was obtained from the staff of laboratory and verified to be negative.

20 mg of decontaminated hair were used for analysis. In order to integrate the analysis of SARMs into the laboratory routine, four extraction methods, commonly used in our laboratory, were tested at the concentration of 100 pg/mg in triplicate.

(1) The first consisted of incubation of hair in 1 mL borate buffer pH 9.5 for 15 h at 40 °C, in the presence of 250 pg of bicalutamide-D_4. After cooling, 5 mL of the mix solution (ether/dichloromethane/hexane/isoamylic alcohol (50/30/20/0.5)) were added. After agitation for 15 min and centrifugation (15 min, 3000 rpm), the organic phase was collected and evaporated to dryness under a nitrogen flow. The dry residue was dissolved in 30 µL of initial mobile phase.

(2) The second method tested was described by Kintz *et al.* for anabolic steroid analysis in hair [6]. 250 pg of bicalutamide-D_4 were used and no derivatization step was applied.

(3) The third method was described by Gheddar *et al.* for stanozolol analysis [7]. Instead of stanozolol-D_3, bicalutamide-D_4 was used as IS.

(4) Finally, a fourth method was tested. It consisted of incubation of hair in 1 mL of MeOH, in the presence of 250 pg of bicalutamide-D_4, in an ultra sound bath for 2 hours. After cooling, MeOH was evaporated and the dry residue was dissolved in 30 µL in MeOH.

Area at 100 pg/mg from methods 2, 3 and 4 were compared for each compound to method 1.

3. Method validation

The validation process was applied to the extraction method chosen by the authors (section 5.1).

3.1. Determination and evaluation of linearity, LOD and LOQ

A nine-point calibration curve (n = 3) was obtained by spiking blank hair at 0.1, 1, 5, 10, 50, 100, 200, 500 and 2000 pg/mg. The limit of quantification (LOQ) was defined as the first point of calibration. The limit of detection (LOD) was the lowest concentration of analyte that could be detected with signal to noise ratio superior to 3.

3.2. Intra and inter – day precision

For the intra-day precision, three quality controls (QC) at 10, 100 and 500 pg/mg were evaluated 5 times. And for the inter-day precision, QCs at 3 levels of concentration are evaluated on three days spread over two weeks. The laboratory will accept coefficients of variation (CV) of less than 30% for the 10 pg/mg controls and less than 20% for the 100 and 500 pg/mg controls.

3.3. Matrix effect

With respect to matrix effect, hair blank specimens collected from different subjects of the laboratory (n = 5) were extracted and 10 µL of standard solution of mixture of SARMs (1 mg/L) was added at the end of extraction. These specimens were compared to 10 µL of standard solution of mixture of SARMs in MeOH (1 mg/L).

3.4. Recovery

It was not possible to establish suitable extraction efficiency in authentic hair due to the lack of reference material available for validation. Nonetheless, authors wanted to approach and control the extraction efficiency. To do that, 5 spiked hair samples at 500 pg/mg of SARMs were extracted and compared to a standard solution of SARMs in MeOH at the same concentration (n=4).

4. Application to an authentic hair specimen

The method developed at the laboratory was applied to the case of a woman top athlete, a practitioner of a combat sport (MMA), following an adverse analytical finding of ostarine in her urine (around 1 ng/mL). The athlete did not admit the anti-doping rules violation and her lawyer requested our laboratory for additional hair analysis in order to find an explanation. A lock of hair was collected by the lawyer and sent to the laboratory. The hair was received in an envelope and stored at room temperature and protected from UV light until the analysis. Hair was brown in colour and 20 cm in longer. The lock of hair was oriented from root to the tip. Hair was segmented (6 x 1 cm to cover the period of the urine control) and analysed according to the protocol of SARMs screening.

5. Results and discussion

5.1. Method validation

Validation of the screening of SARMs was achieved only with UHPLC-MS/MS because this is the gold method for quantification. The choice of the UHPLC-MS/MS system was obvious because it is necessary to use reverse phase to identify all analytes with different chemical structures. Accurate mass spectrometry was used for qualitative confirmation.

Blank hair specimens from laboratory staff, used for validation method, were chosen after checking the absence of response at the retention times of the various SARMs and the IS. It appears that bicalutamide-D_4 is a good internal standard for the analysis of SARMs in hair thanks to its close chemical structure with other SARMs as previously published in urine [8].

The optimization of the mass parameters was not a problem. MK-0773 was purchased on the Internet at the dosage at 10 mg. This tablet was checked using HRMS to verify its composition. HRMS proved the presence of MK-0773 with the mass exact of *m/z* $[M+H]^+$ of 480.2775 with a low mass error (1.1 ppm). No other drug or SARM was detected in MK-0773 tablet. Therefore, theses tablet was used as material reference. Chromatographic separation was tested to obtain the best of resolution and peak separation (resolution superior than 1.5) and the time run faster. All analytes are separated in 6 min.

For each analyte, the area after extraction 2, 3 and 4 were compared with the area from extraction 1. The results are expressed as a gain or loss (in percentage) relative to the results of method 1 and are

summarized in table 2. The second method failed to identify MK-0773 and MK 677. But, it is interesting to note that this method gave better reponses (area and signal/noise ratios) for most compounds, especially for AC262536, ACP-105 and LGD-4033 with gain of 4.5 %, 2.9% and 4.1 % respectively. The third method did not detect SR 9009 and RAD 140. In contrast to methods 2 and 3, the first extraction allowed detecting all analytes.

The fourth method allow to detect all analytes but did not provide to obtain good chromatograms with signal to noise ratios superior than 10 even for concentration at 100 pg/mg.

Therefore, method 1 was chosen for the simultaneous screening SARMs. Only the first method was validated.

The linearity ranges, LOD and LOQ were resumed in table 3 for each compound. The method showed a good linearity in the ranges defined in table 2 with correlation coefficients superior to 0.988. The analysis showed good inter- and intra-day precisions for all analytes with CVs < 20% (table 3). Matrix effect was established for all analytes and was evaluated between 75 and 130 %. However, the matrix effect was similar in the five different tested matrices with a CV < 20%. Finally, recovery extraction was established between 53 and 84% (table 3). It should be noted that the evaluation of the extraction coefficient remains an approximate approach since there is no reference material with certified concentrations.

For all but ACP-105, the limits of quantification were found satisfactory, given the method allows identifying and quantifying simultaneously 11 analytes in hair samples within 6 minutes. The ion chromatograms of 11 analytes and IS in a spiked hair at 100 pg/mg (for ACP-105) and 10 pg/mg (for the other SARMs) are presented in figure 1.

Simultaneous analysis of SARM has been already described in urine and plasma [8-11]. Contrary to urine and plasma, there are few data in literature on the analysis of SARMs in hair. Some authors have reported data in equine hair. For example, Cutler *et al.* have identified AC262536 in equine hair with a LOQ at 1 pg/mg [12]. Another method of quantification in equine hair for several AAS and other products namely andarine, GW501516 and ostarine was published in literature with LOD at 0.2 pg/mg, 0.1 pg/mg and 0.1 pg/mg respectively [13]. Only two groups have reported data in human hair. One was the group of Kintz *et al.* with the identification of ostarine between 3 and 168 pg/mg, andarine between 0.1 and 0.7 pg/mg and LGD-4033 between 14 and 42 pg/mg [14-15]. In another paper, Kintz *et al.* reported GW501516 hair concentrations at 22 and 32 pg/mg after incubation in MeOH [16]. Finally, Rading *et al.* identified GSK2881078 in human hair at 1.7 pg/mg [17].

5.2. Application to an authentic hair specimen

The hair test results of athlete are described in figure 2. Ostarine was detected in all 6 segments, with concentrations increasing from 0-1 cm to 1-2 cm then decreasing from 1-2 cm to the tip, ranging from 1 to 9 pg/mg. Ostarine was the identified agent during the urine control.

The screening revealed the presence of two other SARMs in hair, namely andarine and S23. They were also detected in 6 segments, at concentrations between 120 and 1644 pg/mg for andarine, and 0.6 and 16 pg/mg for S23. The concentration patterns, presented in figure 2, are similar to those observed for ostarine, with increasing concentrations from 0-1 cm to 1-2cm segment then decreasing from 1-2 cm to the tip. The presence of these 3 SARMs was confirmed using UPLC-q-TOF-MS system (figure 3) with mass error from -2.5 to -0.4 ppm. The laboratory accepted mass error < 5.0 ppm and a time retention ± 0.2 min compared to the reference material. Ostarine concentrations are in favour of repeated exposure, but in view of the concentrations described in the literature (12 - 168 pg/mg) [14-15], the concentrations could also be characteristic of environmental or dietary supplement contamination. High concentrations of andarine can be interpreted as a doping practice. For S23, there is no data available in the literature about its identification in hair.

In view of the high concentrations of andarine measured and the information available in the literature, the presence of ostarine and S23 in the hair can be interpreted as a consequence of contamination of andarine by these two products, in an andarine tablet. According to Leaney *et al.*, a single tablet or capsule can contain up to 5 different SARMs in the same item [5]. Switching a SARM to another SARM has also been described for ostarine [18]. It is probably due to the same production line that has also been demonstrated many times for contamination occurring with AAS [19].

Andarine and ostarine drugs are regularly identified as AAF in contrast to S23 that was found in urine for the first time in 2019 by anti-doping laboratories [4]. Nonetheless, the presence of these three molecules in the hair of this top athlete is in favour of doping attitude.

6. Conclusion

In view of the major emergence of SARMs in the field of sport and the interest that this class of drugs has on the general public, the development of an analytical method dedicated to their identification has become necessary. The authors presented a specific and rapid analytic method to 11 “true” and categorized SARMs. This validated method allows in some cases to document the simultaneous presence of several SARMs in the hair of a subject and to trace a possible source of contamination. This is the first time that S23 was reported in human hair.

Conflict of interest and source of funding

The authors declare no conflict of interest and no funding.

Reference

1. M. Thevis, W. Shänzer, Detection of SARMs in doping control analysis, Molecular and cellular endocrinology. 464 (2018) 34-35. doi: 10.1016/j.mce.2017.01.040.

2. J.T. Dalton, A. Mukherjee, Z. Zhu, L. Kirkovsky, D.D. Miller, Discovery of nonsteroidal androgens, Biochemical and biophysical research communications. 224 (1998) 1-4. doi: 10.1006/bbrc.1998.8209.

3. A. R. Christiansen, L.I. Lipshultz, J.M. Hotaling, A.W. Pastuszak, Selective androgen receptor modulators: the future of androgen therapy? Translational Andrology and Urology. 9 (2020) 135-148. doi: 10.21037/tau.2019.11.02.

4. https://www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures-report (accessed on 10 July 2021).

5. A.E. Leaney, P. Beck, S. Biddle, P. Brown, P.B. Grace, S.C. Hudson, D.H. Mawson, Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators, Drug Testing Analysis. 13 (2021) 122-127. doi: 10.1002/dta.2908.

6. P. Kintz, L. Gheddar, J.S. Raul, Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic locations has several advantages when compared with the standard head hair analysis, Drug Test Anal. 13 (2021) 1445-1451. doi: 10.1002/dta.3020.

7. L. Gheddar, J.S. Raul, P. Kintz, Testing for stanozolol, using UPLC-MS/MS and confirmation by UPLC-q-TOF-MS, in hair specimens collected from five different anatomical regions, Journal of Analytical Toxicology. 44 (2020) 834-839. doi: 10.1093/jat/bkaa023.

8. A. Gadaj, E. Ventura, J. Healy, F. Botrè, S.S. Sterk, T. Buckley, M.H. Mooney, Enhanced UHPLC-MS/MS screening of selective androgen receptor modulators following urine hydrolysis, MethodsX. 21 (2020) 7:100926. doi: 10.1016/j.mex.2020.100926.

9. E. Ventura, A. Gadaj, G. Monteith, A. Ripoche, J. Healy, F. Botrè, S.S. Sterk, T. Buckley, M. H. Mooney, Development and validation of a semi-quantitative ultra-high performance liquid chromatography-tandem mass spectrometry method for screening of selective androgen receptor modulators in urine. Journal of Chromatography A. 1600 (2019) 183-196, doi: 10.1016/j.chroma.2019.04.050.

10. M. Polet, W. Van Gansbeke, P. Van Eenoo, Development and validation of an open screening method for doping substances in urine by gas chromatography quadrupole time-of-flight mass spectrometry, Anal. Chim. Acta. 1042 (2018), 52-59. doi: 10.1016/j.aca.2018.08.050.

11. A. Hansson, H. Knych, S. Stanley, M. Thevis, U. Bondesson, M. Hedeland, Investigation of the selective androgen receptor modulators S1, S4 and S22 and their metabolites in equine plasma using high-resolution mass spectrometry, Rapid Commun Mass Spectrom. 30 (2016) 833-842. DOI: 10.1002/rcm.7512.

12. C. Cutler, M. Viljanto, P. Taylor, J. Habershon-Butcher, T. Muir, S. Biddle, P. Van Eenoo, Equine metabolism of the selective androgen receptor modulator AC-262536 in vitro and in urine, plasma and hair following oral administration, Drug Test Anal. 13 (2021) 369-385.

13. B. Gray, M. Viljanto, E. Menzies, L. Vanhaecke, Detection of prohibited substances in equine hair by ultra-high performance liquid chromatography–triple quadrupole mass spectrometry – application to doping control samples First published: 12 February 2018. https://doi.org/10.1002/dta.2367

14. P. Kintz, L. Gheddar, A. Ameline, J.S. Raul, Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases, Drug Test Anal. 12 (2020) 1508-1513. doi: 10.1002/dta.2902.

15. P. Kintz, L. Gheddar, A. Ameline, J.S. Raul, Perspective in evaluating selective androgen receptor modulators in human hair: a short communication, Therapeutic Drug Monitoring. 43 (2021) 298-300. DOI: 10.1097/ftd.0000000000000859.

16. P. Kintz, A. Ameline, L. Gheddar, J.S. Raul, Testing for GW501516 (cardarine) in human hair using LC/MS-MS and confirmation by LC/HRMS, Drug Test Anal. 12 (2020) 980-986. doi: 10.1002/dta.2802.

17. A. Rading, P. Anielski, D. Thieme, A.M. Keiler, Detection of the selective androgen receptor modulator GSK2881078 and metabolites in urine and hair after single oral administration, Drug Test Anal. 13 (2021) 217-222. doi: 10.1002/dta.2943.

18. P. Kintz, A. Ameline, L. Gheddar, J.S. Raul, LGD-4033, S-4 and MK-2866 – Testing for SARMs in hair: about 2 doping cases, Tox Anal Clin. 31 (2019) 56-63. https://doi.org/10.1016/j.toxac.2018.12.001.

19. H. Geyer, M.K. Parr, K. Koehler, U. Mareck, W. Schänzer, M. Thevis, Nutritional supplements cross-contaminated and faked with doping sustances, J. Mass Spectrom. 48 (2008) 892-9022. doi: 10.1002/jms.1452.

Table 1: UPLC-MS/MS conditions for SARMs testing in hair samples

Analyte	Type of compound	TR (min)	Transition (m/z)	Cone (V)	CE (eV)	ESI polarity
AC262536	SARM	2.75	279.1 > 195.0* 279.1 > 235.0	14	22 22	+
Andarine	SARM	2.43	440.2 > 149.9* 440.2 > 261.0	2 36	36 20	-
ACP-105	SARM	2.92	291.2 > 233.0* 279.1 > 79.9	30 44	21 28	+
LGD-4033	SARM	2.80	337.1 > 170.0* 337.1 > 239.0	40	30 20	-
MK-0773	SARM	1.62	480.3 > 132.0* 480.3 > 149.1	88	40 30	+
MK677	SARM	1.80	529.4 > 91.0* 529.4 > 267.2	4	52 18	+
Ostarine	SARM	2.63	388.1 > 117.9* 388.1 > 269.1	8	18 14	-
RAD-140	SARM	2.54	394.2 > 223.1* 394.2 > 169.9	36	10 30	+
S23	SARM	2.91	415.1 > 144.9* 415.1 > 185.0	8 10	18 36	-
GW501516	Metabolic modulator (PPARδ receptor agonist)	3.16	454.0 > 257.0* 452.1 > 137.9	58 18	30 26	+ -
SR9009	Metabolic modulator (Rev-ErbA agonist)	3.32	438.2 > 124.9* 438.2 > 89.2	50	24 78	+
Bicalutamide-D4	SARM	2.55	433.1 > 255.1	14	14	-

Table 2: Gain or loss of areas (%) for method 2, 3 and 4 relative to method 1 (ND = non detected)

	Method 2	Method 3	Method 4
AC262536	+ 4.5 %	+ 1.3%	- 0.3 %
Andarine	- 0.4 %	- 1.0 %	- 1.2 %
ACP-105	+ 2.9 %	+ 0.3 %	- 0.1 %
LGD-4033	+ 4.1 %	+ 0.5 %	- 3.7 %
MK-0773	ND	- 1.0 %	- 2.8 %
MK677	ND	- 0.1 %	- 2.9 %
Ostarine	+ 1.7 %	- 1.0 %	- 0.8 %
RAD-140	- 0.6 %	ND	- 1.7 %
S23	+ 1.9 %	- 1.0 %	- 1.9 %
GW501516	+ 0.9 %	- 0.6 %	+ 0.5 %
SR9009	+ 1.3 %	ND	- 0.6 %

Table 3: Linearity range, LOD, LOQ, intra-inter day precision (%), mean matrix effect (%) and mean extraction coefficient (%)

Analyte	Linearity range (pg/mg)	LOD (pg/mg)	LOQ (pg/mg)	QC concentration (pg/mg)	Intra-inter CV (%)	Matrix effect (%)	Recovery (%)
AC262536	10-1000	5	10	10 100	14.2 - 17.2 14.7 - 17.3	85	56
Andarine	1-1000	0.1	1	10 100	7.4 - 5.2 13.1 - 9.3	130	57
ACP-105	50-1000	20	50	100 500	8.5 - 16 12.3 - 14.6	94	59
LGD-4033	5-1000	3	5	10 100	19 - 18 6.5 - 2.2	125	54
MK-0773	1-1000	0.5	1	10 100	17.5 - 14.9 10.8 - 19.2	112	75
MK677	10-1000	5	10	10 100	19.8 - 11.7 8.2 - 14.6	96	53
Ostarine	0.5-1000	0.1	0.5	10 100	5.9 - 13.5 14.8 - 10.5	117	83
RAD-140	5-1000	3	5	10 100	7.3 - 6.9 5.8 - 4.3	107	68
S23	1-1000	0.5	1	10 100	9.4 - 12.1 15 - 14.9	115	84
GW501516	5-1000	3	5	10 100	5.6 - 3.6 14.8 - 13.0	72	67
SR9009	20-1000	10	20	10 100	18.2 - 17.8 14.6 - 14.1	123	79

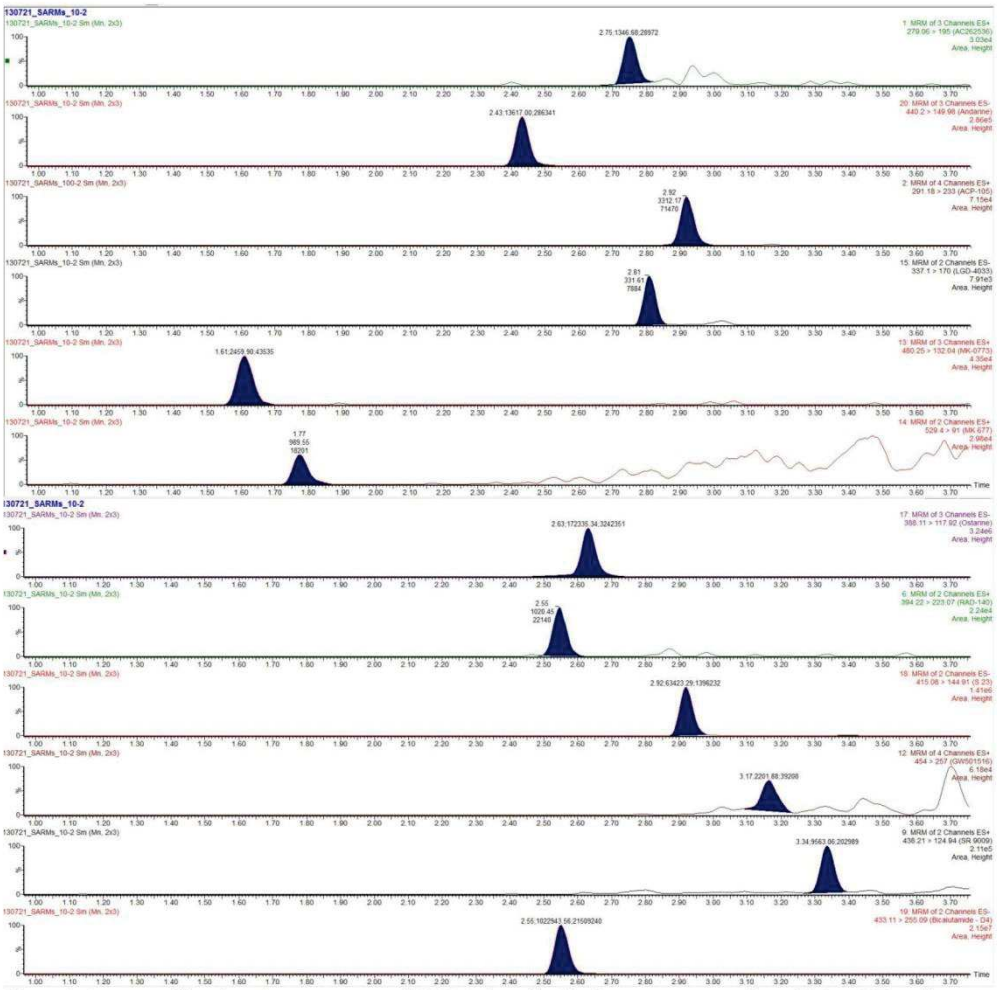


Figure 1: Ion quantification chromatogram of SARMs in spiked hair at a concentration of 10 or 100 pg/mg

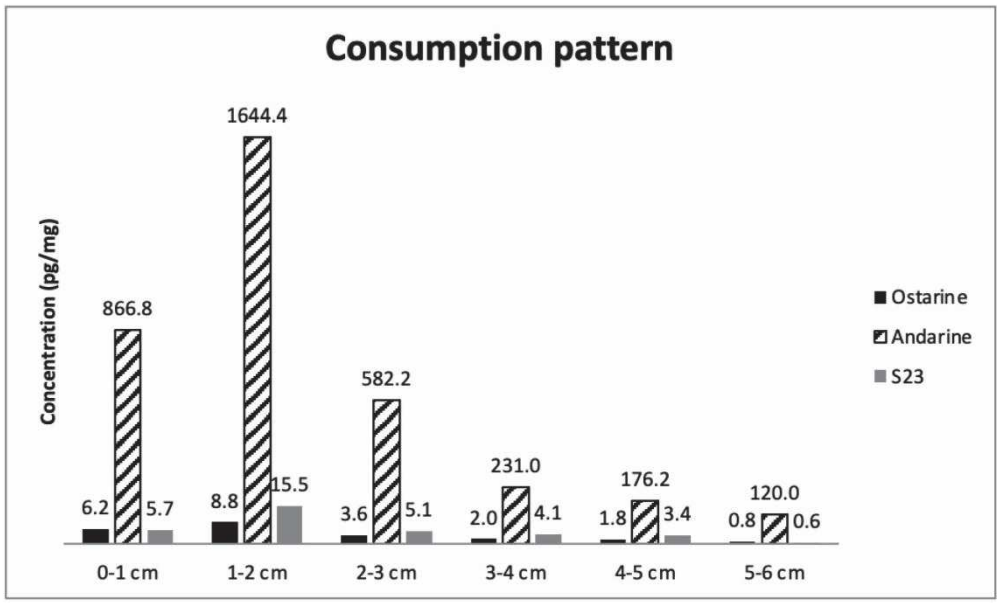


Figure 2: Consumption pattern and concentrations (pg/mg) of ostarine, andarine and S23 in 6 x 1cm segments

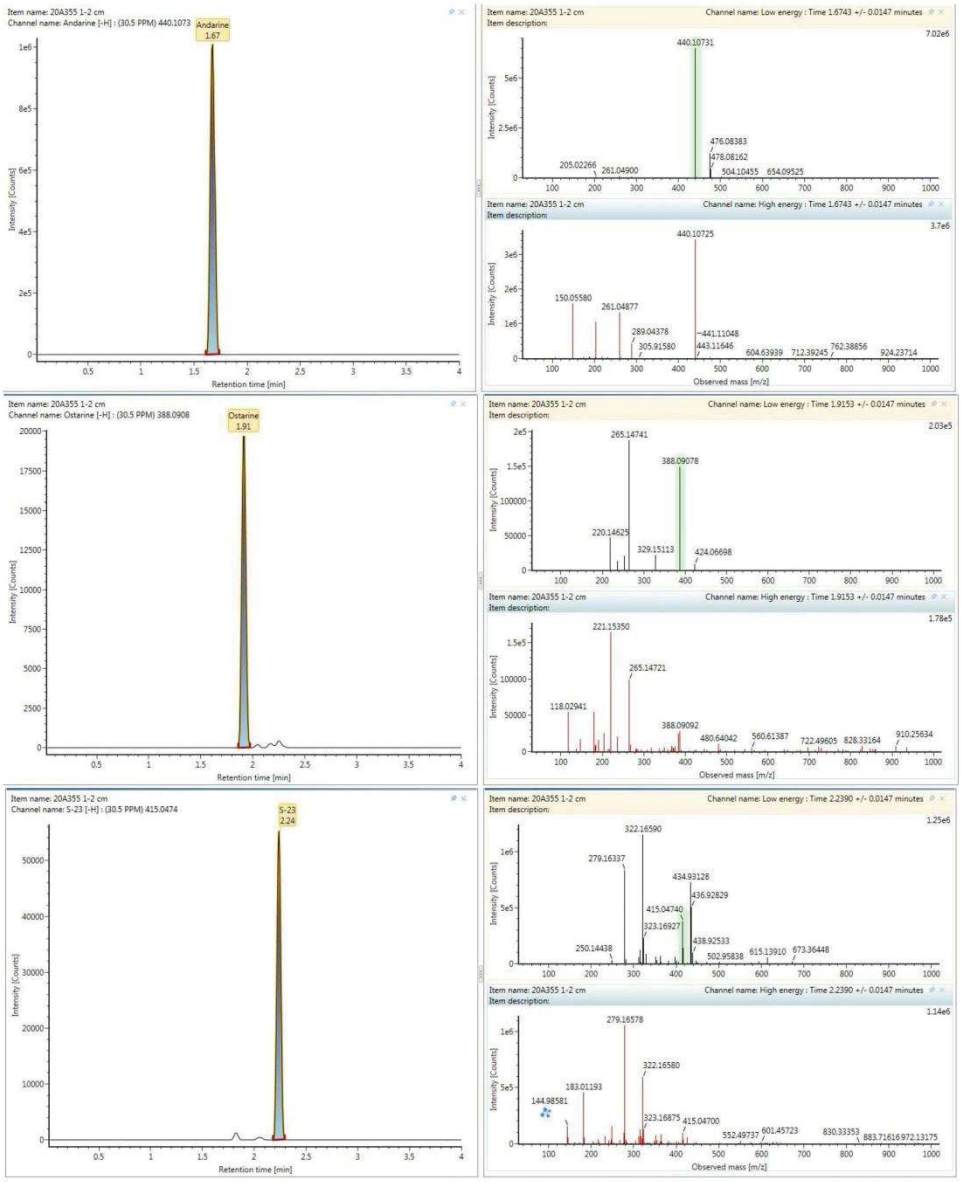


Figure 3: HRMS chromatograms obtained for andarine, ostarine, and S23 with HRMS in authentic hair of athlete (1-2 cm segment)

3.3. Applications

3.3.1. Applications à des cas de dopage et de dossiers cliniques

La méthode développée et décrite dans la section précédente a été appliquée au laboratoire de Strasbourg à plusieurs cas de dopage mais également à un cas clinique suite à une intoxication. La famille des SARMs est en vogue dans le milieu sportif chez les athlètes de haut niveau mais également chez les sportifs amateurs. Ces travaux ont donné lieu à 4 publications :

Article 14 : Dans cet article, les auteurs rapportent 3 cas liés à une exposition répétée de SARMs :

- Un homme de 26 ans a indiqué avoir consommé de l'andarine sur une période de 6 mois. L'homme a craint les conséquences de cette utilisation abusive. Il a donc demandé une analyse de cheveux pour connaître les concentrations dans les cheveux. L'analyse a montré la présence d'andarine à des concentrations de 0,1 et 0,7 pg/mg. L'andarine a été retrouvé dans deux segments consécutifs à des concentrations faibles. Cela peut correspondre à une pratique dopante mais on ne peut connaître ni les doses ni les fréquences d'utilisation.
- Un homme de 24 ans a été arrêté par la douane en possession de plusieurs plaquettes d'ostarine. L'homme a déclaré que c'était pour sa consommation personnelle. Afin de savoir si cela relevait d'un trafic ou d'une consommation personnelle, les douaniers ont demandé des analyses dans les cheveux pour connaître le profil de consommation. L'ostarine a été retrouvé dans toute la mèche du jeune homme avec des concentrations allant de 3 à 21 pg/mg correspondant à de la consommation répétée.
- Un homme de 29 ans a été hospitalisé pour des troubles psychologiques après la consommation de multiples drogues. Il a déclaré consommé de manière fréquente de la cocaïne, du ligandrol et de la 3-MMC. Les psychiatres ont demandé une analyse dans les cheveux afin de vérifier les dires du patient. Le ligandrol a été retrouvé dans tous les segments (14 - 42 pg/mg), ce qui correspond à un usage répété.

Article 15 : Dans cet article, les auteurs rapportent deux cas de dopage. Deux sujets sont arrêtés par la douane en possession de fioles d'ostarine sous forme liquide. Les deux sujets sont des sportifs amateurs et affirment que l'ostarine est dédié à leur usage personnel. L'ostarine n'ayant aucune indication thérapeutique, étant répertorié comme agent anabolisant (interdite depuis 2008 par l'AMA), les douaniers ont considéré que l'ostarine faisant partie d'un trafic. Pour documenter une exposition répétée, le juge a demandé une analyse dans les phanères :

- Sujet 1 : les cheveux n'étant pas disponibles, les ongles de main et de pied ont été analysés. L'ostarine était présente à 111 et 61 pg/mg.

- Sujet 2 : une mèche de cheveux a été prélevée et segmentée en 4 segments de 3 cm. L'ostarine a été quantifiée de 93 à 168 pg/mg dans toute la mèche.

L'analyse des phanères a mis en évidence une pratique dopante et à valider les propos des deux sujets.

Article 16 : Le GW501516 ou cardarine est un agoniste du récepteur delta des prolifélérateurs des peroxysomes (PPAR-δ). L'activation de ce récepteur augmente la capacité de combustion des graisses et la production de muscles. Le GW501516 n'a pas d'utilisation thérapeutique, mais peut être utilisé de manière abusive pour améliorer les performances par voie orale, à des doses de 10 à 20 mg par jour, pendant 6 à 20 jours. Les athlètes et les amateurs peuvent abuser du GW501516 car il est facile de se procurer ce médicament sur Internet. Depuis janvier 2009, la liste des substances et méthodes de dopage interdites, établie par l'AMA, inclut le GW-501516. En quantifiant par LC/MS-MS et en confirmant par LC/HRMS, la cardarine a été identifiée dans les cheveux d'un consommateur à 32 et 22 pg/mg dans deux segments consécutifs de 2 cm. Ce résultat est la première identification de la cardarine et montre que le composé acide est incorporé dans les cheveux.

Article 17 : Un homme de 43 ans, entraîneur sportif, s'est présenté aux urgences d'un hôpital local pour des douleurs épigastriques, des myalgies et des maux de tête sévères. Il a déclaré avoir utilisé pendant quelques jours une combinaison de GW501516 et d'ostarine pour augmenter la masse musculaire. Une cytolyse avec une augmentation marquée de l'ALT (jusqu'à 922 UI/L) et de l'AST (jusqu'à 2558 UI/L) et une rhabdomyolyse massive avec une CPK élevée (jusqu'à 86435 UI/L) étaient les principaux paramètres biochimiques inhabituels observés. En utilisant une méthode spécifique par LC-MS/MS, la cardarine et l'ostarine ont été testées positives dans le sang à 403 et 1 ng/mL, respectivement.
Dans l'urine, en raison d'un métabolisme important, le parent GW501516 n'a pas été identifié, tandis que l'ostarine était à 88 ng/mL. Enfin, les deux produits ont été identifiés dans les cheveux (2 cm, brun), à 146 et 1105 pg/mg pour la cardarine et l'ostarine, respectivement. Les concentrations sont en faveur d'un usage répété au cours des 2 derniers mois. L'asthénie a été persistante pendant deux semaines. Six semaines après l'admission et arrêt de la consommation, le sujet s'est complètement rétabli.

Les cas rencontrés montrent que l'utilisation des SARMs et du clenbuterol ne s'arrête plus aux sportifs de haut niveau mais s'étend au grand public du fait de leur accès facilité par Internet et de leurs faibles effets androgènes, ce qui les rend attractifs. Néanmoins, il y a un risque élevé pour la

santé car les effets sur le long terme sont très mal connus sauf la toxicité hépatique et la rhabdomyolyse qui ont été observés dans le cas présenté dans l'article 17. L'analyse des cheveux a permis de montrer une exposition sur le long terme et de caractériser une contamination par un supplément.

Article 14 : Perspective in evaluating selective androgen receptor modulators in human hair: a short communication

Pascal Kintz, Laurie Gheddar, Alice Ameline, Jean-Sébastien Raul

Therapeutic Drug Monitoring. 2021 ; 43 :298-300.

Perspectives in Evaluating Selective Androgen Receptor Modulators in Human Hair: A Short Communication

Pascal Kintz, PhD,† Laurie Gheddar, MS,† Alice Ameline, PhD,† and Jean-Sébastien Raul, PhD†*

Background: As hair testing increases the window of drug detection and permits the differentiation of long-term use from a single exposure when performing segmental analyses (which also allows establishing the pattern of use), this matrix should be considered as a suitable complement to standard investigations in clinical, forensic, and sport toxicology. The authors were recently involved in 3 cases where hair analysis was used to demonstrate the use of selective androgen receptor modulators (SARMs), including ligandrol (LGD-4033), andarine (S-4), and ostarine (S-22). SARMs are increasingly being abused as "safe" alternatives to steroids.

Methods: After decontamination using dichloromethane, hair specimens were segmented, cut into very small segments (<1 mm), incubated overnight in a buffer, and extracted using a mixture of organic solvents. Drugs were tested using liquid chromatography–tandem mass spectrometry and confirmed using liquid chromatography/HRMS.

Results: The determined concentrations were as follows: ligandrol, 14–42 pg/mg; andarine, 0.1–0.7 pg/mg; and ostarine, 3–21 pg/mg.

Conclusions: To enhance performance, SARMs must be used on a long-term basis, which can have serious clinical consequences, including liver damage, myocardial infarction, and blood clots. Hair testing for SARMs has additional benefits versus urine analysis as it can detect the parent compound and numerous metabolites.

Key Words: SARMs, hair, doping, detection

(*Ther Drug Monit* 2021;43:298–300)

BACKGROUND

Selective androgen receptor modulators (SARMs) are a new class of substances that have a high anabolic potency, in addition to limited androgenic effects; it is this last property that has increased interest in this new pharmacological class. SARMs are known to lack steroid-related side effects on the cardiovascular and hormonal systems, as well as the liver, skin, and mood (psychiatric effects).[1] SARMs have been proposed for the treatment of certain diseases (hypogonadism, osteoporosis, and muscular atrophy); however, to date, no SARM has demonstrated sufficient safety and efficacy to gain clinical approval by either the European Medicines Agency or the US Food and Drug Administration.[2] As SARMs can be potentially misused in sports to enhance performance, they can attract athletes looking for power and strength improvement without experiencing serious side effects. Although SARMs are known to demonstrate few side effects, they can cause liver damage, myocardial infarction, and blood clots.[3] Recently, 2 cases of SARM-induced liver injuries have been documented.[4]

In 2017, the U.S. Anti-Doping Agency published warnings regarding the use of SARMs, highlighting the dangers and risks associated with these drugs.[5] In 2018, according to the World Anti-Doping Agency,[6] 4896 adverse analytical findings (doping cases) were reported; among them, 406 belonged to the S1.2 group (other anabolic agents), including 45 (11%), (6%) and 1 (0.2%) for ostarine (S-22 or MK-2866), ligandrol (LGD-4033), and andarine (S-4), respectively. A marked increase (+800%) was observed when compared with the 2016 report and suggested that regulating their use could pose a challenge. Furthermore, SARMs demonstrate the potential for misuse as growth-promoting agents in livestock-based farming. This can raise 2 issues: false-negative test results in athletes could be observed on consumption of contaminated meat, and in addition, the long-term health consequences after eating such meat have not been established.

SARMs are often illegally marketed as dietary supplements despite their lack of safety approval, and a recent study[7] has indicated that the products present discrepancies between the accuracy of product claims and content labeling. The study has reported discrepancies ranging from a supplement in which no active ingredient was detected to supplements containing undeclared compounds. When SARMs were detected, discrepancies were observed between the concentrations measured and those detailed on the product packaging.

In recent years, hair specimens have been proposed to document drug exposure in any situation in which a history of past rather than recent drug use is desired. It allows the establishment of a retrospective calendar of an individual's drug use. Segmental analysis can document the pattern of drug exposure and allows the distinction between single and repetitive use. A recently published review[8] has discussed the interest and limitations of testing for performance-enhancing agents in human hair. Although some deviations have been observed among sports authorities, hair analyses can provide additional elements to document a specific case, as in clinical or forensic toxicology, where hair testing is a suitable complement to standard investigations in blood and/or urine.

Given that SARMs can be easily obtained on the Internet, one can anticipate an increase in requests for the

Received for publication September 29, 2020; accepted December 1, 2020.
From the *X-Pertise Consulting, University of Strasbourg, Strasbourg, France; and †Institut de Médecine Légale, University of Strasbourg, Strasbourg, France.
The authors declare no conflict of interest.
Correspondence: Pascal Kintz, PhD (e-mail: pascal.kintz@wanadoo.fr).

identification of these drugs in biological fluids, particularly when subjects are presenting with liver disease, skin pathologies, cardiac issues, or psychological disturbances. However, these compounds have numerous urinary metabolites that complicate specific identification. Indeed, for each drug, more than 10 different metabolites have been identified in the urine, with a variable window of detection.[9] Furthermore, most of these metabolites are not commercially available as reference standards, a situation that further complicates their definitive identification. By contrast, in hair, the target analyte is the parent compound, as observed by scientists for 3 decades.

In this article, we presented 3 cases where various SARMs were identified in human head hair.

CASE REPORTS

Case 1

The laboratory received the hair of a male subject (26-year-old) who claimed the use oral andarine (50 mg/mL) on several occasions for 6 months to improve his athletic performance. The subject was afraid of possible misuse of other drugs present in the supplement. The obtained hair sample was dark in color and 7 cm in length.

Case 2

The subject (24-year-old) was arrested by Customs as several blister packs of ostarine tablets (10 mg) were found in his luggage. He claimed the drug was for personal use. To document possible trafficking, a strand of his hair (brown in color and 8 cm in length) was collected.

Case 3

The subject (29-year-old) was hospitalized for psychological disturbances after the consumption of various recreational drugs. The patient indicated repetitive abuse of snorted cocaine, oral ligandrol, and 3-methylmethcathinone to improve his sexual performance over the past year. Psychiatrists collected a hair strand (black in color and 6 cm in length) to verify his claims.

HAIR ANALYSIS

The hair specimens were analyzed according to the procedure previously published by the laboratory.[10,11] Briefly, the whole hair strand was decontaminated with dichloromethane, segmented, and each segment was cut into small pieces of less than 1 mm. Fifty milligrams were incubated overnight in buffer (pH 9.5), and SARMs were extracted using a mixture of organic solvents. Liquid chromatography was performed using a Waters (Milford, MA) Acquity HSS C18 column, with a Xevo TQD triple quadrupole mass spectrometer used for detection. Results were further confirmed using liquid chromatography coupled to high-resolution mass spectrometry (Waters Xevo G2XS Q-TOF). The limits of quantitation were 0.1, 0.1, and 1 pg/mg for ostarine, andarine, and ligandrol, respectively. QC samples (10 pg/mg), analyzed in duplicate in 6 independent experimental assays, were used for determining a coefficient of variation (CV) for precision. Precision CVs were 13.8%, 14.6%, and 16.2% for ostarine, andarine, and ligandrol, respectively.

RESULTS AND DISCUSSION

During the initial stages of implementing the analytical procedure, the method was validated according to the latest recommendations of the Society of Hair Testing.[12] Given that there is no certified reference material for SARM detection in hair, and considering that there exists no laboratory comparison and no proficiency tests for this class of drugs, it was not possible to establish a true measurement uncertainty. Precision using spiked specimens was the only method that accounted for this parameter. This is probably attributed to the current limited scientific interest in hair testing for drugs; however, one can anticipate a rapid growth in focus owing to the expansion of SARMs. In the last weeks, Rading et al[13] have identified GSK2881078, another SARM, in the hair of a subject after a single oral administration of 1.5 mg. The measured hair concentration, 3 weeks postadministration, was 1.7 pg/mg, confirming that a single exposure can be detected in human hair.

All 3 hair specimens tested positive for either one SARM, at extremely low concentrations. Results are presented in Table 1. The toxicological significance of measured concentrations is difficult to determine as considerably limited data are currently available in the medical literature. Only ostarine has been previously identified in hair.[10,11] To date, no report regarding the detection of both ligandrol and andarine in human hair has been published.

The concentrations detected in the subjects' hair were interpreted as those corresponding to repeated exposure because all segments tested positive. Nevertheless, it was not possible to establish the dosage and frequency of consumption to establish the pattern of SARM consumption, owing to a lack of verified patterns of consumption, as well as a scarcity of controlled studies in the available literature.

Testing for drugs in hair to document exposure is receiving increasing attention from clinicians, scientists, and lawyers owing to its long detection window when compared with blood and urine and more convenient conditions for collection, as well as storage at ambient temperature. Hair testing can provide information on exposure to drugs over a prolonged period, depending on the length of the hair lock. The recent widespread availability of new drugs globally, including new psychoactive substances, has revealed the need for analytical approaches to document these new habits. Therefore, implementing a hair test for SARMs seems to be of great importance not only for doping purposes but also for clinical toxicologists or poison centers to accurately establish a diagnosis of addiction. Given the increasing popularity of

TABLE 1. Hair Test Results

Case 1—Andarine	Case 2—Ostarine	Case 3—Ligandrol
Positive (0–2 cm)	3 pg/mg (0–2 cm)	14 pg/mg (0–2 cm)
0.1 pg/mg (2–4 cm)	8 pg/mg (2–4 cm)	42 pg/mg (2–4 cm)
0.7 pg/mg (4–7 cm)	14 pg/mg (4–6 cm)	21 pg/mg (4–6 cm)
	21 pg/mg (6–8 cm)	

Positive: chromatographic signal below the limit of quantitation.

SARM abuse, vigilance and identification of new cases are required. As hair is a unique matrix that allows retrospective and long-term investigations, particularly when compared with blood and urine, this specimen demonstrates all suitable properties to qualify for SARM detection. Only fully validated methods should be applied, and testing methods developed on the spot should be avoided, especially when no information regarding conditions of hair collection, purpose of the test, and potential pitfalls are clearly established.

REFERENCES

1. Zhang X, Sui Z. Deciphering the selective androgen receptor modulators paradigm. *Expert Opin Drug Discov.* 2013;8:191–218.
2. Peixoto Da Fonseca GW, Dworatzek E, Ebner N, et al. Selective androgen receptor modulators (SARMs) as pharmacological treatment for muscle wasting in ongoing clinical trials. *Expert Opin Drug Discov.* 2020;29:881–891.
3. Christiansen A, Lipshultz LI, Hotaling JM, et al. Selective androgen receptor modulators: the future of androgen therapy? *Transl Androl Urol.* 2020;9(suppl 2):S135–S148.
4. Flores JE, Chitturi S, Walker S. Drug-induced liver injury by selective androgenic receptor modulators. *Hepatol Commun.* 2020;4:450–452.
5. Available at: https://www.usada.org/dietary-supplements/usada-supplement-groups-support-action-on-dangerous-sarms/. Accessed September 24, 2020.
6. Available at: https://www.wada-ama.org/sites/default/files/resources/files/2018_testing_figures_report.pdf. Accessed September 24, 2020.
7. Leaney AE, Beck P, Biddle S, et al. Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators. *Drug Test Anal.* 2020 doi: 10.1002/dta.2908.
8. Kintz P, Gheddar L, Ameline A, et al. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal.* 2020;12:316–322.
9. Thevis M, Schänzer W. Detection of SARMs in doping control analysis. *Mol Cell Endocrinol.* 2018;464:34–45.
10. Kintz P, Ameline A, Gheddar L, et al. LGD-4033, S-4 and MK-2866—testing for SARMs in hair: about 2 doping cases. *Toxicol Anal Clin.* 2019;31:56–63.
11. Kintz P, Gheddar L, Ameline A, et al. Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases. *Drug Test Anal.* 2020;12:1508–1513.
12. Cooper GAA, Kronstrand R, Kintz P. Society of hair testing guidelines for drug testing in hair. *Forensic Sci Int.* 2012;218:20–24.
13. Rading A, Anielski P, Thieme D, et al. Detection of the selective androgen receptor modulator GSK2881078 and metabolites in urine and hair after single oral administration. *Drug Test Anal.* 2020;2:1–6.

Article 15 : Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases

Pascal Kintz, Laurie Gheddar, Alice Ameline, Jean-Sébastien Raul

Drug Testing and Analysis. 2020 ; 12 :1508-1513.

Received: 26 May 2020 | Revised: 13 July 2020 | Accepted: 13 July 2020
DOI: 10.1002/dta.2902

SHORT COMMUNICATION

WILEY

Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases

Pascal Kintz[1,2] | Laurie Gheddar[2] | Alice Ameline[2] | Jean-Sébastien Raul[2]

[1]X-Pertise Consulting, 42 rue principale, Mittelhausbergen, F-67206, France
[2]Institut de médecine légale, 11 rue Humann, Strasbourg, F-67000, France

Correspondence
Pascal Kintz, Institut de médecine légale, 11 rue Humann, F-67000, Strasbourg, France.
Email: pascal.kintz@wanadoo.fr

Abstract

Ostarine, also known as S22 or MK2866 and enobosarm, is a selective androgen receptor modulator (SARM). It has high anabolic potency, in addition to limited androgenic effects. At this time, ostarine has no therapeutic use, but can be abused for performance-enhancing purposes using the oral route, at dosages of 10–25 mg per day. As the drug can easily be obtained via the Internet or some fitness centers, athletes and more and more amateurs can use it without undergoing the deleterious physiological side effects that are generally associated with testosterone-related compounds. Since 2008, the World Anti-Doping Agency (WADA) has prohibited SARMs at-all-times in the category of "other anabolic agents" under section S1.2 of the WADA List. In a case of trafficking/abuse, ostarine was identified in nail (subject 1) and hair (subject 2) by LC/HRMS after incubation of 50 mg of matrix in a pH 9.5 buffer, followed by extraction with organic solvents. The drug was quantitated by LC–MS/MS. Ostarine tested positive at 61 pg/mg (toenails) and 111 pg/mg (fingernails) for subject 1. Ostarine was present at 146, 168, 93, and 101 pg/mg in the 4 × 3 cm hair sections of subject 2, clearly demonstrating long-term use.

KEYWORDS
doping, hair, nail, ostarine, SARMs

1 | INTRODUCTION

The "toxicology of victory" has promoted new behaviors, where performance is the key point and the use of performance-enhancing drugs has been described since antiquity. In the past, plant alkaloids or alcohol have been abused for hunting or fighting in large arenas during inter-cities games. Later, with the emergence of synthetic chemistry, students and business people started to abuse stimulants, ß-blockers, or serotonin-reuptake inhibitors. Doping was officially banned late in the 1960s in Olympic sports. However, there is continuous research for new substances that are active at low dosage, easy to purchase on-line, and with limited capacities of detection. Among the most abused performance-enhancing drugs, testosterone and related anabolic steroids have ranked number one for many years. Elite athletes and amateurs abuse steroids because it has been claimed that they increase lean body mass, increase strength, increase aggressiveness, and lead to a shorter recovery time between workouts. However, they are associated with many side effects, which can be life-threatening. Over a dozen years, a new pharmacological class has emerged on the performance market, which are selective androgen receptor modulators (SARMs). These drugs have high anabolic potency, in addition to limited androgenic effects.[1] Initially, SARMs have been subject to evaluation for the treatment of muscle wasting in cancer patients and as therapeutics for cachexia but none has yet been approved.[2] As SARMs have the potential to be misused for performance enhancement in sport due to their anabolic properties as well as in the ability to stimulate androgen receptors in the muscle and the bone, they can attract athletes and amateurs looking for power and strength improvement without undergoing deleterious physiological side effects, given the reduced androgenic properties. As a consequence, since 2008, the World Anti-Doping Agency (WADA) has prohibited SARMs at-all-times in the category of "other anabolic

agents" under section S1.2 of the WADA List.[3] Several SARMs are reported on the list, including ostarine (S-22 or MK-2866), ligandrol (LGD-4033), or andarine (S-4). Although not classified as SARMs but as peroxisome proliferator-activated receptor delta (PPAR-δ) agonists, testolone (RAD140), and cardarine (GW-501516) are sometimes mixed up with them.

Ostarine, or (2S)-3-(4-cyanophenoxy)-N-[4-cyano-3-(trifluorome-thyl)phenyl]-2-hydroxy-2-methyl-propanamide (Figure 1), is one of the most popular SARMs. The drug is also known as S22 or MK2866 and enobosarm. Although no clinical study has been published about ostarine, according to promoters, the daily oral dosage ranges from 10 to 25 mg. The product is available via e-vendors on many websites and can be found in some fitness centers. According to the WADA 2018 testing figures report,[4] there were 4,896 adverse analytical findings (AAF) and among them, 406 were for the S1.2 group, including 45 (11%) for ostarine. WADA accredited laboratories have identified SARMs trafficking over the past decade.[5–7] However, up to now, it seems that these drugs have not been involved in forensic investigations and there is no case report dealing with a SARM-related fatality.

Ostarine and several metabolites have been identified in urine of both humans[8,9] and animals,[10,11] particularly to identify potential targets for routine doping controls.[12,13]

For the past 40 years, hair specimens have been used, mostly in forensic, clinical, and occupational toxicology, to document drug exposure on a long-term basis, when a history of past use was required. Hair test results allow the proof of a retrospective calendar of an individual's drug use. In addition, segmental investigations can document the pattern of drug exposure, particularly when using an average growth rate of 1 cm per month. Recently, a comprehensive literature review has been published,[14] in which the advantages and limitations of performance-enhancing agent detection in human hair have been debated. Clearly, hair test results can generate additional elements to strengthen a specific case, as additional information to standard analyses in blood and/or in urine. To date, ostarine has only been identified in human head hair in one case, with concentrations measured at 12 and 138 pg/mg, in the 2 × 3 cm hair segments.[15] Testing for ostarine in nail, another keratinous matrix, has never been reported.

This publication presents the identification of ostarine in human nail (subject 1) and hair (subject 2) collected during a forensic investigation involving SARMs trafficking. Given the laboratory had already developed a quantitative approach to test for ostarine in hair using LC–MS/MS,[15] the aim of this new method was to specifically identify the drug by LC-HRMS.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99–100%, AnalaR NORMAPUR was purchased from VWR Prolabo (Fontenay-sous-Bois, France). Acetonitrile for LC–MS grade, methanol, and dichloromethane were purchased from Merck (Molsheim, France). Ostarine was purchased from Cayman Chemical (Ann Arbor, MI, USA). The drug was diluted to appropriate concentrations with methanol, using a working 1 mg/L solution.

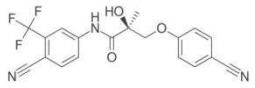


FIGURE 1 Chemical formula of ostarine

2.2 | Nail and hair specimens

Development of the method was achieved using blank fingernail and hair obtained from laboratory personnel, after verification of the absence of any chromatographic response at the retention time of ostarine. Nail specimens were washed with dichloromethane and pulverized in a ball mill (MM200 Retsch). Hair specimens were decontaminated with dichloromethane and cut into small pieces of less than 1 mm. This material was used to spike the vials at appropriate concentrations, since there is no calibrated reference nail or hair available for ostarine.

Two subjects were arrested by Customs. In their luggage, the officers discovered vials of ostarine in the liquid form. Both subjects were amateurs, practicing a lot fitness and weight lifting. They claimed that ostarine was for personal use. Given that ostarine has no therapeutic indication and is listed as a doping agent and a controlled substance, the officers considered the drug to be part of trafficking. To document the pattern of use, the judge in charge of the case requested toxicological analyses. Subject 1 was totally shaved and therefore fingernails and toenails were collected. Two hair strands (black in color, 12 cm in length) were collected from subject 2.

2.3 | Nail and hair preparation

Nail and hair specimens were pulverized for 15 min after dichloromethane decontamination (2 × 5 mL for 2 min) in a ball mill (Retsch MM200). Fifty milligrams of material (nail, hair) were incubated overnight at 50°C in 1 mL borate buffer pH 9.5. Ostarine was extracted by 5 mL of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5).

After centrifugation, separation of the organic phase and its evaporation to dryness, the residue was reconstituted in 30 μL of 5 mM ammonium formate buffer adjusted at pH 3.

2.4 | LC-HRMS instrumentation

Chromatography was performed on an Acquity class I ultra-high performance liquid chromatography and separation was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained

at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 87% phase A for 30 s, then it was increased up to 50% phase A at 10 min, followed by a rinse to decrease to 5% phase A in 0.75 min, kept for 1.5 min and followed by a return to the initial conditions at 13 min, maintained for the last 2 min. An injection volume of 3 μL was used in all cases.

Detection was performed using a high resolution mass spectrometer (XEVO™ G2XS Q-TOF, Waters Corporation, Milford, MA, USA). Leucine-enkephalin was used as the lock mass (m/z 556.2766) for accurate mass calibration and introduced using the LockSpray interface at 10 μL/min and a concentration of 200 pg/μL in 50% aqueous ACN, 0.1% formic acid. The mass spectrometric conditions were as follows: negative electrospray ionization interface (ESI-), ion spray voltage of 25 V, nitrogen as the desolvation gas and argon as the collision gas, source temperature at 150°C and desolvation temperature at 400°C with a desolvation gas (nitrogen) flow rate of 800 L/h. The conditions for the time of flight mass spectrometer scan mode were as follows: scan range 50–1,000 m/z at 6 eV (low energy) and 50–1,000 m/z with a collision energy ramp from 10–40 eV (high energy). Unifi software was used for data, chromatograms, and spectra acquisition.

3 | RESULTS AND DISCUSSION

Given the laboratory has a validated quantitative method[15] to test for ostarine in keratinous matrices, the aim of the present work was to develop a very specific identification procedure that can be accepted by lawyers but also by sport authorities. This is because, both in forensic science and doping control, it is mandatory that a sample showing a suspicious screening result is subjected to a confirmatory analysis using an individual aliquot from the original sample. This allows having two independent methods to qualify for a positive ostarine specimen.

The signal of an ostarine standard solution at 10 ng/mL produced, in the negative mode, an intense signal at m/z 388.0909, consistent with the deprotonated molecule, i.e. ostarine-[H]. The major product ion m/z 269.0536 corresponds to the loss of the cyanophenol moiety, producing an ion at m/z 118.0294. With a loss of $C_{11}H_9NO_3$, ostarine also produces an ion at m/z 185.0323, corresponding to the dissociation of the amide bond. In 2010,[11] Thevis et al had already described this ion production pattern. To qualify for ostarine, its retention time shall not differ by ± 0.1 min from the reference material. With respect to mass spectrometric identification criteria, in addition to the deprotonated molecule, the three diagnostic ions (m/z 269.0536, 118.0294, and 185.0323) must be present at high energy collision with a 20% tolerance window for their relative abundance when compared with a reference standard. A mass error < 5 ppm was considered acceptable.

Although the initial purpose of the method was only qualitative, a limit of detection (LOD) was determined using spiked specimens with decreasing concentrations of ostarine. It was decided to have a signal to noise greater than 5 to account for the LOD, in addition to verification of analytical data (e.g. retention time, ratios of ions, m/z 269.0541 and 118.0297). The LOD was 0.5 pg/mg in both nail and hair.

Figures 2 and 3 represent the chromatograms obtained from the fingernail and toenail extractions from subject 1. When using LC–MS/MS,[15] the ostarine concentrations were 111 and 61 pg/mg, in fingernails and toenails, respectively. Figure 4 represents the chromatogram obtained from the proximal hair segment (0–3 cm) extraction from subject 2. When using LC–MS/MS,[15] ostarine concentrations were 146, 168, 93, and 101 pg/mg in the 4 × 3 cm hair sections.

The final interpretation of the measured concentrations is difficult to establish because there is only one paper[15] in the medical literature

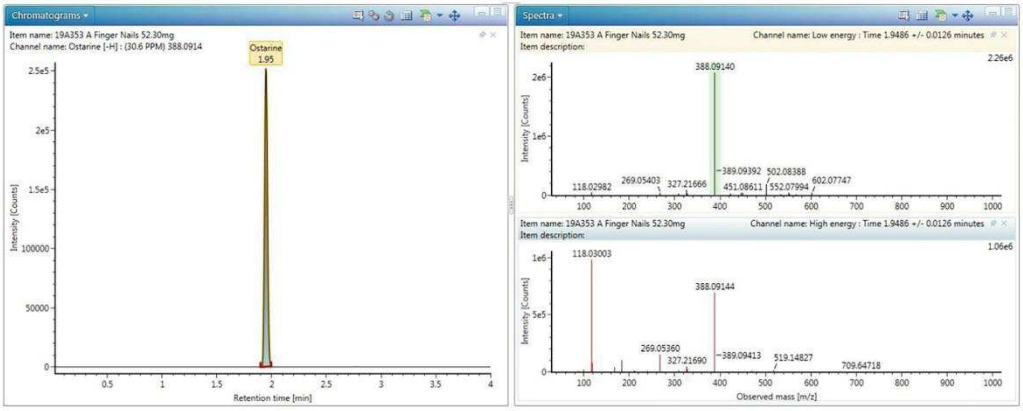


FIGURE 2 LC-HRMS chromatogram obtained after extraction of fingernails [Colour figure can be viewed at wileyonlinelibrary.com]

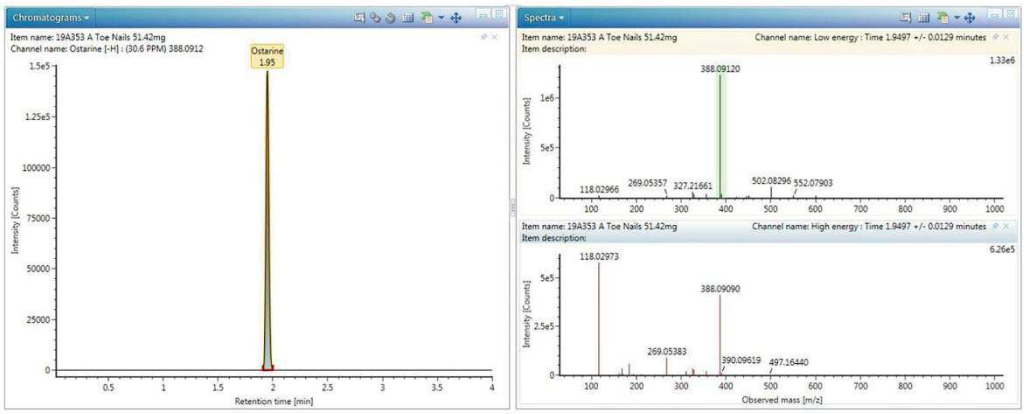


FIGURE 3 LC-HRMS chromatogram obtained after extraction of toenails [Colour figure can be viewed at wileyonlinelibrary.com]

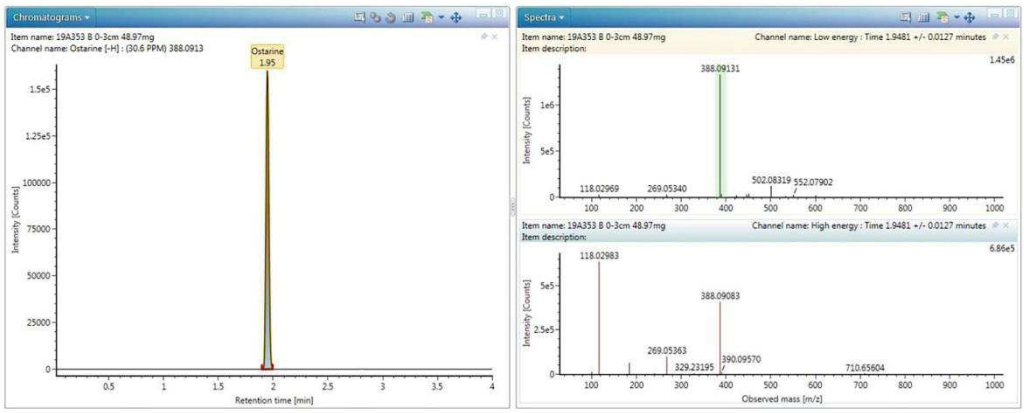


FIGURE 4 LC-HRMS chromatogram obtained after extraction of the 0–3 cm hair segment [Colour figure can be viewed at wileyonlinelibrary.com]

showing the identification of ostarine in hair and nothing has been published for nail.

It can be accepted by the authors that some parameters about the detection of SARMs in nail and hair have not been addressed: the dose necessary to give a positive result, the influence of hair color, the influence of cosmetic treatment, variable incorporation into nail and hair, external factors that may have an impact, etc. The minimum dose of ostarine that had to be ingested by a subject in order to give a "positive" detection in nail or hair is still unknown. The concentrations found in the subject's hair have been interpreted as corresponding to repeated exposures, as the four segments tested positive. Nevertheless, it was not possible to establish the dosage and the frequency of consumption to document the pattern of drug exposure. Although hair applications for performance-enhancing drugs have been well reviewed,[14,16,17] very few data are available for nails.

As is the case for hair, xenobiotics can accumulate in nails over a long period of time, which allows information to be gained about retrospective use. Collecting nails is a non-invasive procedure and specimens can be stored at room temperature. Overseas shipping is easy and there is less risk of destruction in comparison with the liquid blood or urine. Given these practical aspects, nails have been proposed as an alternative to hair in forensic and clinical toxicology.[18] Incorporation of substances into nails mainly occurs through diffusion from blood vessels in both vertically (from the nail bed) and horizontally (from the germinal matrix) during nail formation.[19]

Nails grow at a continuous rate and the average growth rate of fingernails is 3.0 mm per month, while toenails grow slower, at an average rate of 1.1 mm per month. Most scientists accept that the time frame of nail clipping represents what has circulated in blood during the previous 4 to 8 months. However, there is no

agreement on the exact window of detection of nail clipping (which can lead to tedious discussions) and each laboratory has to establish its own range.[20]

It has been advocated that nails can present advantages over hair.[21,22] When hair is not (sufficiently) available (voluntary shaved subjects or cases of alopecia, lupus or due to chemotherapy), nail testing can also provide retrospective long-term information on xenobiotic use. In contrast to hair, nails do not contain melanin, which simplifies the debate on racial bias due to the influence of the pigment on drug incorporation. In contrast to hair, nails grow at a constant rate with no restrictive phases (catagen and telogen), which facilitates the interpretation of the measured concentrations. Finally, nail collection is aesthetically more acceptable to subjects taking care of their personal look. However, segmentation to establish a pattern of drug exposure is much more complicated (if possible) with nail in comparison with head hair.

The concentrations in nails of drugs of abuse are in the same magnitude as in hair, ranging from pg/mg to ng/mg.[22] The same pattern of metabolite(s) distribution is observed in nails versus hair. Data for the detection of performance-enhancing drugs in nail clippings are very limited. Testosterone, testosterone propionate, and stanozolol were screened in fingernails, but the results were disappointing.[23] With respect to endogenous steroids, no significant correlation for cortisone, cortisol, androstenedione, testosterone, and progesterone concentrations were found between hair and nails. These results demonstrate that matrix-dependent value ranges for hair and nail steroid levels should be established and applied for interpretation.[24]

Amphetamines are detected presumably with higher concentrations in toenails than in fingernails. In contrast to amphetamines, cocaine is present in 7- to 20-fold higher concentrations in fingernails compared with toenails.[19] Ostarine was detected in higher amount (about twice) in fingernails when compared with toenails, but there is no reference to discuss the findings.

In the absence of a controlled study, it is not possible to compare the nail versus the hair concentrations to indicate if one of the subjects had consumed more ostarine than the other. This needs further investigation. The authors did not interview the donors of the nails and the hair samples about the dosage.

4 | CONCLUSION

According to the WADA testing figures, it appears that abuse of SARMs is being detected more and more. At this time, there was only one publication dealing with ostarine detection in human hair that has been published in the scientific literature. This double case completes the available data and demonstrates that even with daily use, the measured ostarine concentrations are in the pg/mg range in both nail and hair. These keratinous matrices may be a useful adjunct to conventional drug testing in toxicology. The specimens can be collected easily and can provide a more specific history of drug use, when compared with blood and urine. LC-HRMS was used for the specific identification of ostarine to complement an initial quantitative MS/MS method. This approach, useful in forensic toxicology and in doping control, has the unique advantage of securing the univocal presence of a prohibited drug using two independent methods.

ORCID

Pascal Kintz https://orcid.org/0000-0002-4174-5362
Laurie Gheddar https://orcid.org/0000-0002-7804-3946
Alice Ameline https://orcid.org/0000-0002-1117-0887

REFERENCES

1. Zhang X, Sui Z. Deciphering the selective androgen receptor modulators paradigm. *Expert Opin Drug Discov*. 2013;8(2):191-218.
2. Chen J, Kim J, Dalton J. Discovery and therapeutic promise of selective androgen receptor modulators. *Mol Interv*. 2005;5(3):173-188.
3. https://www.wada-ama.org/en/content/what-is-prohibited/prohibited-at-all-times/anabolic-agents (Accessed 17 May 2020)
4. https://www.wada-ama.org/sites/default/files/resources/files/2018_testing_figures_report.pdf (Accessed 17 May 2020)
5. Thevis M, Geyer H, Thomas A, Schänzer W. Trafficking of drug candidates relevant for sports drug testing: detection of non-approved therapeutics categorized as anabolic and gene doping agents in products distributed via the internet. *Drug Test Anal*. 2011;3(5):331-336.
6. Van Wagoner RM, Eichner A, Bhasin S, Deuster PA, Eichner D. Chemical composition and labelling of substances marketed as selective androgen receptor modulator and sold via the internet. *JAMA*. 2017;318(20):2004-2010.
7. Thevis M, Geyer H, Kamber M, Schanzer W. Detection of the arylpropionamide-derived selective androgen receptor modulator (SARM) S-4 (Andarine) in a black market product. *Drug Test Anal*. 2009;1(8):387-392.
8. Temerdashev A, Dmitrieva E, Azaryan A, Gashimova E. A novel approach to the quantification of urinary aryl-propionamide-derived SARMs by UHPLC-MS/MS. *Biomed Chromatogr*. 2020;34(1):e4700. https://doi.org/10.1002/bmc.4700
9. Ventura E, Gadaj A, Monteith G, et al. Development and validation of a semi-quantitative ultra-high performance liquid chromatography-tandem mass spectrometry method for screening of selective androgen receptor modulators in urine. *J Chromatogr A*. 1600;2019:183-196.
10. Beucher L, Dervilly-Pinel G, Cesbron N, et al. Specific characterization of non-steroidal selective androgen receptor modulators using supercritical fluid chromatography coupled to ion-mobility mass spectrometry: application to the detection of enobosarm in bovine urine. *Drug Test Anal*. 2017;9(2):179-187.
11. Thevis M, Gerace E, Thomas A, et al. Characterization of in vitro generated metabolites of the selective androgen receptor modulators S-22 and S-23 and in vivo comparison to post-administration canine urine specimens. *Drug Test Anal*. 2010;2(11-12):589-598.
12. Thevis M, Thomas A, Möller I, Geyer H, Dalton JT, Schänzer W. Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator S-22 to identify potential targets for routine doping controls. *Rapid Commun Mass Spectrom*. 2011;25(15):2187-2195.
13. Garg N, Hansson A, Knych HK, et al. Structural elucidation of major selective androgen receptor modulator (SARM) metabolites for doping control. *Org Biomol Chem*. 2018;16(5):698-702.
14. Kintz P, Gheddar L, Ameline A, Arbouche N, Raul JS. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal*. 2020;12(3):316-322.
15. Kintz P, Ameline A, Gheddar L, Raul JS. LGD-4033, S-4 and MK-2866 – testing for SARMs in hair: about 2 doping cases. *Toxicol Anal Clin*. 2019;31:56-63.

16. Thieme D. Potential and limitations of alternative specimens in doping control. *Bioanalysis*. 2012;4(13):1613-1622.
17. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complementary matrices: advantages and limitations. *J Pharm Biomed Anal*. 2016;130:220-230.
18. Busardo FP, Gottardi M, Pacifici R, et al. Nails analysis for drugs used in the context of chemsex: a pilot study. *J Anal Toxicol*. 2020;44:69-74.
19. Solimini R, Minutillo A, Kyriakou C, Pichini S, Pacifici R, Busardo FP. Nails in forensic toxicology: an update. *Curr Pharm Des*. 2017;23(36):5468-5479.
20. Kuwayama K, Miyaguchi H, Iwata YT, et al. Time-course measurements of drug concentrations in hair and toenails after single administrations of pharmaceuticals products. *Drug Test Anal*. 2017;9(4):571-577.
21. Baumgartner MR. Nails: an adequate alternative matrix in forensic toxicology for drug analysis. *Bioanalysis*. 2014;6(17):2189-2191.
22. Cappelle D, De Keukeleire S, Neels H, et al. Keratinous matrices for the assessment of drugs of abuse consumption: a correlation study between hair and nails. *Drug Test Anal*. 2018;10(7):1110-1118. https://doi.org/10.1002/dta.2356
23. Brown HG, Perrett D. Detection of doping in sports: detecting anabolic-androgenic steroids in human fingernails clippings. *Med Leg J*. 2011;79(2):67-69.
24. Voegel CD, Hofmann M, Kraemer T, Baumgartner MR, Binz T. Endogenous steroid hormones in hair: investigations on different hair types, pigmentation effects and correlation to nails. *Steroids*. 2020;154:108547. https://doi.org/10.1016/j.steroids.2019.108547

How to cite this article: Kintz P, Gheddar L, Ameline A, Raul J-S. Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases. *Drug Test Anal*. 2020;12:1508–1513. https://doi.org/10.1002/dta.2902

Article 16 : Testing for GW501516 (cardarine) in human hair using LC-MS/MS and confirmation by LC-HRMS

Pascal Kintz, Alice Ameline, Laurie Gheddar, Jean-Sébastien Raul

Drug Testing and Analysis. 2020 ; 12(7) :980-986.

Received: 20 March 2020 | Revised: 8 April 2020 | Accepted: 9 April 2020
DOI: 10.1002/dta.2802

SHORT COMMUNICATION

WILEY

Testing for GW501516 (cardarine) in human hair using LC/MS–MS and confirmation by LC/HRMS

Pascal Kintz[1,2] | Alice Ameline[2] | Laurie Gheddar[2] | Jean-Sébastien Raul[2]

[1]X-Pertise Consulting, Mittelhausbergen, France
[2]Institut de medecine légale, Strasbourg, France

Correspondence
Pascal Kintz, X-Pertise Consulting, 42 rue principale, F-67206 Mittelhausbergen, France.
Email: pascal.kintz@wanadoo.fr

Abstract

GW501516, also known as GW-1516 or cardarine and endurobol, is a peroxisome proliferator-activated receptor delta (PPAR-δ) agonist. Activation of the receptor will increase fat-burning capacity and muscle production, as it changes the body's fuel preference from glucose to lipids. GW501516 has no therapeutic use, but can be abused for performance-enhancing purposes using the oral route, at dosages of 10 to 20 mg per day, for 6 to 8 weeks. Both athletes and amateurs can abuse GW501516 as the drug can be easily obtained via the Internet. Since January 2009, the list of prohibited substances and methods of doping as established by the World Anti-Doping Agency includes GW-501516, first as a gene doping substance and now in the S4.5 Metabolic modulators class. It is prohibited at all times. Using LC/MS–MS and confirmation by LC/HRM, after methanol incubation of 20 mg with ultrasound for 1 hour, GW501516 was identified in the hair of a male abuser at 32 and 22 pg/mg in 2 × 2 cm segments. The result is the first evidence that this compound with a carboxylic acid function is incorporated in human hair.

KEYWORDS
cardarine, doping, GW1516, hair, PPAR- δ agonist

1 | INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors playing important regulatory functions in development and metabolism. Among the PPARs, it has been demonstrated that PPAR-δ agonists play a crucial role in fatty acid metabolism in several tissues. In skeletal muscle and adipose tissue, activation of the receptor promotes fatty acid burning by upregulation of fatty acid uptake, ß-oxidation, and energy uncoupling. In addition, PPAR-δ agonists are involved in the adaptive metabolic responses of skeletal muscle to changes, such as physical exercise by controlling the number of oxidative myofibers.[1] It has also been published that PPAR- δ agonists have a role in the different stages of non-alcoholic fatty liver disease.[2] Rapidly,[3] GlaxoSmithKline and Ligand developed GW-501516, an agonist for the potential treatment of dyslipemia. The drug reached phase II clinical trials but was discontinued in 2007 due to induction of intestinal adenoma in rats, and other highly selective PPAR-δ with an improved safety profile were proposed.[4]

In rats, the induction of oxidative genes as well as a modified substrate preference of skeletal muscles caused a shift from sugar to lipid consumption, resulting in an improvement of endurance performance by about 70%.[5] Despite its bad reputation, GW-501516 started to be misused by athletes but also by amateurs for its performance-enhancing effects, particularly due to its shifting energy consumption from carbohydrates to lipids.[6–8] Since January 2009, the list of prohibited substances and methods of doping as established by the World Anti-Doping Agency (WADA) includes GW-501516, first as a gene doping substance and now in the S4.5 Metabolic modulators class.[9] It is prohibited at all times. In 2018,[10] there were 19 sanctions related to this compound worldwide, while less than 6 in 2016 and 31 in 2017. GW1516 can be abused for performance-enhancing purposes using the oral route. The drug is extremely effective at low dosages of 10 to 20 mg per day, for 6 to 8 weeks.

GW501516 is also known as GW-1516, cardarine, or endurobol. Chemically, the drug is related to {4-[({4-methyl-2-[4-(trifluoromethyl) phenyl]-1,3-thiazol-5-yl}methyl)sulfanyl]-2-methylphenoxy}acetic acid, or $C_{21}H_{18}F_3NO_3S_2$, with a molecular weight of 453.5 daltons. The chemical formula is presented in Figure 1.

GW501516 has been identified in items from the Internet,[11] in plasma,[7] and in urine,[12–14] where several authors have identified two mono-oxygenated and bis-oxygenated urinary metabolites, M1-sulfoxide and M2-sulfone.

In recent years, four review papers have discussed the interest and the limitations of performance-enhancing agent tests in human hair.[15–18] Although some controversies and divergences do exist, hair analyses can provide additional elements to document a specific case, as is done in forensic toxicology. Because hair testing for drugs increases the window of detection and permits the differentiation of long-term use from a single exposure when performing segmental analyses, this matrix should be considered as a suitable complement, and not an alternative, to standard investigations in blood and/or in urine.[19] In addition, hair testing can significantly contribute to exculpation of athletes by demonstrating alternative administration pathways with contamination risks, as explained by scientists from the WADA accredited laboratory in Germany.[16] Under all the above mentioned conditions, the application of hair testing, which is a non-invasive and a non-intrusive matrix, is a viable approach to document a case after an adverse analytical finding, in accordance with the Society of Hair Testing (SoHT) consensus.[20]

Although GW501516 has been already described in equine hair,[21] this paper presents the first identification of GW501516 in human hair.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99–100%, AnalaR NORMAPUR was purchased from VWR Prolabo (Fontenay-sous-Bois, France). Acetonitrile for LC–MS grade, methanol, and dichloromethane were purchased from Merck (Molsheim, France). Diazepam-d_5 was purchased from LipoMed (Arlersheim, Switzerland). GW501516 was purchased from StopAcne as white powder (Leiden, Netherlands). Purity (> 98%) was checked by a standard ^{1}H NMR spectroscopy procedure.[22]

Drugs were diluted to appropriate concentrations with methanol, using a working 1 mg/L solution for GW1516 and a 0.1 mg/L solution for diazepam-d_5.

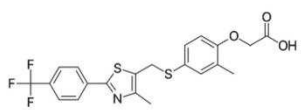


FIGURE 1 Chemical formula of GW1516

2.2 | Hair specimens

Validation of the method was achieved using blank hair obtained from laboratory personnel, after verification of the absence of any chromatographic response at the retention time of GW501516. The hair specimens were decontaminated with dichloromethane, cut into small pieces of less than 1 mm and pooled. This material was used to spike the vials at appropriate concentrations, since there is no reference hair available for GW501516.

The applicability of the method was demonstrated by analyzing the hair of a male subject who claimed to have used GW1516 for several weeks to improve his athletic performance. The hair was dark in color and 4 cm in length, oriented root-tip.

2.3 | Hair preparation

The hair strand was first decontaminated by two baths of 5 mL dichloromethane for 2 min and then segmented in 2 × 2 cm. Each segment was cut into small pieces (< 1 mm). GW501516 was extracted from 20 mg decontaminated cut hair in the presence of 2 ng of diazepam-d_5 (20 µL of a 0.1 mg/L solution in methanol) used as an internal standard, after incubation in 1 mL methanol and placement in an ultrasound bath for 1 h at room temperature. After centrifugation, separation of the supernatant, and evaporation to dryness, the residue was reconstituted in 30 µL of 5 mM ammonium formate buffer adjusted at pH 3.

2.4 | LC/MS–MS instrumentation

Chromatography was performed on an Acquity class I ultra-high performance liquid chromatography and separation was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 87% phase A for 30 sec, then increased up to 50% phase A at 10 min, followed by a rinse to decrease to 5% phase A in 0.75 min, kept for 1.5 min and followed to a return to the initial conditions at 13 min, maintained for the last 2 min. An injection volume of 3 µL was used in all cases. A Xevo TQD triple quadrupole mass spectrometer from Waters (Milford, MA, USA) was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of GW501516 and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple reaction monitoring conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociated of both species was performed. Collision energy was adjusted to optimize the signal

for the two most abundant product ions of GW501516: m/z 454.0 > 257.0 (cone voltage at 58 V and collision energy at 30 eV) and 454.0 > 85.9 (cone voltage at 58 V and collision energy at 30 eV), and the most abundant product ion of internal standard m/z 291.1 > 197.8. Transition m/z 454.0 > 257.0 was used for quantification of GW1516. MassLynx 4.1 software was used for quantification.

2.5 | LC/HRMS instrumentation

Chromatographic conditions (column, temperature, mobile phase, and gradient) were the same as for LC/MS–MS.

Detection was performed using a high resolution (XEVO™ G2XS Q-TOF, Waters Corporation, Milford, MA, USA) mass spectrometer operating in positive-ion mode and in sensitivity mode. The desolvation gas flow was set to 1000 L/h at a temperature of 600°C with the cone gas set to 50 L/h, and the source temperature set to 120°C. The capillary voltage and the cone voltage were set to 500 and 20 V, respectively. Leucine-enkephalin was used as the lock mass (m/z 556.2766) for accurate mass calibration and introduced using the LockSpray interface at 10 μL/min and a concentration of 200 pg/μL in 50% aqueous ACN, 0.1% formic acid. In MS scanning, data were acquired from 50 to 1000 m/z. As for MS/MS fragmentation of target ions, collision energy ranging from 10 to 40 V was applied. Unifi software was used for data, chromatograms, and spectra acquisition.

3 | RESULTS AND DISCUSSION

It has been reported by various authors[7,14,21] that the two most appropriate transitions for GW501516 are m/z 454 > 257 and m/z 454 > 188. However, during the initial implementation of the method at the laboratory with the above mentioned equipment, the second transition resulted in a low signal (less than 0.1% of the total signal), close to the background noise and therefore, the transition m/z 454.0 > 85.9 was considered more suitable for confirmation of the presence of GW1516. However, this transition is also less than 1% of the m/z 454 > 257 transition, as highlighted in Figure 2. This can be a major issue at very low concentration, as the second transition for confirmation can be at a level close to the background noise. Therefore, final confirmation by accurate mass spectrometry appears mandatory, particularly for concentrations below 100 pg/mg.

Although GW501516 and diazepam-d_5 are chemically very different, the rationale for choosing diazepam-d_5 is its use during the initial screening of each hair sample using a procedure for general unknowns and therefore the possibility, in a second time, of direct confirmation of the extract with a more specific method (i.e. this proposed method). This is also due to the fact that in most cases, several tests are requested to be achieved on the same hair material, which is, in general, limited in quantity.

All data of the LC/MS–MS validation are presented in Table 1. Briefly, linearity over eight points was observed for GW501516 concentrations ranging from 1 to 1000 pg/mg, with a correlation coefficient of 0.9993. This wide range of linearity was necessary as there are no data in the literature to estimate the expected concentrations in authentic hair specimens. The extraction time was optimized by comparing the area responses of the authentic positive hair specimen after different incubation times varying from 30 min to 4 hours. A 1 hour incubation was found suitable. The use of methanol as extraction solvent is common,[23] but the chromatograms are generally dirty, due to the lack of further purification. This has an impact on the limit of detection. Figures 3 and 4 represent the chromatograms obtained from the authentic hair extraction. It was not possible to establish a true extraction recovery in authentic hair due to the lack of reference

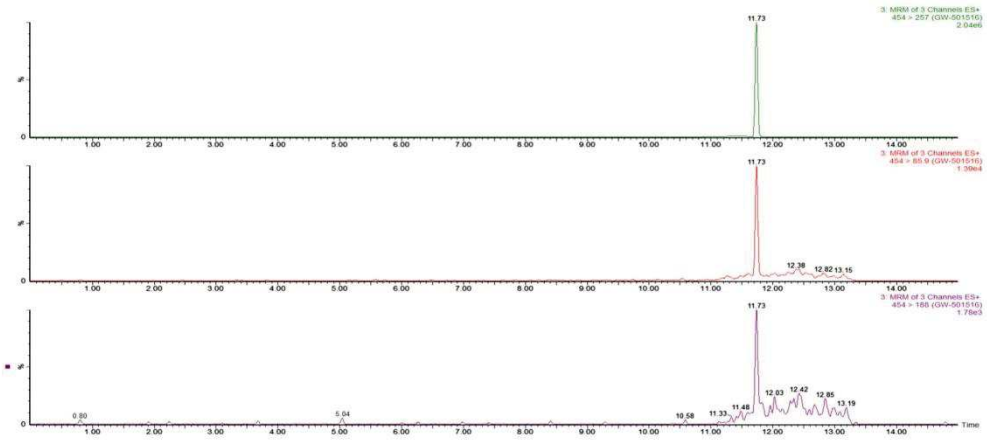


FIGURE 2 Ion transitions for GW501516. The signals of the second (m/z 454 > 85.9) and the third (m/z 454 > 188) transitions were 0.8% and 0.1%, respectively, when compared with the first transition (m/z 454 > 257)

TABLE 1 Validation parameters

Parameters	Results
Linearity	Linear from 1 to 1000 pg/mg, r^2 = 0.9993
Precision (n = 6)	12.4% at 100 pg/mg, 11.9% at 500 pg/mg
Limit of detection	0.3 pg/mg
Limit of quantitation	1 pg/mg
Matrix effects	< 20%
Interferences	None detected, true blanks are negative (n = 10)

material available for validation. Indeed, the only possibility to establish the validation parameters was to use spiked hair. With respect to the incorporation rate, it can be anticipated that it might be poor, due to the presence of an acid function in the chemical structure of GW501516, which is against good incorporation.[19] QC samples (100 pg/mg and 500 pg/mg), analyzed in duplicate in six independent experimental assays, were used to determine a coefficient of variation for precision. Precision CVs were 12.4% and 11.9% for low and high QC samples, respectively. The limit of detection (3 times the background noise) and the lower limit of quantification (first point of calibration) were 0.3 pg/mg and 1 pg/mg, respectively. Under the chromatographic conditions used, there was no interference with the analytes by chemicals or any extractable endogenous materials present in hair. The matrix effect (< 20%) was investigated after extraction of 20 blank hair samples, spiked at 100 pg/mg of GW501516 and compared with a corresponding solution at the same concentration in methanol. There was no GW1516 signal in 10 true negative hair samples collected among laboratory personnel.

The method was used to test for GW501516 in an authentic case. Concentrations were 32 and 22 pg/mg for the 2 × 2 cm segments. The results were confirmed by LC/HRMS (Figure 5). The toxicological significance of the measured concentrations is difficult to determine because there is only one paper in the whole scientific literature presenting GW501516 concentrations in hair, but this was in equine hair.[21] The authors detected GW501516 in a horse's hair at an estimated concentration of 0.2 pg/mg and the results were used to support a previous finding of the same analyte in the corresponding plasma sample.

The measured concentrations of GW501516 are low, in the low pg/mg range. This is probably the consequence of the presence of an acid function in the structure of GW501516, with acid/basic dissociation constants (pKa) of 3.51 and 2.14.[24] Contrary to an alkaline molecule, GW501516, a weak acidic drug, must have a poor ability to penetrate through the follicle membrane, based on the pH gradient between blood and hair follicles.[25] Although drug stability in the hair shaft is generally remarkable once incorporated,[19] nothing is known for GW501516.

When testing for drugs in hair, interpretation of the results is the most critical step, particularly when there are no data in the literature to compare the findings. Unfortunately, nothing is known about the incorporation of PPAR- δ agonists into hair. It can be accepted by the authors that some parameters about the detection of GW501516 in hair have not been addressed in the present manuscript: the dose necessary to give a positive result, influence of external contamination,

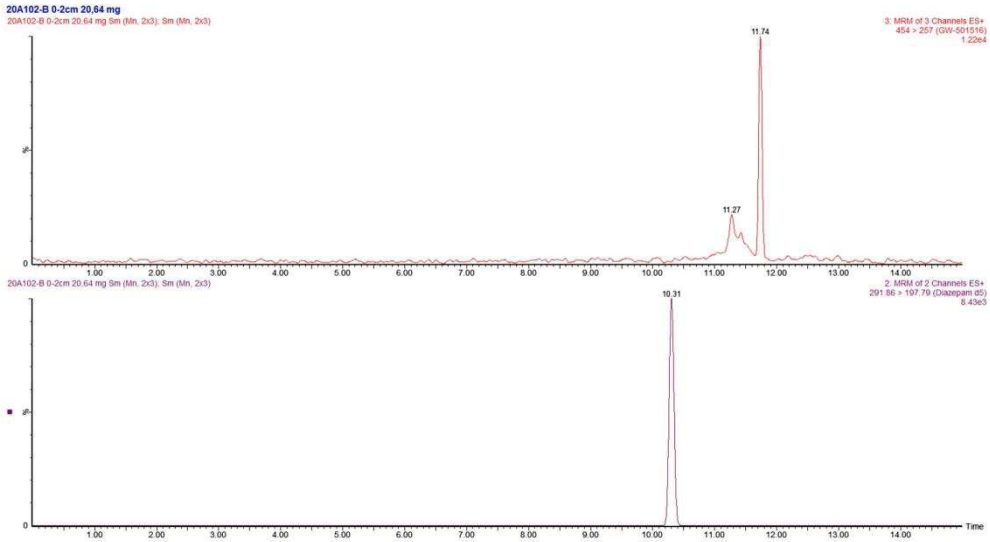


FIGURE 3 Chromatogram obtained after extraction of the 0–2 cm hair segment. From top to bottom: one transition of GW1516 and one transition of the internal standard. Concentration was 32 pg/mg

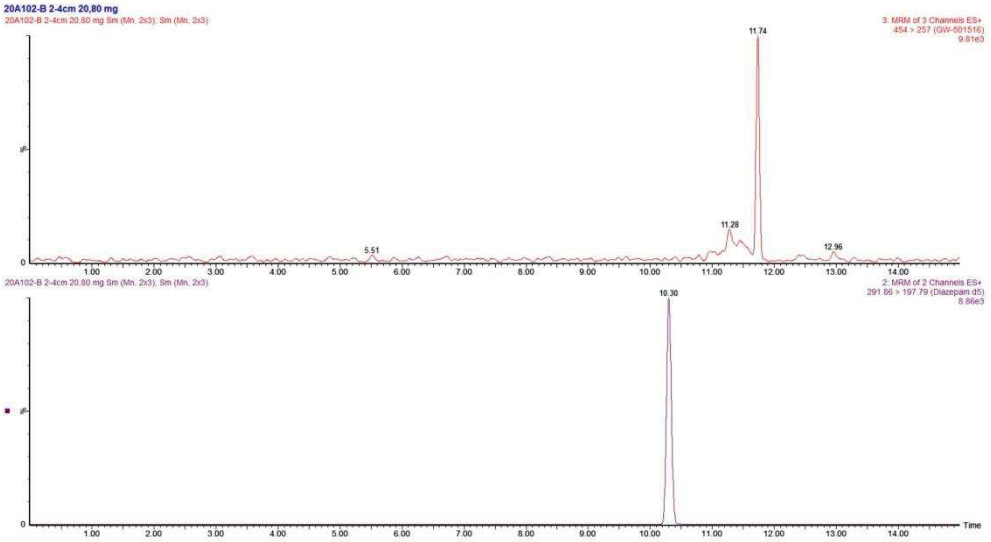


FIGURE 4 Chromatogram obtained after extraction of the 2–4 cm hair segment. From top to bottom: one transition of GW1516 and one transition of the internal standard. Concentration was 22 pg/mg

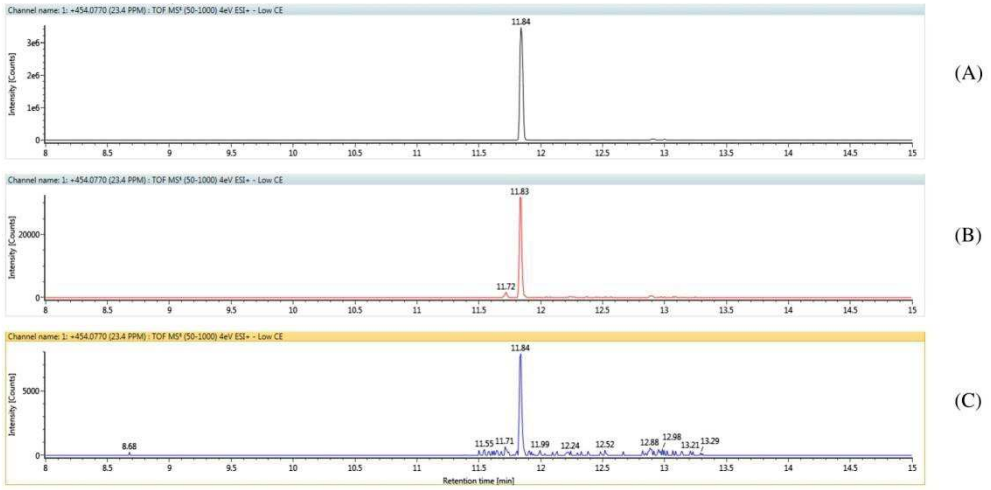


FIGURE 5 LC/HRMS chromatograms obtained after extraction of a GW1516 reference (A), followed by the two hair segments from the abuser (B and C)

variable incorporation into hair, external factors that may have an impact, etc. The minimum dose that had to be ingested by a subject in order to get a "positive" detection in one of the hair sections tested, 1 or 2 months after the collection of a urine sample, is unknown. When using hair analysis as a matrix during analysis, the question of importance is to know whether the analytical procedure is sensitive enough to identify traces of drug (i.e. a single standard dose). The concept of a minimal detectable dosage in hair is of interest to document the findings, but there are no data currently available in the scientific literature about GW501516 or its metabolite(s) hair concentrations. GW501516 was pulled from clinical trials when it was found to cause cancer, and sports authorities present it as a non-safe drug.[26]

Accordingly, pharmaceutical companies will not have undertaken human studies, which means that there will be no response in relation to single use detection in hair. Until laboratories have methodologies sensitive enough to detect the single use of the drug, care should be taken in comparing blood or urine and hair findings. In addition, hair is continuously subjected to natural factors, such as sunlight, weather, water, pollution, etc., which affect and damage the cuticle and hair cosmetic treatments enhance the damage.[19]

The concentrations found in the subject's hair have been interpreted as corresponding to repeated exposures, as two segments tested positive. Nevertheless, it is not possible to establish the dosage and the frequency of consumption. This is also due to the lack of literature. Given the toxicity of GW501516 in animal models,[4] it is obvious that no controlled study will be implemented to address the previously mentioned issues, which have emerged for interpreting the hair findings. This is simply ethically not acceptable.

Testing for drugs in hair to document exposure is receiving more and more attention by scientists and lawyers due to is long detection window, particularly when compared with blood and urine, its less embarrassing conditions of collection and its storage at ambient temperature. Hair testing can provide information on exposure to drugs over a long period of time, depending on the length of the hair lock, and therefore may be useful to establish a pattern of drug exposure in the past months, when performing segmental analyses. Given an average growth rate of 1 cm per month, each cm of hair in the vertex region represents what has circulated in the body during the corresponding month. It also allows verification of self-reported histories of drug use, as was demonstrated in the present case. The application of hair testing is a viable approach to document GW501516 findings during a doping control or survey of a subject presenting health problems after long-term abuse. The side effect of this chemical compound is so serious that WADA has taken the rare step of warning "cheats" to ensure that there is complete awareness of the possible health risks to athletes who succumb to the temptation of using GW501516 for performance enhancement.[27]

4 | CONCLUSION

The application of hair testing seems to be a suitable approach to document GW501516 exposure. However, the method has some limitations. It was not possible to establish the dosage and the frequency of consumption for GW501516 due to a lack of reference data. Due to the expected limited incorporation of the drug into hair linked to its acid properties, very sensitive tools are necessary to test for GW501516. This study represents the first contribution to the identification of a PPAR- δ agonist in human hair.

ORCID

Pascal Kintz https://orcid.org/0000-0002-4174-5362
Alice Ameline https://orcid.org/0000-0002-1117-0887
Laurie Gheddar https://orcid.org/0000-0002-7804-3946

REFERENCES

1. Luquet S, Lopez-Soriano J, Holst D, et al. Roles of peroxisome proliferator-activated receptor delta (PPARdelta) in the control of fatty acid catabolism. A new target for the treatment of metabolic syndrome. *Biochimie*. 2004;86(11):833-837.
2. Tailleux A, Wouters K, Staels B. Roles of PPARs in NAFLD: potential therapeutic targets. *Biochim Biophys Acta*. 1821;2012:809-818.
3. Pelton P. GW-501516 GlaxoSmithKline/ligand. *Curr Opin Investig Drugs*. 2006;7(4):60-370.
4. Lagu B, Kluge AF, Goddeeris MM. Highly selective peroxisome proliferator-activated receptor δ (PPAR- δ) modulator demonstrates improved safety profile compared to GW501516. *Bioorg Med Chem Lett*. 2018;28:533-536.
5. Brunmair B, Staniek K, Dörig J, et al. Activation of PPAR-delta in isolated rat skeletal muscle switches fuel preference from glucose to fatty acid. *Diabetologia*. 2006;49(11):2713-2722.
6. Narkar VA, Downes M, Yu RT, et al. AMPK and PPARdelta agonists are exercise mimetics. *Cell*. 2008;134(3):405-415.
7. Thevis M, Beuck S, Thomas A, et al. Doping control analysis of emerging drugs in human plasma – identification of GW501516, S-107, JTV-519, and S-40503. *Rapid Commun Mass Spectrom*. 2009;23(8): 1139-1146.
8. Pokrywka A, Cholbinski P, Kaliszewski P, Kowalczyk K, Donczak D, Zembron-Lacny A. Metabolic modulators of the exercise response: doping control analysis of an agonist of the peroxisome proliferator-activated receptor δ (GW501516) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). *J Physiol Pharmacol*. 2014; 65(4):469-476.
9. https://www.wada-ama.org/en/resources/science-medicine/prohibited-list-documents. Accessed 19 March 2020
10. https://www.wada-ama.org/sites/default/files/resources/files/2018_testing_figures_report.pdf. Accessed 19 March 2020
11. Thevis M, Geyer H, Thomas A, Schänzer W. Trafficking of drug candidates relevant for sports drug testing: detection of non-approved therapeutics categorized as anabolic and gene doping agents in products distributed via the internet. *Drug Test Anal*. 2011; 3(5):331-336.
12. Thevis M, Thomas A, Kohler M, et al. Mass spectrometry-based characterization of new drugs and methods of performance manipulation in doping control analysis. *Eur J Mass Spectrom*. 2010;16(3):301-312.
13. Sobolevsky T, Dikunets M, Sukhanova I, Virus E, Rodchenkov G. Detection of PPAR- δ agonists GW1516 and GW0742 and their metabolites in urine. *Drug Test Anal*. 2012;4(10):754-760.
14. Thevis M, Möller I, Thomas A, et al. Characterization of two major urinary metabolites of the PPARδ-agonist GW1516 and implementation of the drug in routine doping controls. *Anal Bioanal Chem*. 2010; 396(7):2479-2491.
15. Rivier L. Is there a place for hair analysis in doping controls? *Forensic Sci Int*. 2000;107(1-3):309-323.
16. Thieme D. Potential and limitations of alternative specimens in doping control. *Bioanalysis*. 2012;4(13):1613-1622.
17. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complementary matrices: advantages and limitations. *J Pharm Biomed Anal*. 2016;130:220-230.
18. Kintz P, Gheddar L, Ameline A, Arbouche N, Raul JS. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal*. 2020;12(3):316-322.
19. Kintz P. Hair analysis in forensic toxicology: an update review with a special focus on pitfalls. *Curr Pharm Des*. 2017;23(36):5480-5486.
20. Sachs H, Kintz P. Consensus of the Society of Hair Testing on hair testing for doping agents. *Forensic Sci Int*. 2000;107:3.
21. Gray B, Viljanto M, Menzies E, Vanhaecke L. Detection of prohibited substances in equine hair by ultra-high performance liquid chromatography-triple quadrupole mass spectrometry – application to doping control samples. *Drug Test Anal*. 2018;10:1050-1060.

22. Ameline A, Garnier D, Gheddar L, et al. Identification and analytical characterization of seven NPS, by combination of ^{1}H NMR spectroscopy, GC-MS and UPLC-MS/MS, to resolve a complex toxicological fatal case. *Forensic Sci Int*. 2019;298:140-148.
23. Madry MM, Kraemer T, Baumgartner MR. Systematic assessment of different solvents for the extraction of drugs of abuse and pharmaceuticals from an authentic hair pool. *Forensic Sci Int*. 2018;282:137-143.
24. https://www.drugbank.ca/drugs/DB05416, Accessed 19 March 2020
25. Kronstrand R, Scott K. In: Kintz P, ed. *Drug incorporation into hair. In: Analytical and practical aspects of drug testing in hair*. Forensic Science Series. Boca Raton, FL: Taylor and Francis; 2007:1-19.
26. https://www.usada.org/spirit-of-sport/education/what-should-athletes-know-gw1516/, Accessed 19 March 2020
27. https://www.wada-ama.org/en/media/news/2013-03/wada-issues-alert-on-gw501516, Accessed 19 March 2020

How to cite this article: Kintz P, Ameline A, Gheddar L, Raul J-S. Testing for GW501516 (cardarine) in human hair using LC/MS–MS and confirmation by LC/HRMS. *Drug Test Anal*. 2020;1–7. https://doi.org/10.1002/dta.2802

Article 17 : PPAR-δ and SARM abuse : clinical, analytical and biological data in a case involving a poisonous combination of GW1516 (cardarine) and MK2866 (ostarine)

Pascal Kintz, Laurie Gheddar, Camille Paradis, Mickael Chinellato, Alice Ameline, Jean-Sébastien Raul, Magali Oliva-Labadie

Toxics. 2021; 9(10):251. Doi:10.3390/toxics9100251

toxics

MDPI

Article

Peroxisome Proliferator-Activated Receptor Delta Agonist (PPAR- δ) and Selective Androgen Receptor Modulator (SARM) Abuse: Clinical, Analytical and Biological Data in a Case Involving a Poisonous Combination of GW1516 (Cardarine) and MK2866 (Ostarine)

Pascal Kintz [1,*], Laurie Gheddar [1], Camille Paradis [2], Mickael Chinellato [3], Alice Ameline [1], Jean-Sébastien Raul [1] and Magali Oliva-Labadie [2]

[1] Institut de Médecine Légale, 11 rue Humann, 67000 Strasbourg, France; l.gheddar@gmail.com (L.G.); ameline.alice@gmail.com (A.A.); js.raul@unistra.fr (J.-S.R.)
[2] Centre Antipoison et de Toxicovigilance, Groupe Hospitalier Pellerin, Place Amélie Raba Léon, 33076 Bordeaux, France; camille.paradis@chu-bordeaux.fr (C.P.); magali.labadie@chu-bordeaux.fr (M.O.-L.)
[3] Service des Urgences, Centre Hospitalier de Marmande, 76 rue du Dr Courret, 47200 Marmande, France; n.chinellato@gmail.com
* Correspondence: pascal.kintz@wanadoo.fr

Citation: Kintz, P.; Gheddar, L.; Paradis, C.; Chinellato, M.; Ameline, A.; Raul, J.-S.; Oliva-Labadie, M. Peroxisome Proliferator-Activated Receptor Delta Agonist (PPAR- δ) and Selective Androgen Receptor Modulator (SARM) Abuse: Clinical, Analytical and Biological Data in a Case Involving a Poisonous Combination of GW1516 (Cardarine) and MK2866 (Ostarine). *Toxics* **2021**, *9*, 251. https://doi.org/10.3390/toxics9100251

Academic Editors: Elisabetta Bertol and Claudia Trignano

Received: 8 September 2021
Accepted: 5 October 2021
Published: 7 October 2021

Abstract: A 43-year-old male, sport coach, presented him-self at the Emergency unit of a local hospital for epigastric pain, myalgia pain and severe headache. He claimed having used for some days a combination of GW1516 (cardarine), a peroxisome proliferator-activated receptor delta agonist (PPAR- δ) and MK2866 (ostarine), a selective androgen receptor modulator (SARM) to gain skeletal muscles. Cytolysis with marked increase of alanine aminotransferase or ALT (up to 922 UI/L) and aspartate aminotransferase or AST (up to 2558 UI/L) and massive rhabdomyolysis with elevated creatine phosphokinase or CPK (up to 86435 UI/L) were the main unusual biochemistry parameters. Using a specific liquid chromatography coupled to tandem mass spectrometry method, cardarine and ostarine tested positive in blood at 403 and 1 ng/mL, respectively. In urine, due to extensive metabolism, the parent GW1516 was not identified, while ostarine was at 88 ng/mL. Finally, both drugs were identified in hair (2 cm in length, brown in colour), at 146 and 1105 pg/mg for cardarine and ostarine, respectively. This clearly demonstrates repetitive abuse over the last 2 months. Asthenia was persistent for 2 weeks and 6 weeks after the admission, the subject fully recovered.

Keywords: GW1516; cardarine; MK2866; ostarine; SARM; PPAR- δ; clinical; blood; hair; doping

1. Introduction

The abuse of performance-enhancing drugs is not a recent phenomenon. Since the 60's, anabolic steroid drugs, such as testosterone derivatives, have been used to promote muscle growth. Nandrolone, trenbolone, stanozolol or boldenone and more recently tetrahydrogestrinone are associated with famous doping cases, including state doping programs. These products were manufactured by pharmaceutical groups and were available, as oral tablet forms but mostly as oily injectable preparations. In most cases, they were available from pharmacies in some legal less-regarding countries. With the development of e-shops on the Internet and the continuous research for testosterone substitutes, new pharmacological classes have emerged, including selective androgen receptor modulators

(SARMs) such as ligandrol (LGD-4033), testolone (RAD140), ostarine (MK2866) or andarine (S-4) and peroxisome proliferator-activated receptor delta agonists (PPAR- δ) such as cardarine (GW1516 or GW501516)[1–3].

These drugs are abused because athletes and amateurs have claimed that they increase lean body mass, increase strength, increase aggressiveness and lead to a shorter recovery time between workouts. SARMs present high anabolic potency, in addition to limited androgenic effects [4,5]. PPAR- δ are lipid-activated transcription factors playing important regulatory functions in development and metabolism. Activation of the receptor promotes fatty acid burning by up-regulation of fatty acid uptake, ß-oxidation and energy uncoupling [6,7]. According to the World Anti-Doping Agency (WADA), SARMs and PPAR- δ are prohibited at-all times (in- and out-competition) as they are listed on the prohibited list under sections S1.2 (other anabolic agents) and S4.5 (metabolic modulators), respectively [8]. The number of adverse analytical findings involving SARMs and PPAR- δ during doping controls is continuously increasing in the recent years [9] and many cases of dietary supplements contamination have been reported [10,11].

SARMs have been proposed to treat hypogonadism, muscular atrophy or osteoporosis but the different compounds did not demonstrate enough safety and efficacy to gain clinical approval in the United States or in Europe. Indeed, they can produce heart attack, liver damage and blood clots [12,13]. Due to the induction of intestinal adenoma in rats during the initial clinical trials, Glaxo Smith Kline decided to stop all investigations involving GW1516 [14].

Ostarine and cardarine are mostly available as liquid solutions and tablets (Figure 1), but it is possible to buy larger quantities as bulk material (powders), essentially in China.

Figure 1. Typical material sold on the Internet.

Even though SARMs and PPAR- δ are easily available on the Internet, very few poisoning cases have been reported in the medical literature. Several reasons can account for this under-representation, including the absence of recreational effects but this situation is mainly due to the poor interest of analytical laboratories to test for SARMs and PPAR-δ. Indeed, anti-doping accredited laboratories have published quite most detection methods in urine [15–17] and they do not perform tests for clinical samples. As a consequence, in the limited case reports involving SARMs or PPAR- δ, there is no toxicological data (identification of the substance and evaluation of its concentration). Testing for ostarine [9,18–20] and GW1516 [21] in human hair is the privilege of our laboratory as no other citation is available in the scientific literature.

This technology was used to document a poisoning case after repetitive consumption of ostarine and GW1516, requiring the hospitalization of the abuser.

2. Case Report

A 43-year-old male, sport coach, presented him-self at the Emergency unit of a local hospital for epigastric pain, myalgia pain, severe headache and brown urine. He claimed having used for some days a combination of GW1516 (4 days, 20 mg per day) and MK2866 (1 day, 20 mg) to gain muscle mass. This occurred 10 days before he went to the hospital. Both products were bought on the Internet (10 g of GW1516 and 46 g of MK2866 for a total of 200 euros). The day before he went to the hospital, he cycled 120 km. Vital signs included a blood pressure of 135/70 mmHg and a heart rate of 76 beats per minute and no respiratory distress (SpO_2 at 99%). The ECG was normal. The abdomen was supple but painful. Initial laboratory tests showed serum creatinine of 109 µmol/L (clearance at 76 mL/min), elevated liver enzymes (alanine aminotransferase or ALT 966 UI/L, aspartate aminotransferase or AST 1000 UI/L), normal bilirubin and creatine phosphokinase or CPK elevated at 10,000 UI/L. Blood (green Vacutainer), urine without preservative and hair (2 cm in length, brown in colour) were immediately collected and sent to the laboratory for toxicological investigations. Some hours later, rhabdomyolysis worsened with a rise of CPK at 57,000 UI/L. Treatment consisted of intra-venous (IV) re-hydrating at 3 L per 24 hours. The next day, biochemical tests included CPK at 86435 UI/L, ALT at 922 UI/L and AST at 2558 UI/L. No kidney insufficiency was noticed and urine started to become clearer. Muscle pain remained but abdominal pain was gone. The subject was allowed to turn home, despite the installation of an asthenia that lasted for 15 days. Five days later, during a control, CPK were at 1765 UI/L, ALT at 726 UI/L and AST at 331 UI/L. Normal values were obtained after 2 weeks. Six weeks after the event, the subject totally recovered. Both white powders used by the subject were also submitted to the laboratory for confirmation of identity and determination of purity.

3. Materials and Methods

Powders were diluted in methanol to obtain a 10 mg/mL solution. This solution was compared to reference standards (supplied as powders) obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

The biological specimens (blood, urine and hair) were tested by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), using previously described methods for ostarine [18,19] and cardarine [20]. Briefly, 1 mL of blood or hydrolyzed urine (with 20,000 UI of ß-glucuronidase at pH 5.2) was extracted in 1 mL borate pH 9.5 buffer and 5 mL of diethyl ether/dichloromethane/hexane (50/30/20, *v*/*v*) in presence of 100 ng of bicalutamide-d_4 used as internal standard. After centrifugation, collection of the supernatant and its evaporation to dryness, the residue was reconstituted in 30 µL of 5 mM ammonium formate buffer adjusted at pH 3.

The whole hair strand was first decontaminated by 2 baths of 5 mL dichloromethane for 2 min and then cut into small pieces (< 1 mm) with small scissors. Drugs were extracted from 20 mg decontaminated cut hair in the presence of 1 ng of bicalutamide-d_4, after overnight incubation in 1 mL of pH 9.5 borate buffer at 40 °C. After cooling, the mixture was extracted as blood.

The conditions of use of the equipment (Waters Acquity class I ultra-high performance liquid chromatography and XEVO TQS micro triple quadrupole mass spectrometer) have been described in previous papers [18–20]. Briefly, separation was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 µm) at 50 °C using a gradient elution with pH 3 formate buffer (phase A) and 0.1% formic acid in acetonitrile (phase B) and a flow rate at 0.4 mL/min. The initial gradient was 60% phase A for 30 s to 5% at 1.5 min, kept for 1.5 min and a return at 4 min, maintained for 2 min. The injection volume was 3 µL. Ionization was achieved using electrospray in the negative ionization mode (ES-). Collision energy and cone voltage were adjusted to optimize the signal for the 2 most abundant product ions and are presented in Table 1.

Table 1. Mass spectrometric and chromatographic data for the tested compounds.

Drug	Retention Time min	Transitions *m/z*	Collision Energy eV	Cone Voltage V
Ostarine	2.64	388.1 > 117.9	18	8
		388.1 > 269.1	14	8
Cardarine	3.16	452.1 > 137.9	30	58
		454.0 > 257.0 (+)	26	18
Bicalutamide-d_4	2.57	433.1 > 255.1	14	14

4. Results

The analysis of the powders confirmed the identity of the drug. No other organic chemical was identified in each item. Ostarine purity was 28%. Cardarine purity was 100%. Purity was established by comparing the chromatographic response (area) obtained after injection of a powder solution at 10 mg/L in methanol with a solution of certified standard at the same concentration.

The major validation parameters of the analytical method in blood and hair are presented in Table 2. The validation was achieved using the ISO 17025 guidelines.

Table 2. Validation parameters in blood and hair.

Parameters	Ostarine	Cardarine
Linearity in blood	1 to 1000 ng/mL	5 to 1000 ng/mL
Linearity in hair	0.5 to 1000 pg/mg	1 to 1000 pg/mg
Limit of detection in blood	0.2 ng/mL	1 ng/ml
Limit of detection in hair	0.1 pg/mg	0.3 pg/mg
Limit of quantitation in blood	1 ng/mL	5 ng/mL
Limit of quantitation in hair	0.5 pg/mg	1 pg/mg
Precision in blood (50 ng/mL)	12.8%	14.6%
Precision in hair (100 pg/mg)	11.9%	12.4%

To date, and to the best of the authors' knowledge, ostarine or cardarine detection has not been reported in clinical specimens. The biological samples were submitted to LC-MS/MS and both drugs tested positive in blood and hair, while only ostarine was identified in urine. This is not surprising as it has been reported that cardarine is extensively metabolized and that the parent drug is measurable only for a very short period of time, i.e., no more than 5 days [22,23]. For example, Sobolevsky et al. [22] identified the parent drug for 3 days after oral administration of 15 mg to a volunteer. Given the urine specimen of the present subject was collected 10 days after drug discontinuation, it seems consistent that the parent compound was no more present. Major metabolites include the oxidized GW1516-sulfoxide and the GW1516-sulfone, the later being found in notably higher internal standard than the first one during controlled studies [22]. Unfortunately, these metabolites are not available from reference material suppliers. To date, only WADA accredited laboratories have published data involving these by-products. Concentrations obtained in the submitted biological specimens are reported in Table 3.

Table 3. Measured concentrations in biological specimens from the admission.

Biological Specimens	Ostarine	Cardarine
Blood	1 ng/mL	403 ng/mL
Urine	88 ng/mL	Not detected
Hair	1105 pg/mg	146 pg/mg

Chromatograms obtained after extraction of blood, urine and hair are presented in Figures 2–4, clearly demonstrating suitable sensitivity of the method.

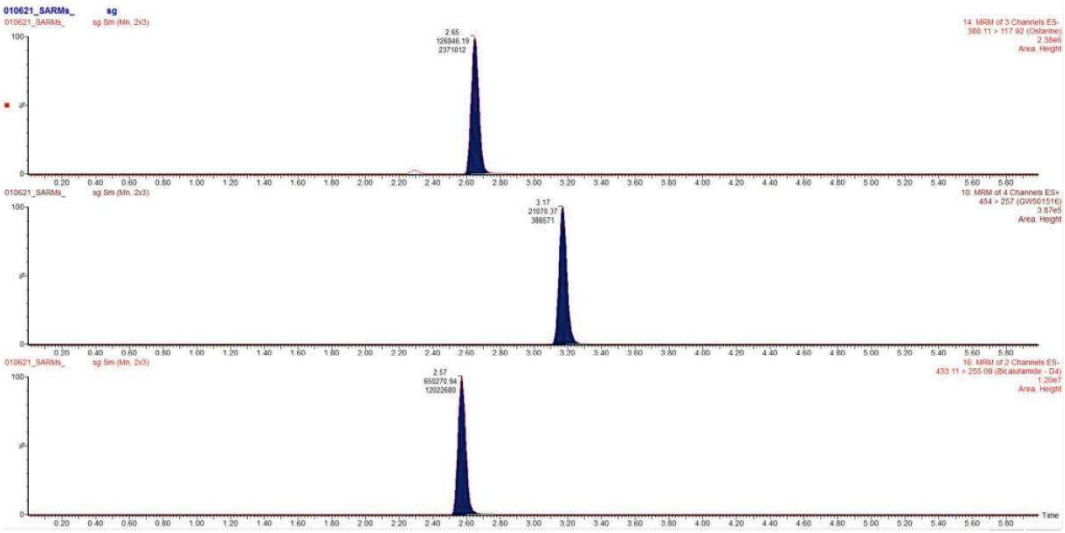


Figure 2. Chromatogram obtained after extraction of the blood. From top to bottom: ostarine (1 ng/mL), cardarine (403 ng/mL) and the internal standard.

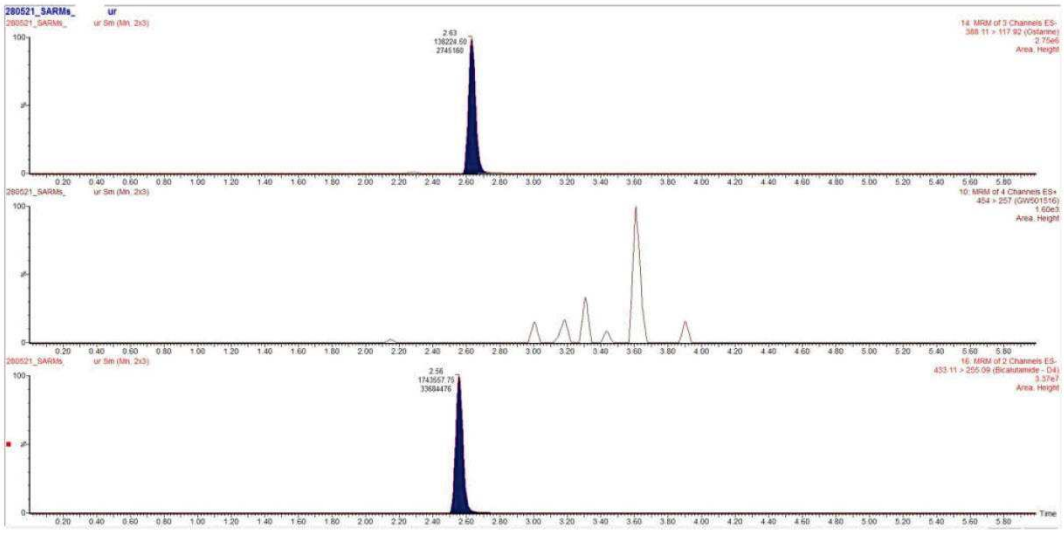


Figure 3. Chromatogram obtained after extraction of the urine. From top to bottom: ostarine (88 ng/mL), cardarine (not detected) and the internal standard.

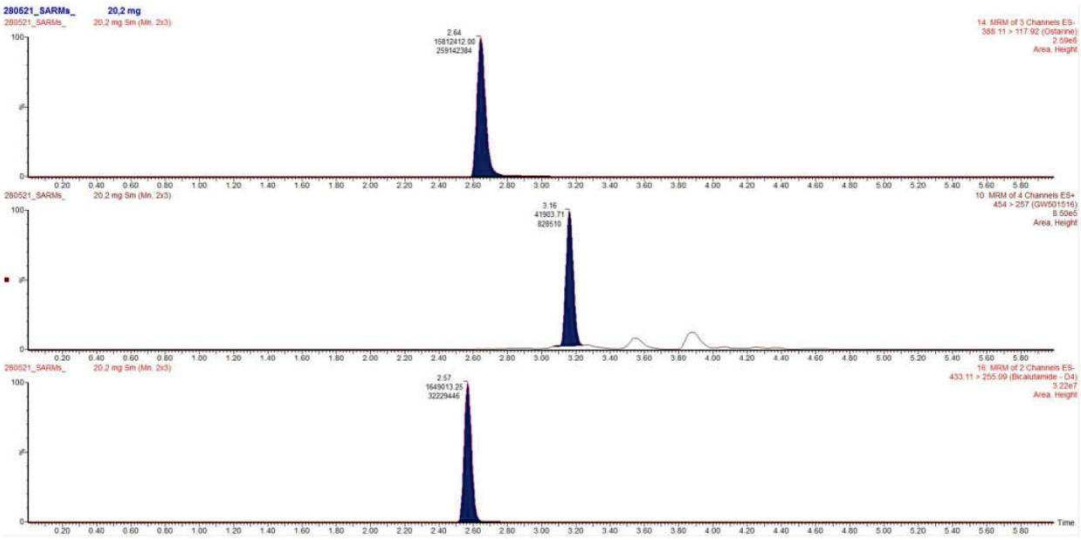


Figure 4. Chromatogram obtained after extraction of the hair. From top to bottom: ostarine (1105 pg/mg), cardarine (146 pg/mg) and the internal standard.

5. Discussion

The subject was admitted to the hospital for severe pain. Major biochemical disruption included rhabdomyolysis and liver cytolysis, as evidenced by enhanced creatine phosphokinases and transaminases. The use of SARMs and PPAR- δ and subsequent rhabdomyolysis has not yet been described. However, it is common to observe rhabdomyolysis in bodybuilders, abusing long-term anabolic steroids, a pharmacological class that is close to SARMs [24,25]. Repetitive abuse of clenbuterol, a ß2-agonist with anabolic properties can also induce rhabdomyolysis [26]. It must be emphasized that rhabdomyolysis is commonly associated with parallel rises in aminotransferases, as these enzymes are also present in muscle.

Even though nothing has been reported for cardarine, human liver injury has been described with ostarine [12,27,28]. However, in these cases, ostarine was always abused on a long-term basis, for several weeks. This is in total contradiction with the claims of the subject, reporting intake of ostarine for 1 day and intake of cardarine for 4 days. The discrimination between these 2 opposite situations was achieved with the hair test results.

Indeed, although hair is not a routine specimen for the WADA, its use as a specimen of investigation is accepted in clinical toxicology [29] and in forensic toxicology [30]. Hair testing is a useful measure of drug intake of an individual, in any situation in which a history of past rather than recent drug use is expected, as it reflects consumption over a long period of time. For practical purposes, it is commonly accepted that each cm of head hair represents the growth, and therefore drug accumulation, for one month. Hair test results allow establishing a retrospective calendar of drug exposure over weeks to months based on a standard head hair growth rate of 1 cm per month. Using segmental hair analysis, statements about the course of drug intake and chronological correlations are possible. While a constant regular profile along the hair shaft is in favour of permanent drug use, any variation of concentration indicates a change in drug intake. Given the submitted material from the subject was limited, there was no attempt to perform segmental analysis in this case. The advantages of hair tests for active substances over blood and/or urine testing include non-invasive and ease of collection to prevent adulteration or substitution.

It must be acknowledged that a single exposure to SARMs is not detectable in hair [9], except for GSK2881078 [31]. There is no data about GW1516 detection after a single exposure. In a case of a subject abusing cardarine for improving athletic performances, concentrations of 32 and 22 pg/mg were measured in 2 × 2 cm segments [21]. Ostarine has been identified on several occasions, including doping challenges or drug trafficking [9,18–20]. Concentrations ranged from 1 to 168 pg/mg and individual results are presented in Table 4.

Table 4. Ostarine concentrations in human hair reported in the literature.

Case	Purpose of the Test	Concentrations	References
1	Doping challenge	26 and 89 pg/mg in 2 × 3 cm	[9]
2	Doping challenge	6, 9, 4, 2, 2 and 1 pg/mg in 6 × 1 cm	[9]
3	Drug trafficking	3, 8, 14 and 21 pg/mg in 4 × 2 cm	[18]
4	Doping challenge	12 and 138 pg/mg, in 2 × 3 cm	[19]
5	Drug trafficking	146, 168, 93 and 101 pg/mg in 4 × 3 cm	[20]

Even though in these published cases, the doses and frequencies of consumption are unknown, it appears clearly that the subject did not use ostarine and cardarine on a limited number of occasions. His measured hair concentrations are much higher than those previously reported. This can be the reason why he experienced clinical troubles that improved after ostarine and cardarine discontinuation.

6. Conclusions

The recent widespread of new drugs all over the world via Internet has demonstrated that there is a need of analytical approaches to document these new habits of consumption. Therefore, implementing tests for SARMs seems of great importance, not only for anti-doping purposes, but also for clinical toxicologists or poison centres, in order to correctly establish a diagnosis of addiction or to document unusual side effects. Given the increasing popularity of SARMs abuse, vigilance and identification of new cases are required. As hair allows retrospective and long-term investigations, these specimens present all suitable properties to be qualified for SARMs or PPAR- δ detection, as the long-term abuse of these drugs can be harmful and potentially life-threatening [32]. Even though liver injury or rhabdomyolysis from SARMs and PPAR- δ have not been reported frequently, one can anticipate that this may be observed more often as the abuse of these drugs increases in the athletic and aesthetic markets.

Author Contributions: Conceptualization, P.K.; methodology, P.K. and A.A.; validation, L.G. and J-S.R.; investigation, C.P., M.C. and M.O-L.; chromatographic analyses, L.G. and A.A.; writing—original draft preparation, P.K. and L.G.; writing—review and editing, P.K. and J-S.R.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable. As the subject was admitted at the hospital for diseases.

Informed Consent Statement: Patient consent was waived due to the fact that biological specimens were collected during hospitalization with a purpose of toxicological diagnostic.

Data Availability Statement: All data are available upon request sent directly to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zhang, X.; Sui, Z. Deciphering the selective androgen receptor modulators paradigm. *Expert Opin. Drug Discov.* **2013**, *8*, 191–218.
2. Bhasin, S.; Jasuja, R. Selective androgen receptor modulators (SARMs) as function promoting therapies. *Curr. Opin. Nutr. Metab. Care* **2009**, *12*, 232–240.
3. Narkar, V.A.; Downes, M.; Yu, R.T.; Embler, E.; Wang, Y.X.; Banayo, E.; Mihaylova, M.M.; Nelson, M.C.; Zou, Y.; Juguilon, H.; et al. AMPK and PPARdelta agonists are exercice mimetics. *Cell* **2008**, *134*, 405–415.
4. Chen, J.; Kim, J.; Dalton, J. Discovery and therapeutic promise of selective androgen receptor modulators. *Mol. Interv.* **2005**, *5*, 173–188.
5. Furuya, K.; Yamamoto, N.; Ohyabu, Y.; Morikyu, T.; Ishige, H.; Albers, M.; Endo, Y. Mechanism of the tissue-specific action of the selective androgen receptor modulator S-101479. *Biol. Pharm. Bull.* **2013**, *36*, 442–451.
6. Luquet, S.; Lopez-Soriano, J.; Holst, D.; Gaudel, C.; Jehl-Pietri, C.; Fredenrich, A.; Grimaldi, P.A. Roles of peroxisome proliferator-activated receptor delta (PPARdelta) in the control of fatty acid catabolism. A new target for the metabolic syndrome. *Biochimie* **2004**, *86*, 833–837.
7. Tailleux, A.; Wouters, K.; Staels, B. Roles of PPARs in NAFLD: Potential targets. *Biochim Biophys Acta* **2012**, *1821*, 809–818.
8. WADA List 2021. Available online: https://www.wada-ama.org/sites/default/files/resources/files/2021list_en.pdf (accessed on 24 August 2021).
9. Kintz, P.; Gheddar, L.; Ameline, A.; Raul, J.-S. Human hair testing for selective androgen receptor modulators (SARMs): Current knowledge and limitations. *Toxicol. Anal. Clin.* **2021**, doi:10.1016/j.toxac.2021.07.002 (in press).
10. Walpurgis, K.; Rubio, A.; Wagener, F.; Krug, O.; Knoop, A.; Görgens, C.; Guddat, S.; Thevis, M. Elimination profiles of microdosed ostarine mimicking contaminated products ingestion. *Drug Test. Anal.* **2020**, *12*, 1570–1580.
11. Leaney, A.E.; Beck, P.; Biddle, S.; Brown, P.; Grace, P.B.; Hudson, S.C.; Mawson, D.H. Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators. *Drug Test. Anal.* **2021**, *13*, 122–127.
12. Flores, J.E.; Chitturi, S.; Walker, S. Drug-induced liver injury by selective androgenic receptor modulators. *Hepatol. Commun.* **2020**, *4*, 450–452.
13. Christiansen, A.; Lipshultz, L.I.; Hotaling, J.M.; Pastuszak, A.W. Selective androgen receptor modulators: The future of androgen therapy? *Transl. Androl. Urol.* **2020**, *9* (Suppl. 2), S135–S148.
14. Lagu, B.; Kluge, A.F.; Goddeeris, M.M. Highly selective peroxisome proliferator-activated receptor δ (PPAR- δ) modulator demonstrates improved safety profile compared to GW501516. *Bioorg. Med. Chem. Lett.* **2018**, *28*, 533–536.
15. Thevis, M.; Möller, I.; Thomas, A.; Beuck, S.; Rodchenkov, G.; Bornatsch, W.; Geyer, H.; Schänzer, W. Characterization of two major urinary metabolites of the PPAR- δ agonist GW1516 and implementation of the drug in routine doping controls. *Anal. Bioanal. Chem.* **2010**, *396*, 2479–2491.
16. Stacchini, C.; Botrè, F.; Comunità, F.; de la Torre, X.; Dima, A.P.; Ricci, M.; Mazzarino, M. Simultaneous detection of different classes of selective androgen receptor modulators in urine by liquid chromatography-mass spectrometry-based techniques. *J. Pharm. Biomed. Anal.* **2021**, *195*, 113849.
17. Sobolevsky, T.; Ahrens, B. High-throuput liquid chromatography tandem mass spectrometry assay as initial testing procedure for analysis of total urine fraction. *Drug Test. Anal.* **2021**, *13*, 283–298.
18. Kintz, P.; Gheddar, A.; Amelie, A.; Raul, J.-S. Perspectives in evaluating selective androgen receptor modulators in human hair: A short communication. *Ther. Drug Monit.* **2021**, *43*, 298–300.
19. Kintz, P.; Ameline, A.; Gheddar, L.; Raul, J.-S. LGD-4033, S-4, and MK-2866—Testing for SARMs in hair: About 2 doping cases. *Toxicol. Anal. Clin.* **2019**, *31*, 56–63.
20. Kintz, P.; Gheddar, A.; Amelie, A.; Raul, J.-S. Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases. *Drug Test. Anal.* **2020**, *12*, 1508–1513.
21. Kintz, P.; Ameline, A.; Gheddar, L.; Raul, J.-S. Testing for GW501516 (cardarine) in human hair using LC/MS-MS and confirmation by LC/HRMS. *Drug Test. Anal.* **2020**, *12*, 980–986.
22. Sobolevsky, T.; Dikunets, M.; Sukhanova, I.; Virus, E.; Rodchenkov, G. Detection of PPARδ agonists GW1516 and GW0742 and their metabolites in human urine. *Drug Test. Anal.* **2012**, *4*, 754–760.
23. Thevis, M.; Möller, I.; Beuck, S.; Schänzer, W. Synthesis, mass spectrometric characterization, and analysis of the PPARδ agonist GW1516 and its major human metabolites: Targets in sports drug testing. *Methods Mol. Biol.* **2013**, *952*, 301–312.
24. Hageloch, W.; Appell, H.J.; Weicker, H. Rhabdomyolysis in a body builder using anabolic steroids. *Sportverletz Sportschaden* **1998**, *2*, 122–125.
25. Kryzak, M.; Elangovan, N. Rhabdomyolysis in a patient taking both oxandrolone and methamphetamine. *Cureus* **2017**, *9*, e1843.
26. Grimmer, N.M.; Gimbar, R.P.; Bursua, A.; Patel, M. Rhabdomyolysis secondary to clenbuterol use and excercice. *J. Emerg. Med.* **2016**, *50*, e71–e74.
27. Bedi, H.; Hammond, C.; Sanders, D.; Yang, H.M.; Yoshida, E.M. Drug-induced liver injury from enobosarm (ostarine), a selective androgen receptor modulator. *ACG Case Rep. J.* **2021**, *8*, e00518.
28. Koller, T.; Vrbova, P.; Meciarova, I.; Molcan, P.; Smitka, M.; Adamcova Selcanova, S.; Skladany, L. Liver injury associated with the use of selective androgen receptors modulators and post-cycle therapy: Two case reports and literature review. *World J. Clin. Cases* **2021**, *9*, 4062–4071.
29. Cuypers, E.; Flanagan, R.J. The interpretation of hair analysis for drugs and drug metabolites. *Clin. Toxicol.* **2018**, *56*, 90–100.

30. Kintz, P. Hair analysis in forensic toxicology: An update review with a special focus on pitfalls. *Curr. Pharm. Des.* **2017**, *23*, 5480–5486.
31. Rading, A.; Anielski, P.; Thieme, D.; Keiler, A.M. Detection of the selective androgen receptor modulator GSK2881078 and metabolites in urine and hair after songle oral administration. *Drug Test. Anal.* **2021**, *13*, 217–222.
32. Richards, J.R.; Scheerlinck, P.H.; Owen, K.P.; Colby, D.K. Bodybuilding supplements leading to copper toxicity, encephalopathy, fulminant hepatic failure and rhabdomyolysis. *Am. J. Emerg. Med.* **2020**, *38*, e1–e5.

3.3.2. Nouveaux outils analytiques (métabolisme)

Les SARMs sont de nouveaux agents anabolisants et sont recherchés par l'AMA seulement depuis 2008. A ce titre de nombreux laboratoires ont développé de nouvelles stratégies afin de les détecter sur le long terme et de régulariser leur consommation par la suite.
Dans la littérature, de nombreuses publications démontrent l'intérêt d'utiliser les microsomes hépatiques humains (HLMs) combinés avec les systèmes spécifiques HRMS afin d'identifier les métabolites des SARMs.

La même approche a été appliquée pour l'identification des métabolites du S23 pour lequel peu de données existent dans la littérature.
Le S23, un SARM non stéroïdien, est apparu très récemment sur le marché du dopage et a été identifié pour la première fois en 2019 par les laboratoires antidopage. De ce fait, très peu de données sont disponibles dans la littérature et rien n'a été publié sur les effets à long terme du S23. Les auteurs se sont concentrés sur la détection du S23 et de ses métabolites dans l'urine humaine, après une administration orale unique d'environ 8 mg à un volontaire, précédé d'un consentement éclairé, en utilisant les systèmes LC-MS/MS et LC-HRMS pour leur identification. Il semble que ce soit la première étude jamais réalisée sur le S23 chez l'homme. Une expérience *in vitro* a été réalisée, en utilisant des HLMs, afin d'étudier les métabolites potentiels du S23 dépendant du CYP et de l'UGT.
Quatre métabolites ont été produits, dont l'hydroxy-S23 ; l'O-déphénylate-S23 ; le S23-glucuronide et l'hydroxy-S23-glucuronide.
Après consommation de S23, la molécule parent était détectable dans l'urine hydrolysée à partir de + 2 heures jusqu'à 28 jours, avec des concentrations variantes entre 0,5 et 93 ng/mL. Dans l'urine, aucun métabolite de cette série n'a été détecté mais l'hydroxy-S-23, identifié uniquement pendant l'étude *in vivo*, a été détecté jusqu'à 28 jours. Néanmoins, il ne semble pas augmenter la fenêtre de détection du S23 puisque le rapport entre l'hydroxy-S23 et la molécule mère était toujours inférieur à 1.
Un mois après la consommation d'un comprimé, une mèche de cheveu a été analysée. Ni le S23, ni l'hydroxy-S23 ont été détecté, avec une LOQ de 0,1 pg/mg. Il semble qu'une prise unique de S23 ne soit pas détectable dans les cheveux ***[Article 18]***.

Article 18 : In vitro characterization of S-23 metabolites produced by human liver microsomes, and subsequent application to urine in a controlled study : first data about this SARM

Alice Ameline, Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz

Drug Testing and Analysis. Soumis le 9 juillet 2021.

DRUG TESTING AND ANALYSIS

In vitro characterization of S-23 metabolites produced by human liver microsomes, and subsequent application to urine in a controlled study: first data about this SARM.

Journal:	*Drug Testing and Analysis*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Ameline, Alice; Institut de médecine légale, Université de Strasbourg , Laboratoire de toxicologie Gheddar, Laurie; Institut de Medecine Legale, Raul, Jean-Sébastien; Institut de Médecine Légale Kintz, Pascal; Institut de Médecine Légale, ; X-Pertise Consulting SAS,
Keywords:	S-23, urine, hair, metabolism, HLMs, LC-HRMS
Abstract:	The selective androgen receptor modulators are a new class of designer steroids, used to improve athletic performance. Among these molecules, there is (2S)-N-(4-cyano-3-trifluoromethylphenyl)-3-(3-fluoro-4-chlorophenoxyl)2-hydroxy-2-methyl-propanamide, commonly known as S-23. This molecule appeared very recently on the doping market. As a result, very few data are available in the literature, and nothing has been published about long-term effects of S-23. The authors focused on the detection of S-23 and its metabolites in human urine, following a single oral administration of approx. 8 mg to a volunteer, using standard ultra-performance liquid chromatography-triple quadrupole-mass spectrometry (UPLC-MS/MS), and ultra-performance liquid chromatography-quadrupole time of flight-mass spectrometry (UPLC-Q-TOF-MS). This seems to be the first study ever achieved on S-23. In vitro experiment was performed, using human liver microsomes, in order to investigate the potential CYP- and UGT-dependent S-23 metabolites. Four metabolites were produced, including hydroxy-S-23 ($C_{18}H_{12}O_4N_2ClF_4$: m/z [M-H-] 431.0423); O-dephenylate-S-23 ($C_{12}H_{10}O_3N_2F_3$: m/z [M-H-] 287.0647); S-23-glucuronide ($C_{24}H_{20}O_9N_2ClF_4$: m/z [M-H-] 591.0794) and hydroxy-S-23-glucuronide ($C_{24}H_{20}O_{10}N_2ClF_4$: m/z [M-H-] 607.0743). After consumption of S-23, the parent drug was detectable in hydrolyzed urine from + 2 hours up to 28 days, with concentrations ranging between 0.5 and 93 ng/mL. In the urine, no metabolite of this series but hydroxy-S-23 was detected up to 28 days. It does not seem to increase the window of detection of S-23 as the ratio between hydroxy-S-23 and the parent drug was always lower than 1. Hair sample, collected one month after the consumption of one tablet, was negative for S-23 and hydroxy-S-23, with a LOQ at 0.1 pg/mg.

In vitro characterization of S-23 metabolites produced by human liver microsomes, and subsequent application to urine in a controlled study: first data about this SARM.

Alice Ameline*, Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz
Institut de médecine légale, 11 rue Humann, 67000 Strasbourg, France

*: author for correspondence
E-mail: ameline.alice@gmail.com

Conflict of interest: the authors declare no conflict of interest, financial or otherwise

Abstract

The selective androgen receptor modulators are a new class of designer steroids, used to improve athletic performance. Among these molecules, there is (2S)-N-(4-cyano-3-trifluoromethylphenyl)-3-(3-fluoro-4-chlorophenoxyl)2-hydroxy-2-methyl-propanamide, commonly known as S-23. This molecule appeared very recently on the doping market. As a result, very few data are available in the literature, and nothing has been published about long-term effects of S-23. The authors focused on the detection of S-23 and its metabolites in human urine, following a single oral administration of approx. 8 mg to a volunteer, using standard ultra-performance liquid chromatography-triple quadrupole-mass spectrometry (UPLC-MS/MS), and ultra-performance liquid chromatography-quadrupole time of flight-mass spectrometry (UPLC-Q-TOF-MS). This seems to be the first study ever achieved on S-23. *In vitro* experiment was performed, using human liver microsomes, in order to investigate the potential CYP- and UGT-dependent S-23 metabolites. Four metabolites were produced, including hydroxy-S-23 ($C_{18}H_{12}O_4N_2ClF_4$: m/z [M-H$^-$] 431.0423); *O*-dephenylate-S-23 ($C_{12}H_{10}O_3N_2F_3$: m/z [M-H$^-$] 287.0647); S-23-glucuronide ($C_{24}H_{20}O_9N_2ClF_4$: m/z [M-H$^-$] 591.0794) and hydroxy-S-23-glucuronide ($C_{24}H_{20}O_{10}N_2ClF_4$: m/z [M-H$^-$] 607.0743). After consumption of S-23, the parent drug was detectable in hydrolyzed urine from + 2 hours up to 28 days, with concentrations ranging between 0.5 and 93 ng/mL. In the urine, no metabolite of this series but hydroxy-S-23 was detected up to 28 days. It does not seem to increase the window of detection of S-23 as the ratio between hydroxy-S-23 and the parent drug was always lower than 1. Hair sample, collected one month after the consumption of one tablet, was negative for S-23 and hydroxy-S-23, with a LOQ at 0.1 pg/mg.

Keywords: S-23; urine; hair; metabolism; HLMs; LC-HRMS

1. Introduction

In the recent years, a new class of "designer steroids" has appeared on the doping market. These agents are selective androgen receptor modulators (SARMs). These products, like anabolic steroids, improve athletic performance and increase muscle mass [1-2]. Indeed, SARMs will mimic the effects of testosterone without reproducing the major negative effects associated with the use of anabolic steroids including liver toxicity, kidney toxicity and testicular atrophy [3]. When SARMs bind to the receptors, they produce anabolic activities both on the muscles and on the bones. In sport, SARMs have the same benefits as anabolic steroids, i.e. more muscles, less fat and good bone density. The use of this class of drugs is banned by the World Anti-Doping Agency (WADA) for human sports and is listed under the section S1.2 (other anabolic agents) [4]. Since 2014, the number of molecules in this category has increased, as well as the number of adverse analytical findings (AAF). In 2014, 15 adverse analytical findings were reported for SARMs by WADA [5]. In its last report, in 2019, WADA reported 142 adverse analytical findings involving the same class [6].

Among these molecules, there is (2S)-N-(4-cyano-3-trifluoromethylphenyl)-3-(3-fluoro-4-chlorophenoxyl)2-hydroxy-2-methyl-propanamide, commonly known as S-23 (Figure 1). This drug is an investigational SARM developed by GTX Incorporation, as a potential male hormonal contraceptive. On the internet, S-23 is described as the strongest SARM available on the market, more powerful than other popular SARMs, such as ligandrol (LGD-4033), ostarine (MK-2866) or andarine (S-4) [7-9]. In 2019, 142 adverse analytical findings were recorder by WADA for SARMs, of which only 1 involved S-23, corresponding to 0.3 % of this class [6]. This was the first AAF involving S-23 as a doping agent. This substance appeared very recently on the doping market. As a result, very few data are available in the literature. Nothing has been published about the long-term effects of S-23. From an analytical point of view, the characterization of S-23 is essential in the view of its easy access via e-shops. Its identification in urine and the characterization of its metabolite(s) is necessary. To date, no study about the identification of metabolites of S-23 has been described in human specimens. However, there are some data in animals. Thevis et al. [10] have simulated the phase -I and -II metabolism of S-22 and S-23 (similar metabolism pattern) using hepatic human enzymes. The authors demonstrated the presence of the glucuronide of S-22 as well as the B-ring-depleted metabolite, in canine urine samples collected up to 72h after oral administration of S-22. So et al. [11] described the study of the *in vitro* biotransformation of S-23, using homogenized horse liver preparations. The authors reported 7 metabolites (3 phase -I and 4 phase -II), but they did not apply it to horses. Other *in vitro* metabolism studies, involving ligandrol, ostarine and andarine, have also been reported in the literature [12-15].

In this study, the authors focused on the detection of S-23 and its metabolites in urine and hair, following a single administration of an oral dose of approx. 8 mg to a human volunteer, using

standard ultra-performance liquid chromatography-triple quadrupole-mass spectrometry (UPLC-MS/MS), and UPLC-quadrupole time of flight-mass spectrometry (UPLC-Q-TOF-MS). In addition, *in vitro* experiment was performed, using human liver microsomes, in order to investigate the potential CYP- and UGT-dependent S-23 metabolites.

2. Materials and methods

2.1 Self-administration study

In order (a) to gain preliminary data on elimination parameters of S-23 in urine, (b) to investigate its metabolism, and (c) to be able to interpret analytical data, a male subject (59-year-old, 83 kg) ingested a pink tablet bought on the Internet with a declare amount of 10 mg of active material (Figure 2). Urine specimens were collected over 28 days in plastic vials without preservative and stored at + 4 °C until analysis. Urine was collected at various times, with no fixed interval. Hair samples (blond, oriented, 3 cm) were collected one month after the ingestion of S-23. Hair sample was stored at room temperature, in an envelope, until analysis. No blood sample was collected, as this requires specific authorization due to the invasive nature of the procedure and specific oxygenotherapy in case of collapse, which was not available at the laboratory.

In France, given the subject was one of the authors, it was not necessary to obtain an ethical committee advise as S-23 is not listed as a drug of abuse.

2.2 Standard and reagents

S-23 tablets were purchased on the Internet, from Dynamic Performance (UK). The reference standards of S-23 and bicalutamide-d_4 were purchased from Toronto Research Chemicals, and were diluted to appropriate concentrations using methanol, according to the manufacturer's recommendations. Ammonium formate 99 % was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99-100 %, AnalaR NORMAPUR and 36 % hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). β-glucuronidase (Helix pomatia), alamethicin (Trichoderma viride), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P) were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at -80°C until use. HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany).

2.3 Urine analysis

Before extraction, urine specimens were hydrolyzed using 200 µL of β-glucuronidase (10.000 UI) at pH 5.2 (200 µL of 1M ammonium acetate buffer), for 15h at 40°C. S-23 was extracted from 1 mL hydrolyzed urine in presence of 5 ng of bicalutamide-d_4 used as internal standard, with 1 mL saturated borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5). After extraction, centrifugation and evaporation to dryness under a nitrogen flow, the residue was reconstituted in 30 µL of ammonium formate buffer adjusted at pH 3.0. Thirty µL were transferred into a vial and 2 µL were injected into the column. A 6-point calibration curve was prepared using the following concentrations: 0.5, 5, 10, 50, 100 and 500 ng/mL of S-23.

2.4 Hair analysis

The analysis of S-23 was conducted on a hair strand of 3 cm, which was segmented into 2 segments (0-1.5 and 1.5-3 cm). S-23 was extracted from 20 mg decontaminated cut hair in presence of 250 pg of bicalutamide-d4 used as internal standard after overnight incubation in 1 mL saturated borate buffer pH 9.5. Then, 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5) was added to extract S-23. After agitation and centrifugation, the organic phase was collected and evaporated to dryness under a nitrogen flow. The residue was reconstituted in 30 µL of ammonium formate buffer adjusted at pH 3.0. Thirty µL were transferred into a vial and 2µL were injected into the column. A 6-point calibration curve was prepared using the following concentrations: 0.1, 1, 5, 10, 50 and 100 pg/mg.

2.5 In vitro metabolism

The formation of S-23 metabolites was investigated by *in vitro* incubation with human liver microsomes (HLMs). Once thawed, HLMs (2 mg protein/mL for a final volume of 100 µL) were pre-activated by alamethicin (at 0.1 mmol/L) on ice in an intermediate volume of 50 µL in 0.1 M Tris-HCl-MgCl2 (10 mM MgCl2 and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50 µL-dried residue of two different concentrations (100 µM and 500 µM) of an S-23 methanolic solution. Fifty µL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37 °C for 60 and 120 min, and stopped by the addition of 200 µL of methanol, subsequently centrifuged at 4 °C for 10 min at 32 000 g. Supernatants were frozen at -20°C until analysis. Thirty µL were transferred into a vial and 1µL was injected and analyzed by LC-HRMS

2.6 LC-MS/MS conditions

Chromatography was performed on an Acquity class I ultra-high performance liquid chromatography and separation was achieved using a Waters Acquity HSS C18 column (150 x 2.1 mm x 1.8µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile LC-MS (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 60 % phase A for 0.5 min, and then it was decreased up to 5 % phase A at 1.5 min, kept for 1.5 min and followed by a return to the initial conditions at 4 min, maintained for the last 2 min. An injection volume of 2 µL was used in all cases.

A Xevo TQS-µ triple-quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) provided with a Z-spray electrospray ionisation source (ESI) used in the negative ionization mode (ESI-) was used for analysis and quantification of the compound. The following conditions were found to be optimal for the analysis of S-23: capillary voltage at 1.5 kV, source block temperature at 150 °C, desolvatation gas nitrogen heated at 550 °C and delivered at a flow rate of 800 L/Hr. The cone voltage and collision energy were adjusted to maximize the intensity of the protonated ion and to optimize the signal of the 2 most abundant transitions of S-23: m/z 415.1 > 144.9 (8V, 18eV) and m/z 415.1 > 185.00 (10V, 36eV), and for the internal standard bicalutamide-d_4: m/z 433.1 > 255.1 (14V, 14eV). Transition m/z 415.1 > 144.9 was used for quantification of S-23. MassLynx 4.1 software was used for quantification. The LC-MS/MS instrument conditions were the same for urine and hair.

2.7 LC-HRMS conditions

The chromatographic separation of samples obtained from incubations with HLMs and from urine and hair extracts, was performed with the same liquid chromatography equipment and the same parameters used with the LC-MS/MS system. Detection was performed using a high resolution (XEVO G2XS Q-TOF, Waters Corporation, Milford, MA, USA) mass spectrometer operation in negative mode and in sensitivity mode. Desolvatation gas flow was set to 1000 L/Hr at a temperature of 600 °C, the cone gas to 50 L/Hr and the source temperature at 150 °C. The capillary voltage and the cone voltage were set to 0.5 and 20 V, respectively. In MS scanning, data were acquired from 50 to 1000 m/z. Collision energy ranged from 10 to 40 V. UNIFI software was used for data, chromatograms and spectra acquisition. UNIFI was also used to predict and to match potential metabolites.

2.8 LC-MS/MS procedure

The method was validated for linearity, repeatability, reproducibility, detection and quantification limits. Three calibrations curves that included six points (concentrations ranging from 0.5 to 500 ng/mL in urine, and from 0.1 to 100 pg/mg in hair), obtained over a three-day period, were established for the verification of linearity. Quantification was achieved by plotting the peak area ratios of S-23 to the IS versus concentration followed by linear regression analysis. Repeatability and

reproducibility were determined for two QC levels, 10 ng/mL and 100 ng/mL for urine, and 1 pg/mg and 100 pg/mg for hair. For the repeatability, six replicates of each QC level were processed the same day. For the reproducibility, each QC level was processed three times, three different days over a period of two weeks. The limit of detection (LOD) is the lowest concentration of the compound that can be detected with a signal-to-noise ratio greater than 3:1 for the specific transition. The limit of quantification (LOQ) was defined as the first point of the calibration curve.

3. Results and discussion

For the method validation, the six-point calibration curve showed good linearity in urine, with a correlation coefficient at 0.9999, and in hair with a correlation coefficient at 0.9944. The LOD and the LOQ of both matrices were established at 0.1 ng/mL and 0.5 ng/mL for urine, and 0.05 pg/mg and 0.1 pg/mg for hair. Repeatability was 13.4 and 17.9 % at 10 ng/mL and 100 ng/mL for urine, and 12.2 and 18.3 % at 1 pg/mg and 100 pg/mg for hair. Reproducibility was 11.9 and 17.1 % at 10 ng/mL and 100 ng/mL for urine, and 12.4 and 16.5 % at 1 pg/mg and 100 pg/mg for hair. Under the chromatographic conditions used, there was no interference with the analytes by chemicals or any endogenous compound (matrix effect < 20 %, irrespective of the tested matrix).

Before starting the self-administration study, three S-23 tablets from Dynamic Performance were analysed by LC-MS/MS to confirm the presence of S-23 and to verify its dosage (announced at 10 mg). The analysis confirmed the presence of S-23, at approx. 8 mg (18 % less than the announced dosage) in all tablets. It was therefore anticipated that the tablet consumed by the subject also contained 8.2 mg of S-23. No trace of other substance was identified in the tablets. After oral administration, the overall behaviour of the subject was not modified. No physical or behavioural impairment was observed.

In the urine of the volunteer, the parent S-23 was detected from + 2 hours up to 28 days (672 hours), using LC-MS/MS. The concentrations ranged from 0.5 to 93 ng/mL (see Table 1), and T_{max} was obtained 8 hours after administration. The excretion pattern of S-23 is presented in Figure 3. The reasons for obtaining a biphasic curve are unknown. A typical chromatogram is presented in Figure 4. Compared to the detection time of the others SARMs, S-23 is detectable for a longer time. For example, Fragkaki et al. [16] detected ligandrol up to 3.5 days in human urine, with a maximum peak of concentration at 4.5 hours. Cutler et al. [17] investigated ligandrol in the horse following oral administration of 50 mg to two thoroughbred horses and urines were collected. Parent ligandrol was detected in all enzyme hydrolysed urine samples up to 12 days post administration. The approximate

maximal concentration in urine was observed 5 hours after the administration for horse 1, and 4 hours after administration for horse 2.

Walpurgis et al. [18] demonstrated that a single dose of as little as 1 µg of ostarine can be detected for up to 9 days, in hydrolysed human urine. A higher dose of 50 µg can be detected for up to 11 days. In the event of an AAF, it is essential to know the maximum detection time of the SARM involved, to evaluate the moment of consumption, in case the result is challenged, and contamination is supposed to have occurred.

In vitro HLMs incubations and LC-HRMS analyses were performed to investigate the appearance of CYP- and UGT- dependent S-23 metabolites. Regarding the two different S-23 concentrations (100 and 500 µM) and the two different incubation times (60 and 120 min), a satisfactory metabolite production (largest chromatographic peak area) was observed after 120 min at 500 µM. Four metabolites were produced in vitro using HLMs under these conditions: hydroxy-S-23 ($C_{18}H_{12}O_4N_2ClF_4$: m/z [M-H$^-$] 431.0423); *O*-dephenylate-S-23 ($C_{12}H_{10}O_3N_2F_3$: m/z [M-H$^-$] 287.0647); S-23-glucuronide ($C_{24}H_{20}O_9N_2ClF_4$: m/z [M-H$^-$] 591.0794) and hydroxy-S-23-glucuronide ($C_{24}H_{20}O_{10}N_2ClF_4$: m/z [M-H$^-$] 607.0743). The spectral and chromatographic data for metabolites are described in Table 2 and Figure 5. These metabolites have already been reported in the literature but have never been observed in human urine. Thevis et al. [10] investigated the phase –I and –II metabolism of S-23, using hepatic human microsomes. Seven metabolites were identified: hydroxyl-S-23, bishydroxy-S-23, *O*-dephenylate-S-23, S-23-glucuronide, hydroxy-S-23-glucuronide, bishydroxy-S-23-glucuronide and hydroxyl-S-23-sulfonide. During our study, bishydroxy-S-23, bishydroxy-S-23-glucuronide and hydroxyl-S-23-sulfonide were not observed. Its production can be anticipated that metabolites depend on the hepatic microsomes used and the enzymes involved.

In the urine of the volunteer, only one of these four metabolites was identified. A hydroxylated was identified and hydroxy-S-23 was detected from +2 hours until +28 days. The profile of this metabolite is presented in Table 3. Since no reference standard for hydroxy-S-23 seems available, this metabolite is expressed as a percentage relative to the chromatographic peak area of S-23. However, the ratio between metabolite and parent drug allows to estimate its analytical response in LC-HRMS. The identification of this metabolite strengthens the specific analysis of S-23, but does not seem to increase the window of detection in case of doping practice, as the ratio between metabolite and parent drug was always lower than 1, except at + 4 hours. A curve representing the peak areas of S-23 and hydroxy-S-23 is presented in figure 6. It is observed that S-23 and its metabolite act in the same way over time, including its biphasic excretion.

Another metabolite, dihydroxy-S-23, was identified in the urine of the volunteer, by LC-HRMS. This metabolite was not detected after *in vitro* HLMs incubation. Dihydroxy-S-23 was detected from + 8 hours up to 5 days, but in very small quantities. The ratio between dihydroxy-S-23 and S-23 were always lower than 1 (see Table 4). In view of these ratios, dihydroxy-S-23 is a minor metabolite. Like hydroxy-S-23, dihydroxy-S-23 does not increase the window of detection of S-23.

A hair sample, collected one month after administration, was analysed by LC-HRMS. Neither S-23, nor any metabolite, were identified in the specimen. The interpretation of this negative hair result is difficult since no data is available in the literature. This was the first attempt to identify S-23 in hair. The minimal dose that must be ingested to get a positive detection in hair remains unknown for S-23. The absence of identification could mean that the consumption of a single dose of S-23 is not detectable in hair at the limit of detection of the method.

Although no data is available for S-23, other SARMs have been identified in hair. Cutler et al. [17] investigated ligandrol and metabolites in horse hair following oral administration of 50 mg. The analyses revealed trace amounts of ligandrol and its mono-hydroxylated metabolite. The responses for the metabolite were stronger than for the parent drug, which is unusual in hair. This analysis indicated that there may be some incorporation of the mono-hydroxylated metabolite into the hair following oral administration. Rading et al. [19] investigated GSK2881078, after administration of a single dose of 1.5 mg to a male volunteer. The authors detected the parent drug at 1.7 pg/mg in the segment collected 3 weeks post administration, representing the time of ingestion. No hydroxy-metabolite was detected. The authors conclude that de detection of GSK2881078 after a single dose is feasible, but at very low concentration. Kintz et al. [20] identified ostarine at 12 and 138 pg/mg, in the 2 x 3 cm hair segments of a male athlete who returned an adverse analytical funding for ostarine. In this case, the hair test results confirmed the anti-doping rules violation. In the hair of a subject arrested by the customs for trafficking, Kintz et al. [21] identified ostarine at 146, 168, 93, and 101 pg/mg in the 4 x 3 cm sections, clearly demonstrating long-term use. Finally, the same group [22], in 3 separate cases, identified ligandrol (14 to 42 pg/mg), andarine (0.1 to 0.7 pg/mg) and ostarine (3 to 21 pg/mg), offering new biological perspectives in SARMs abuse detection.

Although hair is more than a promising matrix that makes it possible to discriminate a single take from a repeated exposure, many parameters that influence data interpretation have to be investigated: dose necessary to give a positive result, influence of gender, variable incorporation according to hair colour, external factors that may have an impact, cosmetic treatment, *etc* [9]. Therefore, at this time hair results for SARMs should be interpreted with caution by experienced forensic toxicologists.

4. Conclusion

S-23 metabolism was studied *in vitro* using human liver microsomes and allowed the identification of 4 metabolites: hydroxy-S-23, *O*-dephenylate-S-23, S-23-glucuronide and hydroxy-S-23-glucuronide. For the first time, one of these metabolites, hydroxy-S-23, was identified in human urine specimens, obtained after ingestion of one tablet of approx. 8 mg. Another metabolite, dihydroxy-S-23, was also identified in the urine of volunteer, with a shorter window of detection. Unfortunately, the ratios between metabolites and parent drug, always lower than 1, show that these metabolites do not increase the time of detection in case of doping practice. The parent S-23 remains the target substance in human urine. In addition, hair analysis revealed that the consumption of a single dose of S-23 is not detectable in hair. Considering all the limitations and difficulties related to the interpretation of the tests, hair results for SARMs should be interpreted with caution by experienced forensic toxicologist.

Reference

1. Mohler ML, Bohl CE, Jones A, Coss CC, Narayanan RN, He Y, Hwang DJ, Dalton JT, Miller DD. Nonsteroidal selective androgen receptor modulators (SARMs): dissociating the anabolic and androgenic activities of the androgen receptor for therapeutic benefit. *J Med Chem*. 2009;52(12):3597-3617.

2. Leaney AE, Beck P, Biddle S, Brown P, Grace PB, Hudson SC, Mawson DH. Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators. *Drug Test Anal.* 2021;13(1):122-157.

3. Dalton JT, Taylor RP, Mohler ML, Steiner MS. (2013) Selective androgen receptor modulators for the prevention and treatment of muscle wasting associated with cancer. *Curr Opin Support Palliat Care*. 2013;7(4):345-351.

4. World Anti-Doping Agency (WADA). 2020 Prohibited List. Available at http://www.wada-ama.org/sites/default/files/wada_2020_english_prohibited_list_0.pdf Accessed July 8, 2021.

5. World Anti-Doping Agency (WADA). 2014 anti-doping testing figures. Available at https://www.wada-ama.org/sites/default/files/wada_2014_anti-doping-testing-figures_full-report_en.pdf Accessed July 8, 2021.

6. World Anti-Doping Agency (WADA). 2019 anti-doping testing figures. Available at https://www.wada-ama.org/sites/default/files/resources/files/2019_antidoping_testing_figures_en.pdf Accessed July 8, 2021.

7. Bodyshock. Available at https://bodyshock.pro/en/products/s-23-10-mg-90-caps-2263.html Accessed July 8, 2021.

8. NanotechProject. Available at https://nanotechproject.org/s23/ Accessed July 8, 2021.

9. Kintz P, Gheddar L, Ameline A, Raul JS. Human hair testing for selective androgen receptor modulators (SARMs): current knowledge and limitations. *Toxicol Anal Clin*. 2021;accepted.

10. Thevis M, Gerace E, Thomas A, Beuck S, Geyer H, Schlörer N, Kearbey JD, Dalton JT, Schänzer W. Characterization of in vitro generated metabolites of the selective androgen receptor modulators S-22 and S-23 and in vivo comparison to post-administration canine urine specimens. *Drug Test Anal*. 2010;2:589-598.

11. So YM, Wong JKY, Choi TLS, Prabhu A, Stewart B, Farrington AF, Robinson P, Wan TSM, Ho ENM. Metabolic studies of selective androgen receptor modulators RAD140 and S-23 in horses. *Drug Test Anal.* 2021;13(2):318-337.

12. Geldof L, Pozo OJ, Lootens L, Morthier W, Van Eenoo P, Deventer K. In vitro metabolism study of a black market product containing SARM LGD-4033. *Drug Test Anal.* 2017;9(2):166-178.

13. Cox HD, Eichner D. Detection of LGD-4033 and its metabolites in athlete urine samples. *Drug Test Anal.* 2016;9(1):127-134.

14. De Rijke E, Essers ML, Rijk JCW, Thevis M, Bovee TFH, Van Ginkel LA, Sterk SS. Selective androgen receptor modulators; in vitro and in vivo metabolism and analysis. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 2013;30(9):1517-1526.

15. Thevis M, Thomas A, Fusshöller G, Beuck S, Geyer H, Schänzer W. Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator andarine (S-4) for routine doping control purposes. *Rapid Commun Mass Spectrom.* 2010;24(15):2245-2254.

16. Fragkaki AG, Sakellariou P, Kiousi P, Kioukia-Fougia N, Tsivou M, Petrou M, Angelis Y. Human in vivo metabolism study of LGD-4033. *Drug Test Anal.* 2018;10(11-12):1635-1645.

17. Culter C, Viljanto M, Hincks P, Habershon-Butcher J, Muir T, Biddle S. Investigation of the metabolites of the selective androgen receptor modulator LGD-4033 in equine urine, plasma and hair following oral administration. *Drug Test Anal.* 2020;12(2):247-260.

18. Walpurgis K, Rubio A, Wagener F, Krug O, Knoop A, Görgens C, Guggat S, Thevis M. Elimination profiles of microdosed ostarine mimicking contaminated products ingestion. *Drug Test Anal.* 2020;12(11-12):1570-1580.

19. Rading A, Anielski P, Thieme D, Keiler AM. Detection of the selective androgen receptor modulator GSK2881078 and metabolites in urine and hair after single oral administration. *Drug Test Anal.* 2021;13(1):217-222.

20. Kintz P, Ameline A, Gheddar L, Raul JS. LGD-4033, S-4 and MK-2866 -Testing for SARMs in hair: about 2 doping cases. *Toxicol Anal Clin.* 2019;31(1):56-36.

21. Kintz P, Gheddar L, Ameline A, Raul JS. Identification of S22(ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases. *Drug Test Anal.* 2020;12:1508-1513.

22. Kintz P, Gheddar L, Ameline A, Raul JS. Perspectives in evaluating selective androgen receptors modulators in human hair: a short communication. *Ther Drug Monit.* 2021;43:298-300.

Table 1: Concentrations of S-23 in urine samples

Moments of urine collection, after administration (hours)	Concentrations (ng/mL)
0	0
2	1.0
4	5.0
6	14.5
8	93.0
12	50.0
24	26.5
48	11.9
72	8.5
96	21.6
120	32.9
168	5.0
240	1.0
336	1.0
672	0.5

Table 2: Metabolites observed in vitro using human liver microsomes (name, elemental composition, mass, characteristic ions and retention time)

Metabolites	Elemental composition	Mass (m/z) [M+H-]	Characteristic ions	RT (min)
S-23	$C_{18}H_{13}ClF_4N_2O_3$	415.0478	144.9867; 185.0335; 269.0551	2.33
hydroxy-S-23	$C_{18}H_{12}O_4N_2ClF_4$	431.0423	183.0123; 265.1481; 297.1531	2.20
***O*-dephenylate-S-23**	$C_{12}H_{10}O_3N_2F_3$	287.0647	126.9049; 185.0331; 241.0592	1.59
S-23-glucuronide	$C_{24}H_{20}O_9N_2ClF_4$	591.0794	144.9863; 241.0594; 287.0650	1.96
hydroxy-S-23-glucuronide	$C_{24}H_{20}O_{10}N_2ClF_4$	607.0743	185.0333; 269.0541; 431.0422	1.75

Table 3: Ratio (peak area) between hydroxy-S-23 and S-23.

Moments of urine collection, after administration (hours)	Ratio (%)
2	62
4	240
6	96
8	74
12	60
24	67
48	77
72	67
96	60
120	48
168	58
240	46
336	50
672	37

Table 4: Ratio (peak area) between dihydroxy-S-23 and S-23.

Moments of urine collection, after administration (hours)	Ratio (%)
8	0.1
12	0.3
24	0.5
48	1
72	0.8
96	0.7
120	0.7

Figure 1: Chemical structure of S-23 ($C_{18}H_{13}ClF_4N_2O_3$)

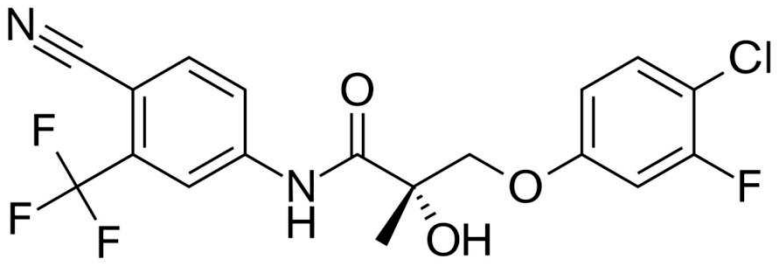


Figure 2: Box of S-23, bought on Internet and announced at 10 mg

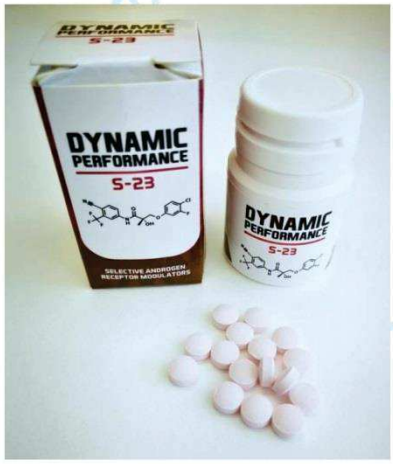


Figure 3: Concentration time profile of S-23 excretion in urine after administration of a single tablet

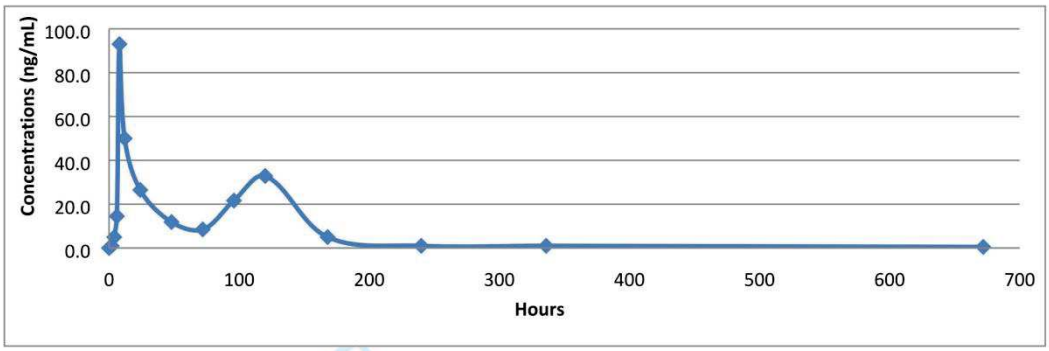


Figure 4: Chromatogram obtained after extraction of urine at + 28 days (672 hours). S-23 concentration was 1 ng/mL. From top to bottom: 2 transitions for S-23 (m/z: 415.1 > 144.9 and 415.1 > 185.0) and 1 transition for bicalutamide-d_4 (m/z: 433.1 > 255.1).

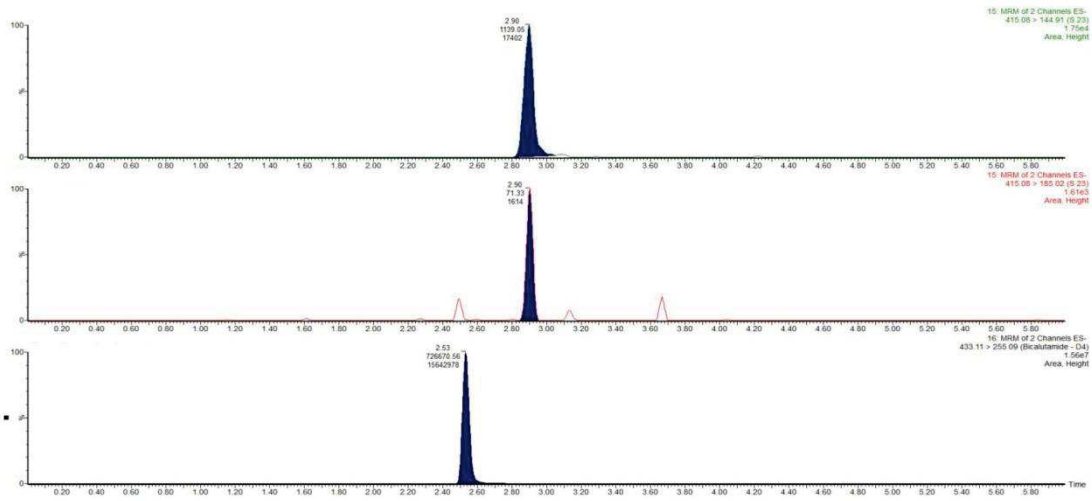


Figure 5: Spectra data information for S-23 and metabolites identified after in vitro incubation, by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). (A) S-23 ($C_{18}H_{13}ClF_4N_2O_3$; m/z [M+H-] 415.0478), (B) hydroxyl-S-23 ($C_{18}H_{12}O_4N_2ClF_4$; m/z [M+H-] 431.0423), (C) O-dephenylate-S-23 ($C_{12}H_{10}O_3N_2F_3$; m/z [M+H-] 287.0647), (D) S-23-glucuronide ($C_{24}H_{20}O_9N_2ClF_4$; m/z [M+H-] 591.0794) and (E) hydroxyl-S-23-glucuronide ($C_{24}H_{20}O_{10}N_2ClF_4$; m/z [M+H-] 607.0743).

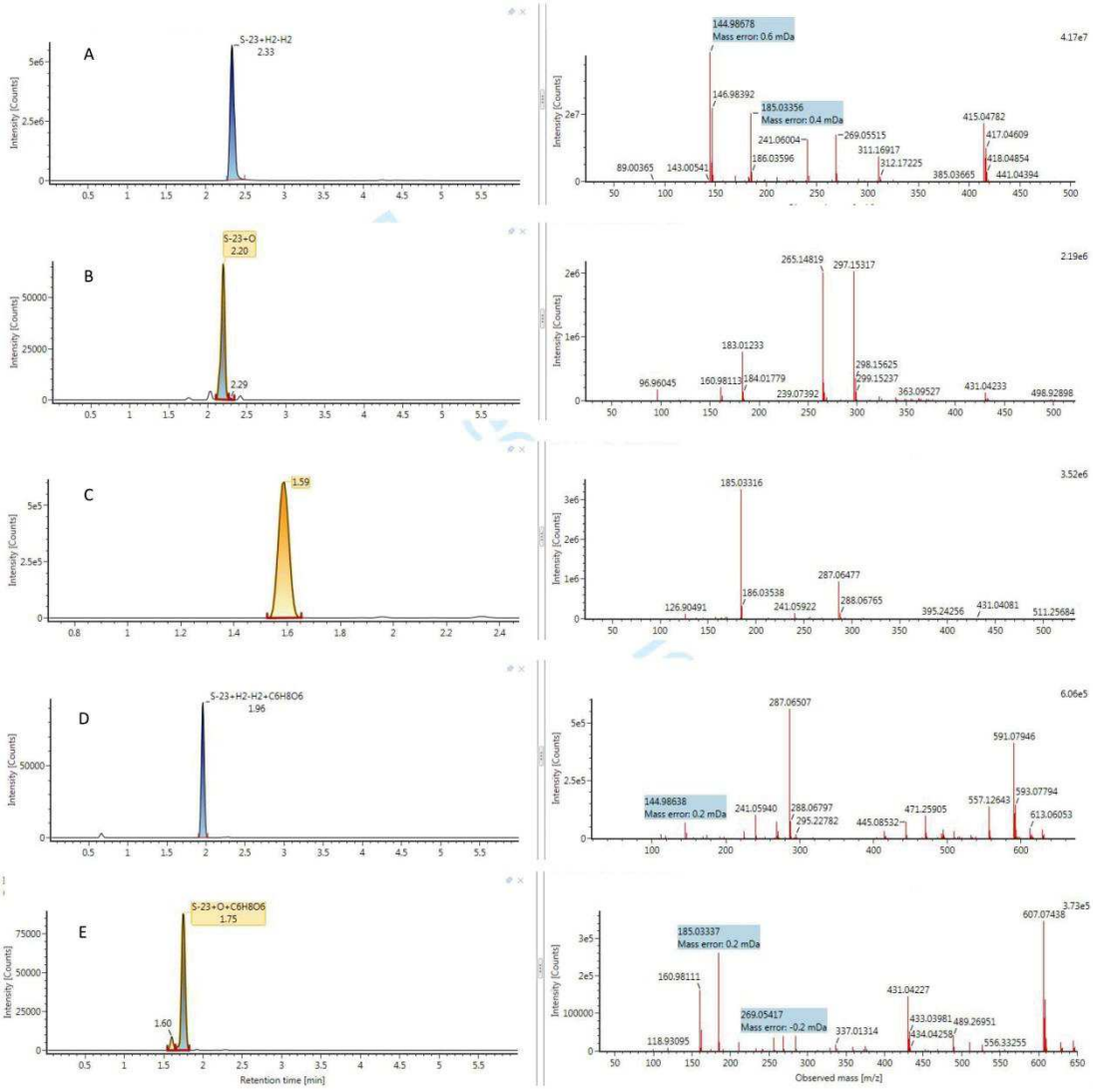
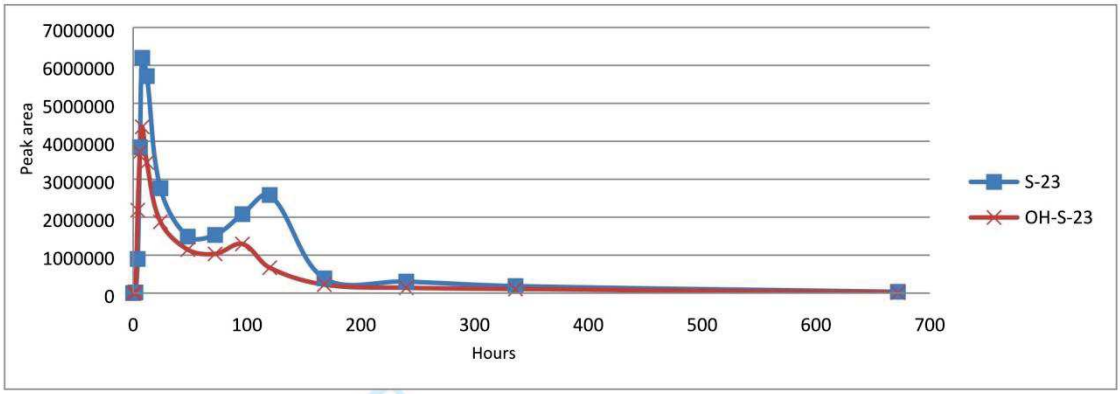


Figure 6: Peak areas of S-23 and hydroxy-S-23



Quatrième partie – Les diurétiques

4.1. Présentation

Les diurétiques sont utilisés dans le traitement de diverses maladies, par exemple dans le traitement de l'hypertension ou de l'insuffisance cardiaque. Ils sont commercialisés seuls ou en association avec d'autres médicaments. Il existe trois grandes classes de diurétiques : les diurétiques de l'anse, les diurétiques thiazidiques et les diurétiques hyperkaliémiants. Ils ont la même propriété, celle d'augmenter l'élimination du sodium et de l'eau. Néanmoins, chacun à son propre mécanisme d'action. Les diurétiques de l'anse (par exemple, le furosémide) inhibent la réabsorption du sodium et du chlorure au niveau de l'anse de Henle, tandis que les thiazidiques (par exemple, l'hydrochlorothiazide) inhibent la réabsorption couplée du sodium et du chlorure au niveau du tube contourné distal. Les deux catégories augmentent la diurèse. Les diurétiques hyperkaliémiants (par exemple, l'acétazolamide) inhibent la réabsorption active du sodium au niveau du tube contourné distal et du tube collecteur.

Dans le milieu sportif, les diurétiques sont mésusés soit pour masquer la prise d'agents dopants soit pour perdre du poids rapidement ; ce qui est recherché par les sportifs pratiquant des sports à catégorie de poids (comme la boxe, ou la lutte). C'est pour cette raison qu'ils sont interdits en permanence par l'AMA *[AMA, 2021].*

Les diurétiques les plus identifiés par les laboratoires antidopage sont le furosémide et l'hydrochlorothiazide, représentant 29 % et 21 % des RAA de classe en 2019 au niveau mondial.

4.2. Développement d'une méthode de screening

Afin de répondre aux demandes hospitalières, de caractériser les consommations de diurétiques pour des expertises médico-légales et dans des cas de dopage, j'ai développé un criblage ciblé des diurétiques à la fois les plus utilisés en thérapeutique et les plus retrouvés par les laboratoires anti-dopage.

Les diurétiques sont identifiés et quantifiés par UHPLC-MS/MS.

Protocole d'extraction

Le type d'extraction choisi est une extraction liquide-liquide.

30 mg de matrices kératinisées (cheveux coupés finement à l'aide d'une paire de ciseaux /ongles pulvérisés par un broyeur à billes Retsch MM200) sont pesés. Les échantillons sont incubés 15 heures dans un tampon pH 5,2 à 40°C en présence de deux SI (1 ng de furosémide-D_5 et d'hydrofluméthiazide). Une extraction liquide-liquide est ensuite effectuée avec 5 mL d'acétate

d'éthyle. Les phases organiques sont évaporées sous flux d'azote à 50°C et repris par 30 µL de MeOH. 2 µL sont ensuite injectés sur un système UHPLC-MS/MS.

Paramètres du système UHPLC-MS/MS

Les échantillons ont été analysés sur un système de chromatographie liquide, Acquity UPLC, de chez Waters (Waters Corporation, Milford, MA, USA).
La séparation des analytes est réalisée en phase inverse sur une colonne Acquity UPLC© HSS C18 (150 × 2,1 mm, 1,8 µm), thermostatée à 50 °C.
Le débit de travail est fixé à 0,3 mL/min pendant toute l'analyse. Une élution en gradient a été réalisée en utilisant un tampon acétate 1,0 mM de tampon acétate et 1,0 mM dans du méthanol de qualité LC-MS. Le temps d'analyse est de 9,0 min. Le gradient d'élution a été optimisé (tableau 4).

Temps (min)	Tampon acétate d'ammonium (%)	Méthanol, 1mM d'acétate d'ammonium
0	95	5
2	95	5
4	0	100
4,5	0	100
4,6	95	5
9	95	5

Tableau 4 : Gradient d'analyse UHPLC – méthode des diurétiques

La détection a été effectuée à l'aide d'un triple quadripôle (XEVO™ TQ-S Micro, Waters Corporation, Milford, MA, USA) équipé d'une source d'ionisation Z-spray-TM-électrospray (ESI) et utilisé en mode positif et négatif (ES+/ES-). L'azote a été utilisé comme gaz de nébulisation avec un débit de 50 L/hr. Les paramètres de masse ont été optimisés : température de la source : 150°C ; débit du gaz de désolvatation : 800 L/h ; température de désolvatation : 500°C. La. La tension du capillaire est réglée à 3,0 kV. La tension du cône et l'énergie de collision ont été automatiquement ajustées avec Intellistart pour optimiser le signal de tous les analytes. Le tableau 5 présente les transitions de quantification et de confirmation, les temps de rétention ainsi que les limites de détection et de quantification pour chaque analyte.

Analytes	Transition de quantification	Transition de confirmation	T_R (min)	LD (pg/mg)	LQ (pg/mg)
Acétazolamide	223,0 > 180,9	223,0 > 163,9	4,72	10	20
Bumétanide	365,1 > 184,1	365,1 > 240,2	5,65	3	5
Hydrochlorothiazide	296,1 > 77,8	296,1 > 125,9	4,79	3	5
Furosémide	329,1 > 204,9	329,1 > 125,9	5,32	3	5
Indapamide	366,0 > 132,0	366,0 > 90,9	5,97	3	5
Benzthiazide	429,9 > 307,9	429,9 > 228,0	5,73	5	10
Chlortalidone	336,9 > 145,9	336,9 > 189,9	5,62	5	10
Chlorothiazide	293,9 > 214	293,9 > 178,9	3,00	80	100
Spironolactone	341,3 > 107	341,3 > 187,1	6,30	20	50
ISTD					
Hydrofluméthiazide	329,8 > 238,9	/	5,19	/	/
Furosémide-D5	334,1 > 205,9	/	5,31	/	/

Tableau 5 : Liste des diurétiques – transitions - temps de rétention - LD et LQ

4.3. Applications

La méthode a été appliquée à des cas de dopage et à des affaires médico-légales.
Elle a été adaptée et légèrement modifiée lorsqu'un seul diurétique était demandé et recherché afin d'augmenter la sensibilité de la méthode.

Il n'y avait au début de ma thèse, aucune donnée dans la littérature concernant l'incorporation des diurétiques dans les cheveux. Ces molécules présentent des propriétés acides pour la plupart et de ce fait s'incorporent mal dans les cheveux ***[Article 19]***.
Toutefois afin d'interpréter les résultats dans les cheveux, j'ai mis en place deux études contrôlées afin d'établir des critères d'interprétation pour les deux diurétiques les plus identifiées par l'AMA et pour lesquels le laboratoire de toxicologie reçoit des demandes cliniques : l'hydrochlorothiazide et le furosémide.
Les travaux de ma thèse au sujet des diurétiques ont donné lieu à 4 publications décrivant des applications à des cas de dopage et à une application médico-légale :

Article 19 : Un athlète a contesté un résultat anormal impliquant de l'hydrochlorothiazide, et a demandé à notre laboratoire de tester ses cheveux. Du fait de l'absence de données dans les cheveux dans la littérature, les auteurs ont mis en place une étude contrôlée en deux étapes. Afin d'établir la base de l'interprétation, plusieurs volontaires ont été testés (quatre après une seule administration de 25 mg et dix sous traitement quotidien).
L'analyse du segment 0 - 2 cm des 4 volontaires ayant reçu une dose unique, prélevé 1 mois après l'administration, a été négative à la limite de quantification (LQ = 5 pg/mg). Les cheveux des 10 patients (2 cm proximaux) sous traitement quotidien étaient positifs avec des concentrations allant de 12 à 1845 pg/mg, sans corrélation entre la dose quotidienne et la concentration.
Les cheveux de l'athlète se sont révélés positifs à l'hydrochlorothiazide à 36 pg/mg dans le segment correspondant à la période du contrôle urinaire. Étant donné qu'une exposition unique à l'hydrochlorothiazide n'est pas détectable dans les cheveux et sur la base des résultats des patients sous traitement quotidien, la concentration trouvée chez l'athlète a été interprétée comme correspondant à des expositions répétées, dont il n'a pas été possible d'établir la dose et la fréquence.

Article 20 : Les auteurs rapportent deux cas de dopage présentant des résultats anormaux dans les urines pour l'hydrochlorothiazide :

- Cas 1 : il s'agit d'un pratiquant d'arts martiaux mixtes. Des poils de bras ont été prélevés en l'absence de cheveux. Les poils se sont révélés négatifs mais ne contredisent pas les résultats urinaires. En effet, de la méthyltestostérone a été retrouvée dans les poils. Il est tout à fait possible que l'athlète ait consommé de l'hydrochlorothiazide de façon unique pour éliminer plus rapidement le SAA retrouvé. En effet, une prise unique d'HCT n'est pas détectable dans les cheveux et poils.
- Cas 2 : il s'agit d'un rugbyman. Une mèche de cheveux de 6cm a été prélevée, segmentée (6 x 1 cm) et analysée. 4 segments consécutifs dont celui qui recouvre la période du contrôle urinaire étaient positifs (6 - 15 pg/mg). Ces résultats sont en faveur d'une pratique dopante.

Article 21 : L'acétazolamide est un inhibiteur efficace de l'anhydrase carbonique, principalement utilisé pour traiter le glaucome. Il peut être utilisé par les athlètes pour modifier l'excrétion et le métabolisme des agents dopants.

L'équipe juridique d'un athlète a contacté le laboratoire après un RAA due à l'acétazolamide. La concentration était inférieure à 5 ng/mL. Il s'agissait du premier contrôle de l'athlète depuis 9 mois, en raison de la pandémie de COVID-19. L'athlète a nié toute violation des règles antidopage mais a déclaré utiliser quotidiennement des capsules contenant 135 mg de citrate et de maléate de magnésium. Pour démontrer son honnêteté, l'athlète a soumis un échantillon de cheveux (noire, 4 cm, orientée), qui a été prélevé environ 6 semaines après la collecte d'urine. Une boîte non scellée de 15 gélules blanches (sur 200) et une boîte scellée mais portant un autre numéro de lot ont été soumises par l'athlète pour analyse. Selon l'athlète, il prenait une gélule par jour depuis, au moins, les 8 derniers mois.

Malgré la forte dénégation de l'athlète, l'acétazolamide a été retrouvé dans les quatre segments de cheveux avec des concentrations allant de 24 à 31 pg/mg. Ces résultats peuvent correspondre soit à une pratique dopante soit à une contamination par des suppléments diététiques. Néanmoins, ils ne peuvent pas être correctement interprétés car il n'y a aucune donnée dans la littérature.

Par ailleurs, l'acétazolamide a également été identifié dans la boîte de gélules non scellée à 42 ± 7 µg /capsule (n = 6).

Les auteurs estiment donc que l'hypothèse du sportif est probable, car elle est étayée par les résultats des analyses de cheveux et les résultats des suppléments.

Cependant, on ne peut pas totalement exclure que les gélules contaminées non scellées aient pu être manipulées par l'athlète après la révélation du résultat anormal.

Article 22 : La recherche de xénobiotiques dans les cheveux soulève plusieurs difficultés. Parmi elles, l'interprétation de la ou des concentrations finales. Dans un cas post-mortem, les analyses ont révélé la présence de furosémide (12 ng/mL) dans le sang fémoral alors qu'il ne faisait pas partie du traitement de la victime. Le procureur a demandé de procéder à une analyse complémentaire dans les cheveux pour obtenir des informations sur l'utilisation du furosémide.

Le furosémide a été trouvé dans les cheveux de la victime à 225 pg/mg. Cependant, il n'a pas été possible d'interpréter cette concentration en raison de l'absence de données dans la littérature. Par conséquent, les auteurs ont réalisé une étude contrôlée en deux parties. Afin d'établir la base de l'interprétation, plusieurs volontaires ont été testés (quatre après une seule administration de 20 mg et vingt-quatre sous traitement quotidien). La première série a montré qu'une dose unique n'est pas détectable dans les cheveux par notre méthode. La deuxième série a mis en évidence des concentrations allant de 5 à 1110 pg/mg sans qu'il y ait de corrélation entre la dose et la concentration dans les cheveux. Le résultat dans les cheveux de la défunte a été interprété comme une exposition répétée.

Dans le cas de l'analyse du furosémide, les cheveux peuvent fournir des informations sur sa présence mais ne peuvent pas donner d'informations sur le dosage ou la fréquence d'utilisation.

Mes travaux de thèse ont permis d'établir les critères d'interprétation dans les cheveux pour le furosémide et l'hydrochlorothiazide. L'acétazolamide a été identifié pour la première fois dans les cheveux dans un cas de dopage authentique.

A ce jour, le laboratoire reçoit des demandes d'analyse dans les cheveux de la part des services cliniques permettant ainsi de vérifier si le traitement est correctement suivi et non interrompu et si un mésusage à visée amaigrissante est caractérisé.

Article 19 : First identification of a diuretic, hydrochlorothiazide, in hair : application to a doping case and interpretation of the results

Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz

Drug Testing and Analysis. 2019 ;11 :157-161.

Received: 12 March 2018 | Revised: 9 June 2018 | Accepted: 11 June 2018
DOI: 10.1002/dta.2445

SHORT COMMUNICATION

WILEY

First identification of a diuretic, hydrochlorothiazide, in hair: Application to a doping case and interpretation of the results

L. Gheddar | JS. Raul | P. Kintz

Institut de médecine légale, laboratoire de toxicologie, Strasbourg, France

Correspondence
L. Gheddar, Institut de médecine légale, 11 rue Humann, 67000 Strasbourg, France.
Email: l.gheddar@gmail.com

Abstract

An athlete contested an adverse analytical finding involving hydrochlorothiazide, and requested our laboratory for testing his hair. As there is no reference in the literature about identification of hydrochlorothiazide in hair, several volunteers were first enrolled (4 after a single 25 mg administration and 10 with daily therapeutic treatment). A specific method was developed by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS. Hair samples were decontaminated with dichloromethane and 30 mg were incubated in buffer at pH 7.0 for 15 hours at 50°C. Then, 5 mL ethyl acetate was added for extraction. Linearity was observed for hydrochlorothiazide concentrations ranging from 5 to 2000 pg/mg. The limit of quantification was 5 pg/mg. The coefficients of variation (CVs) of repeatability and matrix effect were lower than 20%. Analysis of the 0–2-cm segment of the 4 volunteers having received a single dose, collected 1 month after administration, was negative at the limit of quantification. The hair of the 10 patients (proximal 2 cm) on daily treatment was positive with concentrations ranging from 12 to 1845 pg/mg, with no correlation between daily dose and concentration. The athlete's hair tested positive for hydrochlorothiazide at 36 pg/mg in the segment corresponding to the period of the urinary control. Since a single exposure to hydrochlorothiazide is not detectable in hair and based on the results of the patients on daily treatment, the concentration found in the athlete has been interpreted as corresponding to repeated exposures, where it was not possible to establish the dosage and the frequency.

KEYWORDS

Diuretic, doping, hair, hydrochlorothiazide, LC–MS/MS

1 | INTRODUCTION

Hydrochlorothiazide (HCT) is marketed alone (Esidrex®, Novartis) or in combination with other molecules. This drug is available in 6.5, 12.5, and 25 mg tablets. It is a diuretic of the benzothiadiazine family, used since 1957 in the treatment of control of high blood pressure. It inhibits the renal tubular sodium and chloride reabsorption and increases urinary excretion. It is a weak acid substance with 2 dissociation constants at 7.9 and 9.2 and has a half-life of 6 to 15 hours.[1]

Due to its diuretic properties, HCT can be used by athletes to lose weight (weight class sport such as judo or wrestling) or to increase the elimination rate of anabolics or stimulating agents (used as masking agent). It is classified[2] as a prohibited substance by the World Anti-Doping Agency (WADA) in and out-of-competition (class S5: Diuretics and masking agents) and is screened in anti-doping laboratories using urine. An adverse analytical finding (AAF) leads to the immediate suspension of the athlete as the presence of a banned substance in urine is generally enough to be eligible to a sanction.[3] However, in urine, it is impossible to establish the pattern of drug exposure (single or repetitive use). At the opposite, in forensic science, it can be useful to document the initial findings using a hair test. Thanks to its large window of detection, depending on the hair length, and the possibility to segment hair strands, it is generally possible to determine the pattern of drug exposure in the months before collection. For these reasons, it seems interesting to test for drugs in hair, even in doping cases. Moreover, hair analysis was approved as an alternative matrix according to

International Standard for Laboratories[3] but this analysis cannot be the standard method for a general screening in doping situation.[3,4] For example, a negative hair result does not always mean that urine was negative.[4] Indeed, the consensus of the Society of Hair Testing (SoHT) has fixed the applications of hair testing for doping agents, including the possible interpretation. In the case of positive urine results, the negative hair result cannot exclude the administration of drug. The negative hair results can be explained by the absence of a sensitive enough analytical method or limited drug incorporation. This can be explained[5] by the chemical properties of the molecules such as the presence of a nitrogen atom (good for incorporation) or the presence of an acidic function (bad for incorporation).

A review of the literature has indicated that there are some articles about testing for HCT in urine and blood but there is no reference for hair. In fact, the detection of diuretics in hair has never been reported. It is not known if diuretics are incorporated in hair. This paper aims to present a specific method for testing HCT in hair and to propose data for suitable interpretation of the measured concentrations.

2 | MATERIALS AND METHODS

2.1 | Case report and hair specimens

During an out-of-competition period, an anti-doping laboratory tested the urine of a top athlete (17 years old) and returned an AAF for HCT (about 300 pg/mL). The athlete challenged the result and denied any voluntary intake of the drug. He admitted, however, that inadvertent consumption was possible since one of his friends was treated with HCT [sic]. The athlete confirmed to us that he had not treated his hair in the 3 months before sampling. At the request of his lawyer, our laboratory received 3 hair strands from the athlete to establish the pattern of HCT use at the time of the urinary control. The hair was collected by a US point of collection with suitable chain of custody about 3 months after the urinary control and the athlete declared a hair growth rate of about 1.5 cm/month. The hair was 10 cm in length, and blond in colour. The orientation root to tip was maintained.

To interpret the results of the athlete's test and to obtain information about the detectability of HCT in hair, 2 surveys were carried out.

2.1.1 | Survey 1

One tablet containing 25 mg of HCT was taken orally by 4 healthy male volunteers (20–57 years old). Written consent was obtained from each subject after the study was explained. For each volunteer, 2 hair strands were collected 1 month after administration. The strands were segmented in 2 segments of 2 cm in length.

2.1.2 | Survey 2

Ten patients (men and women aged 45–70 years old) under daily medical treatment with HCT since more than 6 months (6.25, 12.5, or 25 mg/daily) participated in the study. A written consent was obtained from each subject after the study has been explained. For each person 2 hair strands were collected. The hair length was variable (2–10 cm), but only the proximal segment (2 cm) was tested.

The hair strands were received in an envelope and stored at ambient temperature in the laboratory before analysis.

Blank hair for method validation was obtained from laboratory staff.

2.2 | Chemicals and reagents

HCT and hydroflumethiazide (HFT) were obtained from Ciba-Ceigy (Groot-Bijgaarden, Belgium) and from Sigma Aldrich (Steinheim, Germany), respectively. Ammonium acetate, dichloromethane, and ethyl acetate (HLPC grade) were purchased from Merck (Darmstadt, Germany) and methanol for HPLC Isocratic Grade (MeOH) from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Aqueous buffer pH 7.0 was purchased from Hanna Instruments (Woosocket, RI, USA).

The standard solutions, prepared in MeOH, were stored at 4°C.

2.3 | Sample treatment

2.3.1 | Decontamination

The hair strands were decontaminated twice with 5 mL of dichloromethane for 2 minutes at room temperature and dried.

2.3.2 | Preparation and extraction of hair

The hair was segmented and cut into very short segments. Thirty mg of decontaminated hair was incubated in 1 mL buffer pH 7.0 for 15 hour at 50°C, in the presence of 30 ng of HFT used as internal standard. Then, 5 mL of ethyl acetate were added. After agitation for 15 minutes and centrifugation (15 minutes, 3000 rpm), the organic phase was collected, and evaporated to dryness under a nitrogen flow. The dry residue was dissolved in 50 µL of the initial mobile phase, and 10 µL was injected into the UHPLC-system.

2.4 | Instrumentation

Chromatographic separations were performed with a liquid chromatography Waters Acquity UPLC™ system (Waters corporation, Milford, MA, USA) using an Acquity UPLC HSS C18 column (150 mm x 2.1 mm, i.d. 1.8 µm particle size). The column temperature was set to 50°C, and the flow rate at 0.3 mL/min. The mobile phase consisted of 1.0mM ammonium acetate in water (eluent A) and 1.0mM ammonium acetate in MeOH (eluent B). Analysis time was 6.0 minutes and the following gradient pattern (eluent A) was used for the separation of HCT and HFT: 0 minutes, 95%; 2.00 minutes, 95%; 4.00 minutes, 0%; 4.10 minutes, 0%; 4.30 minutes, 95%; 6.00 minutes, 95%. HCT and HFT were eluted in 3.87 and 4.08 minutes, respectively.

Detection was performed using a triple quadrupole (XEVO™ TQD, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionisation source (ESI) and used in the negative mode (ES-). Nitrogen was used as drying gas as well as nebulising gas. The desolvation gas flow was set to 800 L/h and the desolvation temperature was 400°C. The source temperature was 150°C. The capillary voltage was set at 1.50 kV. Cone voltage and collision energy were adjusted to optimise the signal of the 2 most abundant product ions of HCT: m/z 296.1 > 268.9 and 296.1 > 204.9 and for HFT: m/z 329.8 > 302.9 (Table 1). MassLynx 4.1 software was used for quantification.

TABLE 1 MS–MS transitions

Molecule	MW (g/mol)	Parent Ion	Cone Voltage (V)	Daughter Ions	Collision Energy (eV)	Rt (min)
Hydrochlorothiazide	297.74	296.1	78	268.9 204.9	20 24	3.87
Hydroflumethiazide	331.29	329.8	42	302.9	22	4.08

2.5 | Validation method

A 9-point calibration curve (n = 3) was obtained by spiking blank hair at 5, 10, 20, 50, 200, 500, 1000, and 2000 pg/mg. The limit of quantification (LOQ) was defined as the first point of calibration. The limit of detection (LOD) was the lowest concentration of analyte that could be detected with signal to noise ratio superior than 3. The repeatability, matrix effect, and extraction efficiency were evaluated. For the repeatability, 2 calibration points at 50 and 500 pg/mg were evaluated 8 times. With respect to matrix effect, 17 different hair blank specimens were extracted and 50 µL of standard solution of HCT (1 mg/L) was added at the end of extraction. These specimens were compared to 50 µL of standard solution of HCT in MeOH (1 mg/L). For the extraction efficiency, 8 spiked hair samples at 1 mg/L of HCT were extracted and compared to a standard solution of HCT in MeOH (1 mg/L) (n = 4).

3 | RESULTS AND DISCUSSION

3.1 | Validation

Three calibration curves based on analyte/internal standard peak area ratios were obtained on 3 separate days and analysed by weighted linear regression to validate the linearity of the method. The assay was linear in the concentration range 5–2000 pg/mg (r^2 > 0.990) with an LOQ of 5 pg/mg. The LOD was 3 pg/mg. A typical standard curve of a validation assay (after weighted linear regression 1/x) is y = 0.000006x-0.00004, r^2 = 0.9998, where x represents the mass, and y is the peak area ratio of HCT to IS. The concentration is calculated with the exact weighed mass.

QC samples (50 pg/mg and 500 pg/mg), analysed in duplicate in 8 independent experimental assay, were used to determine a coefficient of variation (CV) for precision and accuracy. These CVs were lower than 20%. The matrix effect was lower than 20%. Finally, the average extraction efficiency was 61% (n = 8). It was not possible to establish a suitable extraction efficiency in authentic hair due to the lack of reference material available for validation.

3.2 | Hair results

When testing for drugs in hair, interpretation of the results is the most critical step, particularly when there is no data in the literature to compare the findings. For example, in the case of HCT, there is a lack of knowledge about the minimal detectable dosage in hair or about what can be expected after daily treatment. In some cases,[5] it might be necessary to test a marker or a minor metabolite rather than the administered molecule itself; for example, ethyl glucuronide instead of ethanol is the compound of interest in hair. Unfortunately, nothing is known about diuretic incorporation into hair.

The first step of this study was therefore to determine whether HCT was detectable in hair after a single administration. Four subjects (specimens 11 to 14) received a single 25 mg of HCT by the oral route. At the LOQ of 5 pg/mg, the 4 specimens were negative. A very small signal was observed for 2 subjects, but this was under the 5 pg/mg cut-off. Table 2 presents the results of this study, including dosage, hair colour, and concentrations of HCT. It was concluded that a single administration of HCT, even at the highest available dosage, is undetectable in human hair. This has also been published for other drugs. For example, Kintz[5] reported that a single dose of 2.5 mg of lorazepam is undetectable, while a 2-mg dose of clonazepam can be detected. In a study by Segura et al.,[6] testosterone esters could not be detected after intramuscular administration of 250 mg. Furthermore, Kintz et al[7] reported in a study that nandrolone was not detectable in hair while urine tested positive for its metabolites (NA and NE) for at least 8 months, after a single intramuscular administration of 50 mg of nandrolone undecanoate. The problem of detectability can be accounted for either by the molecules properties or by the instrument which may not be sensitive enough. If the latter applies, it can be anticipated that the chances HCT will be detected in hair should only occur if larger doses are taken by the patients.[8]

In light of the first experiment, the second step of this study was to determine the range of concentration of HCT in hair obtained from subjects under daily treatment. For this purpose, 10 subjects under daily therapeutic HCT treatment, with daily dosage ranging from 6.25 to 25 mg were enrolled in this experiment. Concentrations of HCT in the proximal 2-cm segments are reported in Figure 1 and range from 12 to 1845 pg/mg. Although HCT was detected in all hair segments, the measurement points are very scattered which demonstrates that there is no apparent correlation between the concentrations found into

TABLE 2 Hair specimens and results

Subject	Dose	Hair colour	Hair concentration (pg/mg)
1	25 mg/daily	Dark-grey	12
2	12.5 mg/daily	Dark-grey	35
3	6.25 mg/daily	White	185
4	12.5 mg/daily	Grey	111
5	12.5 mg/daily	White	1261
6	25 mg/daily	White	393
7	12.5 mg/daily	Dark-grey	1058
8	12.5 mg/daily	Grey	1845
9	12.5 mg/daily	Dark	123
10	Not known	Brown	406
11	Single 25 mg	Blond	Not detected
12	Single 25 mg	Brown	Not quantified
13	Single 25 mg	Blond	Not detected
14	Single 25 mg	Brown	Not detected

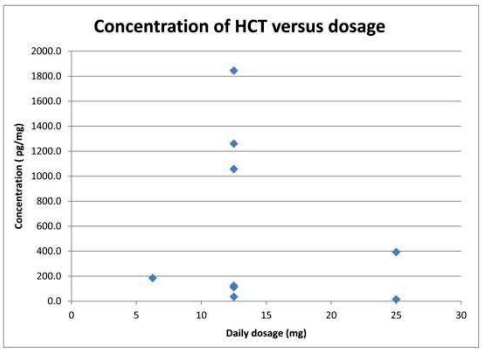


FIGURE 1 Concentration of HCT in hair versus daily dosage [Colour figure can be viewed at wileyonlinelibrary.com]

hair and the daily dose (R^2 = 0.04). For example, a 12.5 mg/day dose gave concentrations ranging from 35 to 1845 pg/mg. The absolute lack of relationship between dosage and concentration is not common.[8] For example, Jurado[9] reported that the concentration of cocaine found in hair is proportionate to the dose with a good correlation factor of 0.86.

The incorporation of drug into hair is controlled by some factors such as melanin affinity or lipophilicity. It can be anticipated that HCT will be badly incorporated into hair because there is no electric charge binding possibility between melanin which is negatively charged and HCT which is not positively charged at pH 5.0 of the hair. Contrary to an alkaline molecule, HCT, a weak acidic drug, must have a poor ability to penetrate through the membrane, based on the pH gradient between blood and hair follicles.[10]

This study showed that hair colour has no influence for incorporation of HC into hair (Survey 1 results). Indeed, pigmented hair and unpigmented hair were the same results. Colour can be an important paramount for the incorporation of alkaline molecules but not for neutral or acidic molecules. Indeed, Henderson et al[11] conducted a study on the incorporation of cocaine (alkaline molecule) on subjects with light and dark hair. They reported that cocaine was better incorporated in dark hair than in light hair.[11] For neutral and acid molecules, several studies report that hair colour has no influence. Mieczkowski et al.

TABLE 3 Case results

Segment	0–2 cm	2–4 cm	4–6 cm	6–8 cm	8–10 cm
Hair concentration	Not detected	Not detected	36 pg/mg	Not detected	Not detected

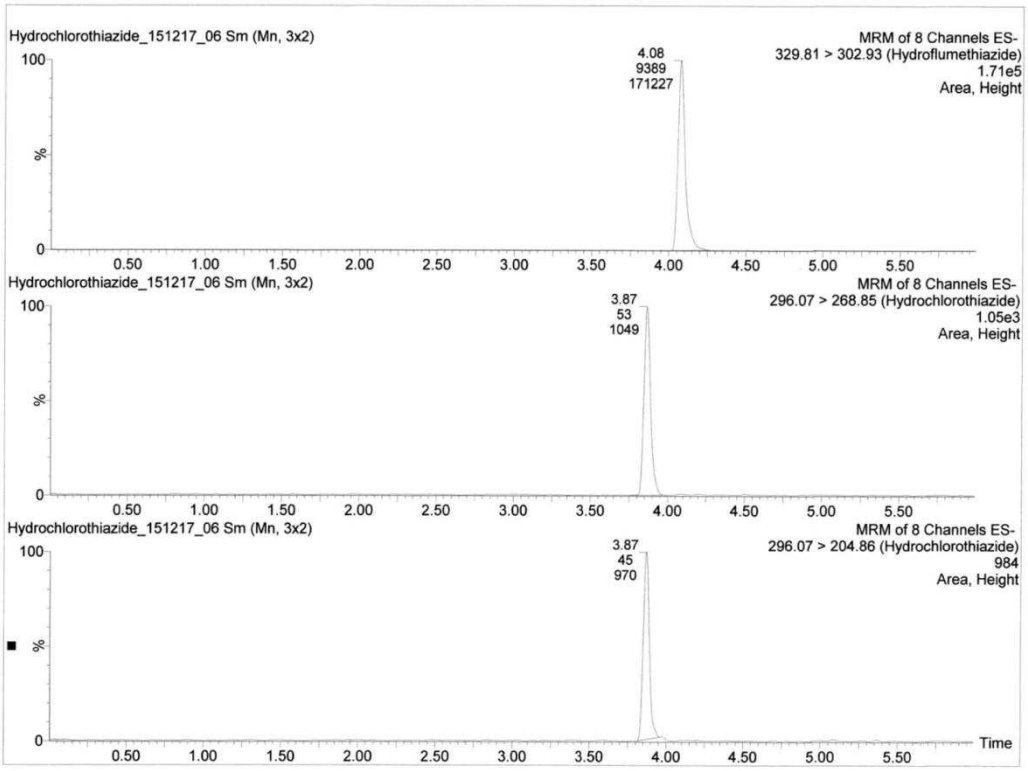


FIGURE 2 Chromatogram of top athlete's hair 4–6-cm segment. A, transition of the internal standard; B, C, transitions of HCT. Concentration was 36 pg/mg

showed that there was no relationship between carbamazepine concentration and hair colour.[12] Kintz et al showed that the hair colour had no influence on the Gamma-Hydroxybutyric acid (GHB) concentration.[13] And finally, Gygi et al compared the incorporation of codeine (alkaline) and phenobarbital (acid) into the hair.[14] It showed that for codeine, concentrations were 44 times higher in pigmented hair than in unpigmented hair, while for phenobarbital, the results were identical.[14]

In doping cases, urinalysis for HCT does not allow for distinguishing between single and multi-doses (which is not the purpose of the test). This study has demonstrated that a single HCT dose is not detectable in hair at this time. However, repetitive doses of HCT, administered to patients suffering from high blood pressure, were always detectable.

The test result for HCT in the athlete's hair is presented in Table 3. The analysis of the combined dichloromethane washes was negative. Figure 2 shows the chromatogram obtained after extraction of the segment 4–6 cm of the athlete. This segment corresponds to the period of the AAF in urine. This result (36 pg/mg) is in the range of what has been observed in daily treated patients (low limit of range). Since a single exposure is not identifiable in the hair and based on the results of the patients on daily treatment, the concentration found in the athlete's hair has been interpreted as corresponding to repeated exposures. Nevertheless, it is not possible to establish the dosage and the frequency of consumption. This is also due to the obvious lack of literature. Given that a single 25 mg dose seems not to be detectable in hair, it is therefore anticipated that residual contamination by supplements should not be detectable in hair. This is the opposite with clenbuterol, where the authors were able to discriminate therapeutic use from contamination.[15]

4 | CONCLUSION

The application of hair testing seems to be a suitable approach to document HCT findings in doping control. However, the method has some limitations. It was not possible to establish the dosage and the frequency of consumption for HCT due to the weak incorporation of the drug into the hair and the lack of relationship between dosage and hair concentrations. Unlike urine testing, this study demonstrated it is possible in hair to discriminate between single take and repetitive exposure. This study represents the first contribution to the identification of a diuretic in hair.

ORCID

L. Gheddar http://orcid.org/0000-0002-7804-3946
P. Kintz http://orcid.org/0000-0002-4174-5362

REFERENCES

1. Baselt RC. *Disposition of Toxic Drug and Chemicals in Man*. 11th ed. Seal Beach, CA: Biomedical Publications; 2017:1049-1050.
2. World Anti-Doping Agency. What is prohibited. https://www.wada-ama.org/en/content/what-is-prohibited/prohibited-at-all-times/diuretics-and-masking-agents. Accessed February 27, 2018.
3. World Anti-Doping Agency. World Anti-Doping Code International Standard Laboratories. June 2016. Montreal Version 9. https://www.wada-ama.org/sites/default/files/resources/files/isl_june_2016.pdf. Accessed May 05, 2018.
4. Sachs H, Kintz P. Consensus of the society of hair testing on hair testing for doping agents. *Forensic Sci Int*. 2000;107:3.
5. Kintz P. Value of the concept of minimal detectable dosage in human hair. *Forensic Sci Int*. 2012;218(1-3):28-30.
6. Segura J, Pichini S, Peng SH, de la Torre X. Hair analysis and detectability of single dose administration of androgenic steroid esters. *Forensic Sci Int*. 2000;107(1-3):347-359.
7. Kintz P, Cirimele V, Ludes B. Pharmacological criteria that can affect the detection of doping agents in hair. *Forensic Sci Int*. 2000;107(1-3):325-334.
8. Kintz P. Hair analysis in forensic toxicology: an updated review with special focus on pitfalls. *Curr Pharm Des*. 2017;23(36):5480-5486.
9. Jurado C. Hair Analysis for cocaine. In: Kintz P, ed. Analytical and Practical Aspects of Drug Testing in Hair. *Forensic science series*. Boca Raton, FL: Taylor and Francis; 2007:95-120.
10. Kronstrand R, Scott K. Drug Incorporation into Hair. In: Kintz P, ed. Analytical and Practical Aspects of Drug Testing in Hair. *Forensic science series*. Boca Raton, FL: Taylor and Francis; 2007:1-19.
11. Henderson GL, Harkey MR, Zhou C, Jones RT, Jacob P. Incorporation of isotopically labelled cocaine and metabolites into human hair: 1. Dose-response relationships. *J Anal Toxicol*. 1996;20(1):1-12.
12. Mieczkowski T, Tsatsakis AM, Kruger M, Psillakis T. The concentration of three anti-seizure medications in hair: the effects of hair color, controlling for dose and age. *BMC Clin Pharmacol*. 2001;1(1):2. http://doi.org/10.1186/1472-6904-1-2
13. Kintz P, Cirimele V, Jamey C, Ludes B. Testing for GHB in hair by GC/MS/MS after a single exposure. Application to document sexual assault. *J Forensic Sci*. 2003;48(1):195-200.
14. Gygi SP, Wilkins DG, Rollins DE. A comparison of phenobarbital and codeine incorporation into pigmented and nonpigmented rat hair. *J Pharm Sci*. 1997;86(2):209-214.
15. Krumbholz A, Anielski P, Gfrerer L, et al. Statistical significance of hair analysis of clenbuterol to discriminate therapeutic use from contamination. *Drug Test Anal*. 2014;6(11-12):1108-1116.

How to cite this article: Gheddar L, Raul JS, Kintz P. First identification of a diuretic, hydrochlorothiazide, in hair: Application to a doping case and interpretation of the results. *Drug Test Anal*. 2019;11:157–161. https://doi.org/10.1002/dta.2445

Article 20 : Recherche d'hydrochlorothiazide dans les phanères après deux contrôles antidopage

Laurie Gheddar, Jean-Sébastien Raul, Pascal Kintz

Toxicologie analytique et clinique. 2018 ;30 :268-272.

Toxicologie Analytique & Clinique (2018) 30, 268—272

Disponible en ligne sur ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com

ÉTUDE DE CAS

Recherche d'hydrochlorothiazide dans les phanères après deux contrôles antidopage

Testing for hydrochlorothiazide in human hair after two antidoping controls

Laurie Gheddar[a,*], **Jean-Sébastien Raul**[a], **Pascal Kintz**[a,b]

[a] *Institut de médecine légale, 11, rue Humann, 67000 Strasbourg, France*
[b] *X-Pertise Consulting, 84, route de Saverne, 67205 Oberhausbergen, France*

Reçu le 8 août 2018 ; reçu sous la forme révisée le 19 septembre 2018 ; accepté le 19 septembre 2018
Disponible sur Internet le 9 octobre 2018

MOTS CLÉS
Hydrochlorothiazide ;
Cheveux ;
Dopage ;
Diurétique

Résumé Commercialisé sous le nom Esidrex®, l'hydrochlorothiazide est de plus en plus utilisé dans le sport. Du fait de son pouvoir diurétique, les sportifs l'utilisent pour perdre du poids ou pour éliminer plus rapidement des substances anabolisantes ou encore stimulantes. L'analyse des cheveux permet de discriminer une prise unique d'une prise répétée. Les auteurs rapportent ici deux cas de dopage. Le cas 1 concerne un sportif pratiquant les arts martiaux mixtes et le cas 2 un rugbyman. Les urines des deux athlètes ont montré des résultats anormaux pour l'hydrochlorothiazide. Des poils de bras et des cheveux ont été prélevés respectivement pour les cas 1 et 2. Les phanères ont été analysés selon une méthode précédemment publiée faisant appel à la chromatographie liquide couplée à de la spectrométrie de masse en tandem, avec une limite de quantification (LOQ) à 5 pg/mg. Les analyses pour le cas 1 (poils de bras) n'ont pas mis en évidence la présence d'hydrochlorothiazide. Ce résultat ne contredit pas les analyses urinaires. Il pourrait s'agir d'une prise unique. Pour le cas 2 (cheveux), l'hydrochlorothiazide est retrouvé dans plusieurs segments consécutifs avec des concentrations supérieures à la limite de quantification dans trois segments : 15 pg/mg (0—1 cm), 7 pg/mg (1—2 cm), < LOQ (2—3 cm) et 6 pg/mg (3—4 cm). Ces résultats peuvent être interprétés en tant qu'expositions répétées. Ces observations permettent de montrer que l'analyse des phanères est un bon complément de l'analyse urinaire.

* Auteur correspondant.
Adresse e-mail : l.gheddar@gmail.com (L. Gheddar).

https://doi.org/10.1016/j.toxac.2018.09.003
2352-0078/

KEYWORDS
Hydrochlorothiazide;
Hair;
Doping;
Diuretic

Summary Marketed under the name Esidrex®, hydrochlorothiazide is increasingly used in sport. Because of its diuretic power, athletes use it to loose weight or to eliminate more rapidly anabolic or stimulant substances. Hair analysis enables to discriminate a single dose from repeated exposures. The authors report two cases of doping. Case 1 concerns an athlete practicing mixed martial arts and case 2 a rugby player. The analysis of the urine of both top athletes produced adverse analytical results for hydrochlorothiazide. Arm hair and head hair were analyzed for case 1 and 2, respectively. The hair was analyzed using a recently published procedure, using liquid chromatography coupled to tandem mass spectrometry, with a limit of quantification (LOQ) at 5 pg/mg. The test for case 1 (arm hair) didn't show the presence of hydrochlorothiazide. However, his result does not contradict urinalysis. It could be a single intake. For case 2 (head hair), hydrochlorothiazide was found in several consecutive segments with concentrations higher than the limit of quantification in three segments: 15 pg/mg (0—1 cm), 7 pg/mg (1—2 cm), < LQ (2—3 cm) and 6 pg/mg (3—4 cm). These results can be interpreted as repeated exposures. This short study confirms that hair analysis is a good complement to urinalysis in sport.

Introduction

L'utilisation des produits diurétiques et agents masquants est interdite par l'agence mondiale antidopage (AMA) en compétition et hors compétition. En 2017, la classe S5 (diurétiques et agents masquants) représentait 17,4 % des résultats anormaux au niveau national (donnée de l'agence française de lutte contre le dopage) et 15 % au niveau mondial (donnée de l'AMA), soit une augmentation respective de 34 et 25 % par rapport à 2016 [1,2]. Les diurétiques sont utilisés par les sportifs pour deux raisons. La première est pour perdre du poids et la seconde pour éliminer plus rapidement des substances comme les anabolisants, les stimulants ou encore les corticoïdes. L'hydrochlorothiazide (HCT), diurétique thiazidique, est classé comme substance S5 diurétiques et agents masquants [3]. Ce médicament, commercialisé sous le nom ESIDREX® (Novartis), est proposé dans le traitement de l'hypertension artérielle et est disponible sous forme de comprimés dosés à 6,25—12,5 et 25 mg. Il agit en tant qu'inhibiteur de la réabsorption du sodium par le tubule au niveau du segment cortical de dilution et permet l'augmentation de l'excrétion urinaire. C'est un acide faible avec des constantes d'acidité de 7,9 et 9,2 [4].

La recherche des diurétiques par les laboratoires antidopage se fait généralement dans les urines. Néanmoins, lorsqu'il est nécessaire de documenter le cas (lors d'une contestation par exemple) cette matrice ne permet pas de discriminer une prise unique d'une prise répétée. Les cheveux ont été acceptés par le tribunal arbitral du sport dans des dossiers sensibles comme l'affaire Richard Gasquet [5]. Cette matrice permet, dans un cadre médico-légal, de rechercher des xénobiotiques sur une plus longue durée, c'est-à-dire d'augmenter la fenêtre de détection selon la longueur des cheveux disponible mais également de différencier une prise unique d'une prise répétée en segmentant les cheveux. Cette analyse peut être un bon complément de l'analyse urinaire dans des cas de dopage [6].

Dans une précédente publication, les auteurs ont rapporté les critères d'interprétation des concentrations d'hydrochlorothiazide dans les cheveux [7]. Dans cette nouvelle étude, ils rapportent deux nouveaux cas de résultats anormaux après identification dans les urines de l'hydrochlorothiazide. L'objectif de ce travail est donc de présenter et d'enrichir la littérature avec deux nouvelles observations.

Présentation des cas

Cas 1. Un sportif américain pratiquant les arts martiaux mixtes (MMA) a été contrôlé par un laboratoire antidopage, hors compétition. Ses urines ont présenté un résultat anormal pour la méthyltestostérone et l'hydrochlorothiazide, sans que soit indiquée une concentration. Des poils de bras (brun, 2 cm) ont été prélevés 2 mois après le contrôle urinaire et ont été envoyés au laboratoire. La totalité de la longueur des poils a été analysée.

Cas 2. Un rugbyman anglais est contrôlé par un laboratoire antidopage, en dehors d'une compétition. Ses urines ont présenté un résultat anormal pour l'hydrochlorothiazide. Deux analyses urinaires ont été réalisées ; l'échantillon A a donné une concentration de 60 ng/mL et l'échantillon B en contre-expertise, une concentration de 30 ng/mL. Trois mèches de cheveux (brun, 21 cm) ont été prélevées 2 mois après le contrôle urinaire et ont été envoyés au laboratoire. La vitesse de pousse prise en compte

est de 1 cm/mois. Les segments suivants sont analysés : 0—1 cm, 1—2 cm, 2—3 cm, 3—4 cm, 4—5 cm et 5—6 cm.

Les deux athlètes ont déclaré ne pas avoir utilisé de traitement cosmétique.

Matériel et méthode

La méthode analytique citée précédemment et publiée [7] fait appel à la chromatographie liquide (Waters Corporation, Milford, MA, USA) couplée à de la spectrométrie de masse en tandem (XEVO^{TM} TQD, Waters Corporation, Milford, MA, USA). Brièvement, la séparation des analytes est réalisée sur une colonne Acquity HSS C18 (1,8 µm, 2,1 × 150 mm) en utilisant le mélange de phases suivant : A (solution aqueuse à 1 mM acétate d'ammonium) et B (méthanol à 1 mM acétate d'ammonium). L'hydrofluméthiazide (HFT) a été utilisé comme standard interne. L'HCT et l'HFT sont élués respectivement à 3,87 minutes et à 4,08 minutes. Deux transitions ont été retenues pour l'HCT : m/z 296,1 > 268,9 (quantification) et m/z 296,1 > 204,9 (confirmation) et une transition a été retenue pour l'HFT : m/z 329,8 > 302,9. L'acquisition est réalisée en mode MRM avec une source électrospray en mode négatif [7].

Après préparation de 30 mg de cheveux, 30 ng du standard interne (HFT) sont ajoutés. Les cheveux sont incubés à 50 °C pendant 16 h à pH 7,0 et 5 mL d'acétate d'éthyle sont ajoutés au mélange. La phase organique est récupérée et évaporée sous flux d'azote. Le résidu sec est repris dans 50 µL du mélange de phase mobile initiale et 10 µL sont injectés dans le système de chromatographie liquide.

Résultats et discussion

Les critères d'interprétation [7] pour une dose unique de 25 mg et une dose journalière d'HCT ont été établis dans la littérature. Les limites de détection et de quantification ont été déterminées à 3 et à 5 pg/mg. Une prise unique n'est pas identifiable avec une limite de quantification à 5 pg/mg. Par ailleurs, il a été démontré qu'il n'y a pas de corrélation entre la posologie quotidienne et la concentration d'HCT retrouvée dans les cheveux. Les auteurs ont montré qu'après des expositions répétées, les concentrations pouvaient varier entre 12 et 1845 pg/mg. De plus, il a été montré que la couleur des cheveux n'a pas d'impact sur l'incorporation de l'HCT dans les cheveux. Dans cette étude, un cas de dopage a été reporté. Les cheveux ont été analysés et la mèche correspondant à la période du contrôle urinaire avait montré la présence d'HCT avec une concentration de 36 pg/mg. Ce résultat a été interprété en tant que prise répétée [7].

Les Fig. 1 et 2 présentent les chromatogrammes obtenus pour le cas 1 et pour le cas 2 (segment 1—2 cm).

Pour le cas 1, la méthyltestostérone a été mise en évidence à la concentration de 142 pg/mg alors que l'hydrochlorothiazide n'a pas été retrouvé, au seuil de 5 pg/mg (LOQ), dans les poils de bras. Ce résultat négatif ne contredit pas le résultat obtenu dans les urines car un résultat négatif dans les phanères peut s'expliquer par une mauvaise incorporation de la molécule mais aussi par le manque de sensibilité de la méthode analytique [6]. Selon Gheddar et al. [7], l'absence de réponse analytique peut correspondre à une prise unique. En effet, il a été démontré qu'une prise unique donnait un résultat inférieur à 5 pg/mg voire négatif.

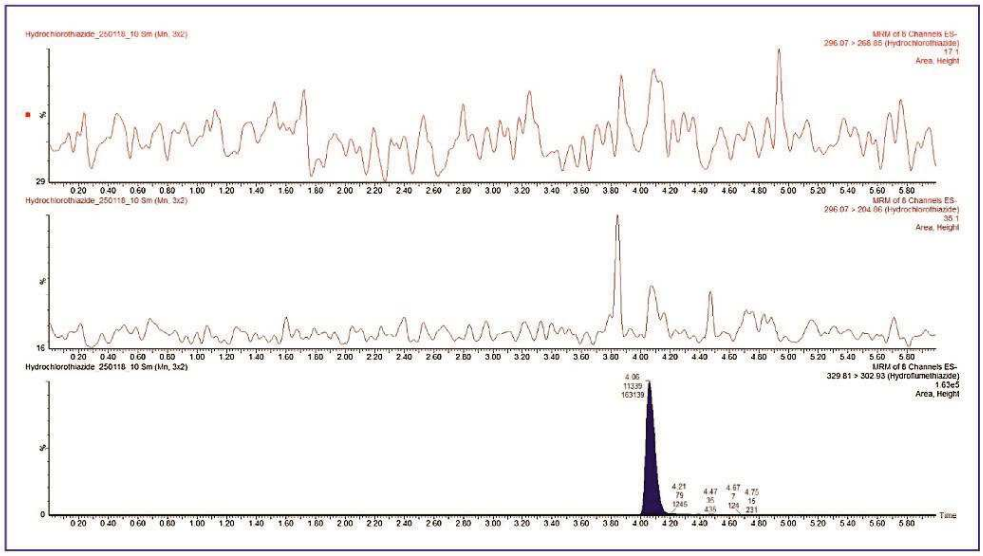


Figure 1. Chromatogramme du cas 1 - poils de bras.

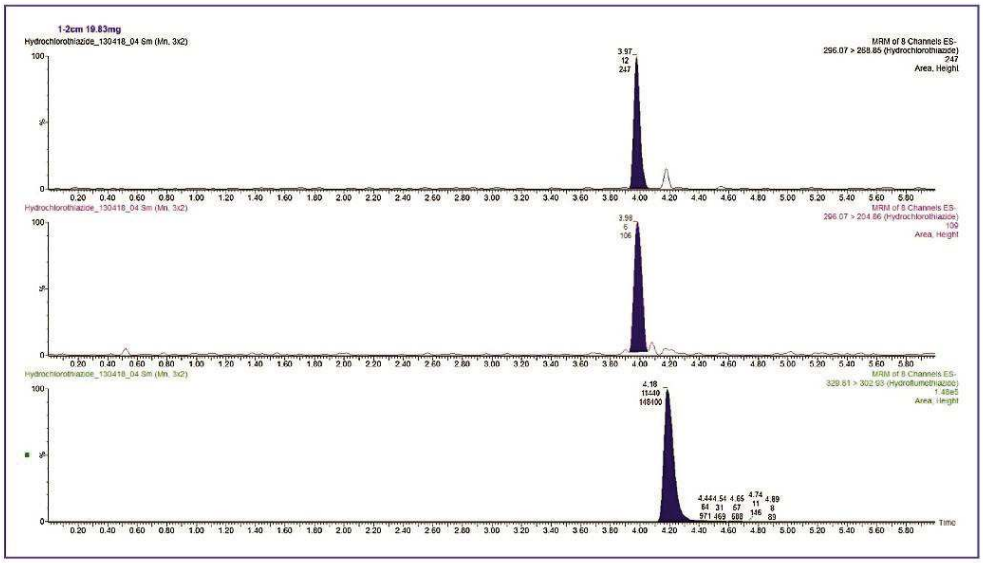


Figure 2. Chromatogramme du cas 2 - segment 1—2 cm.

De nombreuses études décrivent l'incorporation des xénobiotiques dans les poils axillaires, pubiens et les cheveux. Han et al ont montré que la méthamphétamine s'incorporait mieux dans les poils pubiens et axillaires que dans les cheveux [8]. Mais ce résultat varie d'un xénobiotique à l'autre. Mangin et Kintz ont montré que la morphine s'incorporait mieux dans les poils pubiens que dans les cheveux et dans les cheveux que dans les poils axillaires [9]. Néanmoins il n'existe pas d'étude concernant l'incorporation des xénobiotiques dans les poils de bras. La sueur, proposée pour expliquer l'incorporation des molécules qui ont un gradient de pH défavorable (comme les acides et donc les diurétiques), pourrait être un argument dans la discussion des résultats. En effet, les glandes sudoripares sont présentes sur l'ensemble du corps mais essentiellement sur le front, les aisselles, les paumes de mains [9,10]. Il est donc envisageable que l'hydrochlorothiazide se concentre moins dans les poils de bras par défaut de glandes sudoripares, en opposition aux cheveux.

Pour le cas 2, l'hydrochlorothiazide a été identifié et quantifié dans 4 segments consécutifs. Les segments 4—5 cm et 5—6 cm se sont révélés négatifs (cf. Tableau 1). Le segment 1—2 cm correspondant à la période du contrôle urinaire peut être interprété en tant que prise répétée selon les critères d'interprétation précédemment établis par Gheddar et al (> 5 pg/mg) [7]. De plus, 4 segments consécutifs sont positifs à l'hydrochlorothiazide. Ce profil peut donc correspondre à un profil d'expositions répétées.

Au vu des résultats, les analyses des phanères ont été favorable pour le cas 1 mais défavorables pour le cas 2. L'analyse des cheveux permet d'agrandir la fenêtre de détection des molécules mais également de différencier une prise unique d'une exposition répétée en segmentant une mèche. Dans le cas de l'analyse de produits dopants ou masquants comme l'hydrochlorothiazide dans les cheveux, il est nécessaire de travailler avec des techniques sensibles comme la spectrométrie de masse en tandem car les concentrations retrouvées sont de l'ordre du pg/mg. Cette analyse est un complément de l'analyse urinaire dans des cas de contre-expertise par exemple et pourrait être utile dans la lutte contre la fraude [11].

Tableau 1 Résultats des cheveux du cas 2.

Segments (cm)	Hydrochlorothiazide (pg/mg)
0—1	15
1—2	7
2—3	Compris entre 3 et 5
3—4	6
4—5	ND
5—6	ND

Récemment, Favretto et al. ont démontré que des compléments alimentaires contaminés par l'HCT pouvaient contaminer jusqu'à 10 ng/mL les urines, ce qui ne devrait pas se vérifier pour les cheveux [12].

Conclusion

Cette étude permet d'enrichir la littérature avec de nouveaux cas consécutifs à un résultat anormal dans les urines en hydrochlorothiazide. En accord avec le consensus de la

Society of Hair Testing, un résultat négatif dans les phanères, quel que soit leur localisation (cheveux, axillaires, pubiens, poils de bras,.) ne peut pas contredire un résultat positif dans les urines [13].

Déclaration de liens d'intérêts

Les auteurs déclarent ne pas avoir de liens d'intérêts.

Références

[1] World Anti-Doping Agency. 2017 Anti-Doping Testing Figures; 2018 https://www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures-report le 26 juillet 2018.

[2] Agence française de la lutte contre le dopage. Rapport d'activité 2017; 2018 https://www.afld.fr/doc_categorie/rapports-dactivite le 26 juillet 2018.

[3] World anti-doping agency. What is prohibited; 2018 https://www.wada-ama.org/en/content/what-is-prohibited/prohibited-at-all-times/diuretics-and-masking-agents le 27 février 2018.

[4] Baselt RC. Disposition of toxic drug and chemicals in man. 11th ed. Seal Beach, CA: Biomedical publications; 2017. p. 1049—50.

[5] Tribunal Arbitral du Sport. Cas/2009/A/1930; 2009 https://www.wada-ama.org/en/resources/legal/cas-wada-itf-v-gasquet le 6 août 2018.

[6] Kintz P. Hair analysis in forensic toxicology: an updated review with special focus on pittfalls. Curr Pharm Des 2017;23:5480—6.

[7] Gheddar L, Raul JS, Kintz P. First identification of a diuretic, hydrochlorothiazide, in hair: application to a doping case and interpretation of the results. Drug Test Anal 2018, http://dx.doi.org/10.1002/dta.2445.

[8] Eunyoung H, Wonkyung Y, Jaesin L, Yonghoon P, Eunmi K, Miae L, et al. Correlation of methamphetamine results and concentrations between head, axillary, and pubic hair. Forensic Sci Int 2005;147:21—4.

[9] Mangin P, Kintz P. Variability of opiates concentrations in human hair according to their anatomical origin: head, axillary and pubic regions. Forensic Sci Int 1993;63:77—83.

[10] Offidani C, Strano Rossi S, Chiarotti M. Drug distribution in the head, axillary and pubic hair of chronic addicts. Forensic Sci Int 1993;63:105—8.

[11] Kintz P. Quelle est la place pour les cheveux dans la lutte contre le dopage ? Ann Tox Anal 2000;12(1):49—55.

[12] Favretto D, Visentin S, Scrivano S, Roselli E, Mattiazzi F, Pertile R, et al. Multiple incidence of the prescription diuretic hydrochlorothiazide in compounded nutritional supplements. Drug Test Anal 2018, http://dx.doi.org/10.1002/dta.2499.

[13] Sachs H, Kintz P. Consensus of the society of hair testing on hair testing for doping agents. Forensic Sci Int 2000;107:3.

Article 21 : Hair testing for acetazolamide as an evidence of the use of a contaminated dietary supplement

Pascal Kintz, Laurie Gheddar, Alice Ameline, Jean-Sébastien Raul

Drug Testing and Analysis. 2021 ;1584-1588.

DOI: 10.1002/dta.3100

Hair testing for acetazolamide as an evidence of the use of a contaminated dietary supplement

Pascal Kintz[1,2] | **Laurie Gheddar**[2] | **Alice Ameline**[2] | **Jean-Sébastien Raul**[2]

[1]X-Pertise Consulting, Mittelhausbergen, France

[2]Laboratory of Toxicology, Institut de Medecine Légale, Strasbourg, France

Correspondence
Pascal Kintz, Laboratory of Toxicology, Institut de Medecine Légale, 11 Rue Humann, F-67000 Strasbourg, France.
Email: pascal.kintz@wanadoo.fr

In the recent years, it has been increasingly published that dietary supplements can be falsified.[1] They may contain no active ingredient or other active ingredient(s). In addition, the announced active ingredient can be associated to other active substances or present a variable dose. These supplements can produce numerous adverse events, ranging from absence of therapeutic efficacy up to serious health effects, which can involve deaths.[2,3] In sport, contaminated supplements can lead to adverse analytical findings, with complicated consequences for the athletes to justify their behaviour.[4,5] Although theoretically all doping agents can contaminate supplements, in most cases, anabolic steroids, stimulants, selective androgen receptor modulators (SARMs), clenbuterol or diuretics have been identified.[6–9] With respect to this last pharmacological class, hydrochlorothiazide[10,11] and furosemide[12,13] have been identified. It seems that acetazolamide, another diuretic, was never reported as a contaminant of a dietary supplement.

Acetazolamide is an effective carbonic anhydrase inhibitor, mainly used to treat glaucoma. It can be used by athletes to modify the excretion and metabolism of doping agents[14] but has also been promoted as prophylaxis for altitude sickness.[15] The list of prohibited substances and methods of doping, as established by the World Anti-Doping Agency (WADA), includes acetazolamide in the S5 class (diuretics and masking agents).[16] It is prohibited at all times. In 2019,[17] there were 13 adverse analytical findings (over a total of 4180) related to this compound. Acetazolamide does not seem to be popular in the S5 class, ranking 12 in the most detected diuretics, far after furosemide and hydrochlorothiazide.

The legal team of an athlete who returned a violation of the anti-doping rules contacted the laboratory after an adverse analytical finding due to acetazolamide. Urine acetazolamide concentration was less than 5 ng/mL. This was the first control of the athlete since 9 months, due to the COVID-19 pandemic. The athlete denied any doping violation but declared the daily use of capsules containing 135 mg of magnesium citrate and maleate. To demonstrate his honesty, the athlete submitted a hair specimen, which was collected about 6 weeks after urine collection. The hair was black in colour and 4 cm in length. The orientation root to tip was maintained. An unsealed box of 15 white capsules (from 200) and a sealed box but with another batch number were submitted by the athlete for analysis. According to the athlete, he was taking one capsule per day since, at least, the last 8 months. No other supplement or medication was reported. For legal reasons, the name and the manufacturer of this product cannot be shared. The capsules were bought in a local shop, specialized in dietary supplements. The white powder contained in the capsules was diluted with

methanol, and using liquid chromatography coupled to tandem mass spectrometry, acetazolamide was identified in the unsealed box at 42 ± 7 μg/capsule ($n = 6$). Acetazolamide was not identified in six capsules sampled from the sealed box. The batch numbers of these two sets of products were different, as well as the use-by dates.

Identification in hair of acetazolamide has been very recently published by Lo Faro et al.[18] The authors measured 2300 pg/mg in the hair of a subject treated daily with acetazolamide for systemic hypertension. However, diuretic detection in hair has been seldom reported. Gheddar et al. identified hydrochlorothiazide[19] and furosemide[20] in human hair. In both cases, a single-dose administration was undetectable, and concentrations in the range 12–1845 and 5–1110 pg/mg were measured in subjects under daily treatment with hydrochlorothiazide and furosemide, respectively. Finally, hair concentrations of 230, 1360 and 16,210 pg/mg for dorzolamide ($n = 3$) and of 290 and 6240 pg/mg for brinzolamide ($n = 2$) were published by Lo Faro et al.[18] in patients treated for ocular diseases or for systemic hypertension, respectively.

The hair of the athlete was first decontaminated with 2 × 5 mL dichloromethane for 2 min and then cut for 4 × 1 cm segments, thus covering the period of the control. Applying a standard growth rate of 1 cm/month, one can anticipate that the 1- to 2-cm segment corresponds to the time the urine was collected. The extracts were analysed by liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis on an Acquity class I ultra-high performance liquid chromatography coupled to a Xevo TQS micro tandem mass spectrometer (UPLC–MS/MS) from Waters (Milford, MA, USA), using a specific method for the detection of diuretics. Briefly, 30-mg cut hair were incubated for 10 h in 1 mL of pH 5.2 ammonium acetate buffer at 40°C, in presence of 1 ng of furosemide-d_5 used as internal standard (IS). Then, 5 mL of ethyl acetate were added. After agitation for 15 min, centrifugation and evaporation of the organic phase to dryness at room temperature, the residue was reconstituted in 30 μL of methanol. Chromatography was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 μm). The temperature of the column was maintained at 50°C in a thermostatically controlled oven. A gradient elution was used with 1-mM ammonium acetate in water (mobile phase A) and 1-mM ammonium acetate in methanol (mobile phase B). The flow rate of the mobile phase was 0.3 mL/min. The initial gradient was 95% phase A. At 5 min, 100% phase B was reached, which returned at 95% phase A at 7 min. The injection volume was 2 μL. Ionization was achieved using electrospray in the positive ionization mode for acetazolamide, while in the negative ionization mode for the IS. Although the choice of the IS furosemide-d_5 (in negative ionization) is semi-optimal in order to control the positive ionization conditions of the analyte acetazolamide, its major interest is the frequency of detection when the method is used as a screening test. The following apparatus conditions were found suitable for the analysis of acetazolamide and the IS: capillary voltage at 3 kV; source block temperature at 150°C; desolvation gas nitrogen heated at 500°C and delivered at a flow rate of 800 L/h. Cone voltage and collision energy were adjusted to optimize the signal for the two most abundant product ions of acetazolamide: m/z 223.0 > 180.9 (28 V and 12 eV) and 223.0 > 163.9 (28 V and 12 eV) and the most abundant product ion of IS m/z 334.1 > 206.0 (32 V and 20 eV). MassLynx 4.1 software was used for quantification.

Retention times were 4.53 and 5.16 min for acetazolamide and the IS, respectively. Linearity of the method was verified for acetazolamide concentrations ranging from 20 to 500 pg/mg, with a correlation coefficient of 0.9996. Quality control (QC) samples (50 pg/mg) were tested in duplicate in six independent experimental assays. The coefficient of variation for precision and accuracy was lower than 20%. The limit of detection and the lower limit of quantification were 10 and 20 pg/mg, respectively. These parameters are due to a weak signal-to-noise ratio of the confirmation transition (m/z 223.0 > 163.9). Under the used chromatographic conditions, no interference was observed between the analytes and extractable endogenous material present in hair. The matrix effect (<20%) was investigated with spiked 10 blank hair samples with acetazolamide at 50 pg/mg.

Despite the strong deny of the athlete, acetazolamide tested positive in the four hair segments with concentrations ranging from 24 to 31 pg/mg (Table 1). Although the measured concentrations are in the low pg/mg range, suitable chromatograms were obtained. The chromatogram obtained after the extraction of the proximal segment is presented in Figure 1. In addition, the chromatograms from a blank negative hair specimen (Figure 2) and from a hair sample spiked with acetazolamide at the limit of detection (Figure 3) are provided to thus prove the integrity and the very low sensitivity of the employed analytical method.

The interpretation of the measured concentrations is complicated as this is the first case involving hair test results for acetazolamide in a context of anti-doping rules violation. Due to the absence of controlled study presenting acetazolamide incorporation into hair, it is not possible to interpret the data in terms of dosage and frequency of consumption. However, the paper of Lo Faro et al.[18]is useful to demonstrate the different hair concentration of acetazolamide in chronic consumers assuming a pharmacological treatment and an athlete consuming a dietary supplement.

With respect to the concentrations already published for subjects under treatment with diuretics,[18–20] the measured concentrations are much lower. However, during two anti-doping violations, Gheddar et al. identified hydrochlorothiazide at 6–15 pg/mg in the head hair of a rugby player[21] and at 36 pg/mg in the head hair of a top athlete[20] who challenged his adverse analytical finding, demonstrating that in athletes, the diuretic concentrations can be much lower to the ones measured in clinical patients.

Given the four hair segments were positive, the standard interpretation would be to consider that the donor had repetitively

TABLE 1 Hair test results for acetazolamide

	Acetazolamide concentrations
Segment 0 to 1 cm	24 pg/mg
Segment 1 to 2 cm *	31 pg/mg
Segment 2 to 3 cm	30 pg/mg
Segment 3 to 4 cm	26 pg/mg

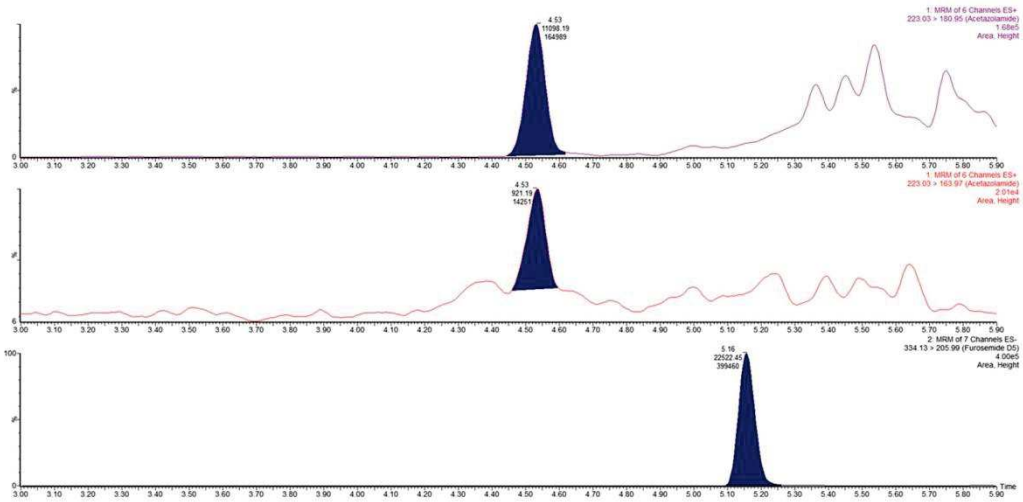


FIGURE 1 Chromatogram obtained after extraction of the 0- to 1-cm hair segment. From top to bottom: Two transitions of acetazolamide and one transition of the internal standard. Drug concentration was 24 pg/mg [Colour figure can be viewed at wileyonlinelibrary.com]

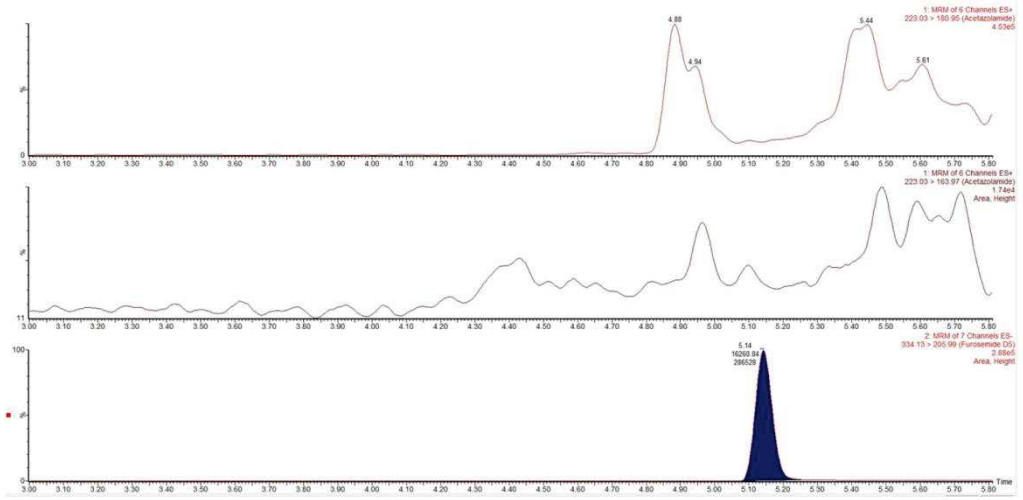


FIGURE 2 Chromatogram obtained after extraction of a blank hair segment. From top to bottom: Two transitions of acetazolamide and one transition of the internal standard [Colour figure can be viewed at wileyonlinelibrary.com]

consumed acetazolamide, thus confirming the anti-doping violation. However, it was obvious that the measured acetazolamide concentrations in the four different segments were close, if not identical at the precision of the method. This is a typical pattern observed in subjects under long-term pharmaceutical(s) therapy and has been the basis of using hair as a suitable matrix for therapeutic drug monitoring.[22] Similar drug concentrations along the hair shaft are highly indicative of an identical regimen of drug exposure, both in terms of dose and frequency. As the athlete was using on a daily basis contaminated capsules, it seems scientifically acceptable that the source of acetazolamide has been identified, which could account for his adverse analytical finding. The contamination scenario is supported by the positive hair tests and their specific pattern of drug exposure.

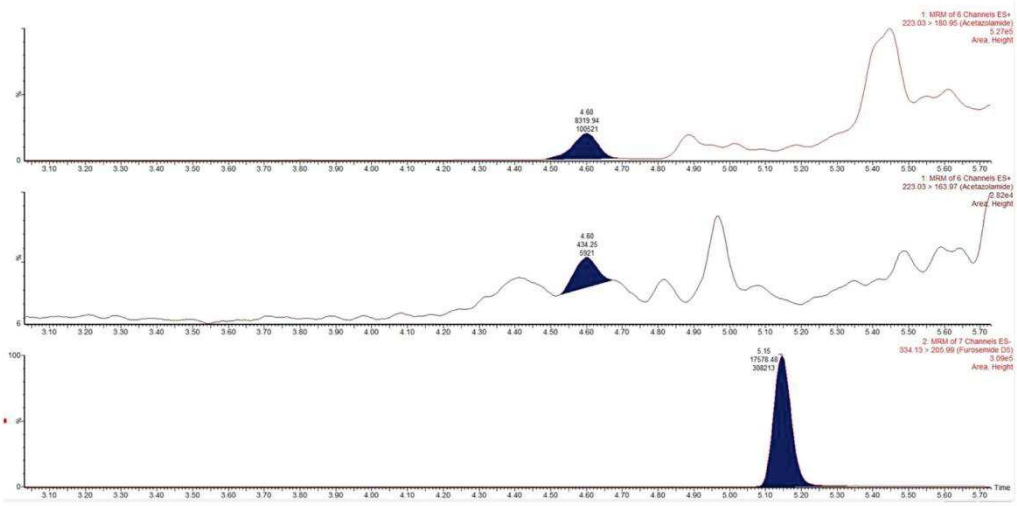


FIGURE 3 Chromatogram obtained after extraction of a blank hair segment spiked with acetazolamide at the limit of detection (10 pg/mg). From top to bottom: Two transitions of acetazolamide and one transition of the internal standard [Colour figure can be viewed at wileyonlinelibrary.com]

At this time, the final outcome of the case is not known. With respect to acetazolamide contamination of the capsules used daily by the athlete that has been proposed to account to the adverse analytical finding, the authors would rate this very likely, as it is supported by his hair test results. However, one cannot totally exclude that acetazolamide was not present in the sealed products and that the contaminated unsealed capsules could be manipulated by the athlete after the disclosure of the adverse analytical finding. In such a scenario, one would expect that the measured acetazolamide concentrations would be much higher (given what has been measured by Lo Faro et al.) and with a different drug pattern of distribution.

ORCID

Pascal Kintz https://orcid.org/0000-0002-4174-5362
Laurie Gheddar https://orcid.org/0000-0002-7804-3946
Alice Ameline https://orcid.org/0000-0002-1117-0887

REFERENCES

1. Fabresse N, Gheddar L, Kintz P, Larabi IA, Alvarez JC. Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders. *Forensic Sci Int*. 2021;322:110771. https://doi.org/10.1016/j.forsciint.2021.110771
2. Pena-Acevedo L, Zuluaga AF, Aristizabal-Solis A. A counterfeit multivitamin product inducing severe bleeding disorders in humans. *Clin Toxicol*. 2020. https://doi.org/10.1080/15563650.1703999
3. Venhuis BJ, Zwaagstra ME, Keizers PH, de Kaste D. Dose-to-dose variations with single packages of counterfeit medicines and adulterated dietary supplements as a potential source of false negatives and inaccurate health risk assessments. *J Pharm Biomed Anal*. 2014;89:158-165.
4. Walpurgis K, Thomas A, Geyer H, Mareck U, Thevis M. Dietary supplement and food contaminations and their implications for doping controls. *Foods*. 2020;9(8):1012. https://doi.org/10.3390/foods9081012
5. Martinez-Sanz JM, Sospedra I, Ortiz CM, Baladia E, Gil-Izquierdo A, Ortiz-Moncada R. Intended or unintended doping? A review of the presence of doping substances in dietary supplements used in sports. *Nutrients*. 2017;9(10):1093. https://doi.org/10.3390/nu9101093
6. Denham BE. Dietary supplements in the USA: problematic trends. *Public Health Nutr*. 2021;1-5. https://doi.org/10.1017/S1368980021000665
7. Al-Khdhra RS. The determination of common anabolic steroid and stimulants in nutritional supplements by HPLC-DAD and LC-MS. *J Chromatogr Sci*. 2020;58(4):355-361.
8. Geyer H, Schänzer W, Thevis M. Anabolic agents: recent strategies for their detection and protection from inadvertent doping. *Br J Sports Med*. 2014;48(10):820-826.
9. Pellegrini M, Rotolo MC, Busardò FP, Pacifici R, Pichini S. Non-allowed pharmacologically active substances in physical and sexual performance enhancing products. *Curr Neuropharmacol*. 2017;15:724-730.
10. Helmlin HJ, Mürner A, Steiner S, et al. Detection of the diuretic hydrochlorothiazide in a doping control urine sample as the result of a non-steroidal anti-inflammatory drug (NSAID) tablet contamination. *Forensic Sci Int*. 2016;267:166-172.
11. Favretto D, Visentin S, Scrvano S, et al. Multiple incidence of the prescription diuretic hydrochlorothiazide in compounded nutritional supplements. *Drug Test Anal*. 2019;11(3):512-522.
12. Zeng Y, Xu Y, Kee CL, Low MY, Ge X. Analysis of 40 weight loss compounds adultered in health supplements by liquid chromatography quadrupole linear ion trap mass spectrometry. *Drug Test Anal*. 2016;8(3-4):351-356.
13. Lu Y, Chen J, Chen B, Yao S. Simultaneous determination of 8 illegal synthetic drugs in antihypertensive dietary supplements by high performance liquid chromatography-mass spectrometry. *Se Pu*. 2009;27(1):44-49.

14. Delbeke FT, Debackere M. The influence of diuretics on the excretion and metabolism of doping agents—I. Mephentermine. *J Pharm Biomed Anal*. 1985;3(2):141-148.
15. Dawadi S. Acetazolamide use in ultra-runners at altitude: issues with doping. *Wilderness Environ Med*. 2018;29(1):140.
16. https://www.wada-ama.org/sites/default/files/resources/files/2021list_en.pdf. Accessed April 22, 2021.
17. https://www.wada-ama.org/sites/default/files/resources/files/2019_anti-doping_testing_figures_en.pdf. Accessed April 22, 2021.
18. Lo Faro AF, Tini A, Gottardi M, et al. Development and validation of a fast ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method for determining carbonic anhydrase inhibitors and their metabolites in urine and hair. *Drug Test Anal*. 2021. https://doi.org/10.1002/dta.3055
19. Gheddar L, Raul JS, Kintz P. First identification of a diuretic, hydrochlorothiazide, in hair: application to a doping case and interpretation of the results. *Drug Test Anal*. 2019;11(1):157-161.
20. Gheddar L, Batt MO, Raul JS, Kintz P. Identification of furosemide in hair in a post-mortem case by UHPLC-MS/MS with guidance on interpretation. *J Forensic Sci*. 2021;66(1):272-277.
21. Gheddar L, Raul JS, Kintz P. Recherche d'hydrochlorothiazide dans les phanères après deux contrôles antidopage. *Toxicol Anal Clin*. 2018;30:268-272.
22. Avataneo V, D'Avolio A, Cusato J, Cantù M, de Nicolo A. LC-MS application for therapeutic drug monitoring in alternative matrices. *J Pharm Biomed Anal*. 2019;166:40-51.

Article 22 : Identification of furosemide in hair in a post-mortem case by UHPLC-MS/MS with guidance on interpretation

Laurie Gheddar, Marie-Odile Batt, Jean-Sébastien Raul, Pascal Kintz

Journal of Forensic Sciences. 2021 ; 66 (1) : 272 - 277.

Received: 17 June 2020 | Revised: 3 September 2020 | Accepted: 9 September 2020

DOI: 10.1111/1556-4029.14585

JOURNAL OF FORENSIC SCIENCES

PAPER

Toxicology

Identification of furosemide in hair in a post-mortem case by UHPLC-MS/MS with guidance on interpretation

Laurie Gheddar MS[1] | Marie-Odile Batt MD[2] | Jean-Sébastien Raul PhD[1] | Pascal Kintz PhD[1]

[1]Institut de Médecine Légale, Strasbourg, France

[2]Centre hospitalier de Molsheim, Molsheim, France

Correspondence
Laurie Gheddar, Institut de Médecine Légale, 11 rue Humann, 67000 Strasbourg, France.
Email: l.gheddar@gmail.com

Abstract

Testing for drugs in hair raises several difficulties. Among them is the interpretation of the final concentration(s). In a post-mortem case, analyses revealed the presence of furosemide (12 ng/mL) in femoral blood, although it was not part of the victim's treatment. The prosecutor requested our laboratory to undertake an additional analysis in hair to obtain information about the use of furosemide. A specific method was therefore developed and validated to identify and quantify furosemide in hair by UHPLC-MS/MS. After decontamination of 30 mg of hair, incubation in acidic condition, extraction with ethyl acetate, the samples were analyzed by UHPLC-MS/MS. Furosemide was found in the victim's hair at 225 pg/mg. However, it was not possible to interpret this concentration due to the absence of data in the literature. Therefore, the authors performed a controlled study in two parts. In order to establish the basis of interpretation, several volunteers were tested (four after a single 20 mg administration and twenty-four under daily treatment). The first part indicated that a single dose is not detectable in hair using our method. The second part demonstrated concentrations ranging from 5 to 1110 pg/mg with no correlation between dosage and hair concentrations. The decedent's hair result was interpreted as repeated exposures. In the case of furosemide analysis, hair can provide information about its presence but cannot give information about dosage or frequency of use.

KEYWORDS

diuretic, forensic toxicology, furosemide, hair, UHPLC-MS/MS, ultra-high liquid chromatography-mass spectrometry-mass spectrometry

1 | INTRODUCTION

In toxicology, hair specimens are used to complement urine and blood analyses and provide more information about the pattern of consumption of a drug. Hair analysis can detect chronic drug exposure depending on the available hair length. For some drugs, a single dose may also be detected [1–3]. Given an average growth rate of 1 cm per month, each cm of hair strand allows estimating drug exposure for 1 month. For example, a hair length of 6 cm allows for drug exposure history during the last 6 months. Moreover, hair segmentation can give the pattern of exposure by providing the variation in concentration over time [4].

However, testing hair for drugs raises several difficulties. First, the variable chemical structure of each compound will influence their incorporation into hair, and therefore will impact their concentration. For instance, the presence of a nitrogen atom and the absence of acidic function are considered suitable for the incorporation of a molecule due to its binding ability with melanin [5]. Most acidic molecules do not incorporate well into hair. They do not ionize due to the hair's physiological pH of 5 and cannot bind

the negative charge of melanin [5]. In addition, acid drugs poorly pass through the follicle membrane, based on the pH gradient between blood (pH 7.4) and the acidic hair matrix (pH 5). External factors (e.g., UV) and cosmetic treatment can modify the concentration of molecules in hair. It can result in a loss of 50-80% of the initial hair concentrations of a molecule [6–8]. Finally, the interpretation of hair concentrations can be difficult due to the lack of information in the scientific literature. For example, an isolated hair concentration of drug is difficult to interpret. This is often the case for new psychoactive substances or drugs which are seldom tested in hair [9].

With respect to diuretics, Gheddar et al attempted to address these difficulties after testing for hydrochlorothiazide (HCT) in hair [10]. In this paper, the authors indicated that there was no correlation between measured concentration and hair color or dose. The authors also found that a single intake of 25 mg of HCT was not detected in hair and that a daily therapeutic treatment (6.25-12.5 or 25 mg/daily) can produce concentrations ranging from 12 to 1845 pg/mg.

This same approach was used to document the hair concentrations of letrozole after single and chronic administration. In this later study, the authors concluded that discrimination between single dosage and repeated exposure was possible [11].

In this paper, the authors applied the same strategy to a post-mortem case.

2 | CASE REPORT

A 56-year-old man (Mr. X) was found dead at home, in bed. Alcohol and drugs were found in the house, including lormetazepam and hydroxyzine. The deceased had a history of depression and alcoholism. No traumatic lesion was visible at the autopsy.

Standard toxicological analyses, including alcohol and screening of drugs, were performed by another laboratory. Hydroxyzine (5800 ng/mL), its metabolite, cetirizine (2805 ng/mL) and furosemide (12 ng/mL) were identified in femoral blood from the decedent.

Furosemide was not an expected drug, because it was not in Mr. X's treatment. The public prosecutor requested our laboratory to further explore this finding. For this, a bulk hair lock stored in an envelope was send to our laboratory. The hair of the deceased was 4 cm in length, and gray in color.

3 | MATERIAL AND METHODS

3.1 | Human hair testing

A literature search, using the keywords "hair" and "furosemide" did not produce any suitable citation. Therefore, based on the HCT study, which is the only literature reference for diuretic testing in hair [10], a controlled study was carried out in two parts. Each volunteer and regular user provided written consent after explanation of the study.

The first part consisted of the administration of 20 mg tablet of furosemide to four healthy volunteers (23-58 years old) (Table 1). Two hair strands (2-20 cm in length) were collected 1 month after administration. Only one hair strand was analyzed. The second hair strand is collected in case of a second opinion or in case of analytical issues. Only the proximal segment (2 cm) was analyzed for each volunteer. This proximal segment was expected to contain the spot of exposure, based on the assumed hair growth rate of 1 cm per month.

The second part consisted of the evaluation of furosemide concentration in regular users. Two hair strands were collected by one of the authors in a local hospital. Twenty-four patients (males and females aged 50-80 years old) under daily medical treatment with furosemide (20-500 mg/daily) participated in the study. The duration of the patient treatments ranged from 1 month to 7 years. Twenty-six hair specimens (24 head hair and 2 chest hair) were tested. The hair specimens were ranging from 1 to 22 cm in length, and white, gray, brown, or black in color. One patient had colored hair (Table 2). Only the proximal segment was analyzed and the tested hair length was either 1 cm (only for patients who started their treatment 1 month ago) or 2 cm. All samples were received in envelopes and stored at ambient temperature.

3.2 | Chemicals and reagents

Furosemide and furosemide-D5 were obtained from Cerilliant (Round Rock, USA) and Santa Cruz Biotechnology (Dallas, USA) respectively. Ammonium acetate and ethyl acetate (HPLC grade) were purchased from Merck (Darmstadt, Germany) and methanol (HPLC MS Grade, MeOH) from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Acetic acid was purchased from Prolabo (Fontenay-sous-Bois, France).

TABLE 1 Hair test results – part 1-single dose

Head hair specimens	Sex	Dose	Hair color	Concentration (pg/mg)	Duration of treatment
1	Male	Single dose 20 mg	Blond	ND	/
2	Female	Single dose 20 mg	Brown	ND	/
3	Female	Single dose 20 mg	Blond	ND	/
4	Female	Single dose 20 mg	Blond	ND	/

Abbreviation: ND, No detected.

TABLE 2 Hair test results – part 2

Head hair specimens	Sex	Dose	Hair color	Concentration (pg/mg)	Duration of treatment
5	Female	20 mg/daily	Gray	30	/
6	Male	20 mg/daily	White	25	8 months
7	Male	20 mg/daily	Gray	60	1 year
8	Female	20 mg/daily	Brown-gray	275	2 years
9	Female	20 mg/daily	Red coloring (cosmetic treatment)	ND	1 month
10	Female	20 mg/daily	Dark-gray	110	1 month
11	Female	20 mg/daily	Gray	195	2 years
12	Male	40 mg/daily	White	5	/
13	Male	40 mg/daily	Brown	40	7 years
14	Male	40 mg/daily	Brown	10	1 month
15	Male	40 mg/daily	Brown	115	2 years
16 head hair	Male	40 mg/daily	Brown	955	8 months
16 chest hair	Male	40 mg/daily	Brown	670	8 months
17	Male	40 mg/daily	Gray	1060	7 years
18	Female	40 mg/daily	White	1110	5 years
19	Female	40 mg/daily	Brown	115	1 month
20	Female	40 mg/daily	Dark	65	2 years
21	Male	40 mg/daily	Dark-gray	430	2 years
22 head hair	Male	40 mg/daily	Gray	250	2 years
22 chest hair	Male	40 mg/daily	Dark	535	2 years
23	Female	40 mg/daily	Brown	40	5 years
24	Female	60 mg/daily	Brown	50	/
25	Male	60 mg/daily	Gray	25	1 year
26	Female	80 mg/daily	Gray	185	/
27	Male	250 mg/daily	Dark-gray	915	/
28	Female	500 mg/daily	Brown	670	1 month

Abbreviation: ND, No detected.

The standard solutions, prepared in MeOH, were stored at 4°C. Blank hair for method validation was obtained from laboratory staff.

3.3 | Furosemide analysis by UHPLC-MS/MS

Hair samples were decontaminated twice with 5 mL of dichloromethane for 2 min at room temperature and dried. The wash solutions were mixed, evaporated to dryness, reconstituted in 30 µL of MeOH, and tested for furosemide. The decedent's hair was not segmented, because hair specimens were received at the laboratory as bulk material. The hair was cut into very short segments (<1 mm) and 30 mg were incubated in 1 mL ammonium acetate buffer pH 5.2 for 15 h at 50°C, in the presence of 20 ng of furosemide-D5, used as internal standard. After incubation, 5 mL of ethyl acetate was added. After agitation for 15 min and centrifugation (15 min, 3000 rpm), the organic phase was collected and evaporated. The dry residue was dissolved in 30 µL of MeOH, and 2 µL was injected into the UHPLC-system.

Chromatographic separations were achieved with a liquid chromatography Waters Acquity UPLC™ system (Waters corporation, Milford, MA, USA) using an Acquity UPLC HSS C18 column (150 mm × 2.1 mm, i.d. 1.8 µm particle size). The column temperature was set to 50°C, and the flow rate at 0.3 mL/min. The mobile phase consisted of 1.0 mmol/L ammonium acetate in water (eluent A) and 1.0 mmol/L ammonium acetate in MeOH (eluent B). Analysis time was 6.0 min with the following gradient pattern (eluent A): 0 min, 95%; 0.10 min, 95%; 3.00 min, 2.0%; 4.00 min, 2.0%; 4.10 min, 95.0%; 6.00 min, 95%. Furosemide and furosemide-D5 were eluted in 2.91 and 2.90 min respectively.

Detection was performed using a triple quadrupole (XEVO™ TQD, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionization source (ESI) and used in the negative mode (ES-). Nitrogen was used as the drying gas as well as the nebulizing gas. The desolvation gas flow was set to 800 L/h and the desolvation temperature was 500°C. The source temperature was 150°C. The capillary voltage was set at 3.0 kV.

For the detection, cone voltage and collision energy were adjusted to optimize the signal of the 2 most abundant product ions

of furosemide: m/z 329.1 > 204.9 and 329.1 > 285.0 and for furosemide-D5: m/z 334.1 > 290.0. MassLynx 4.1 software was used for quantification.

Ion ratio and retention time were verified for all samples with an acceptable precision inferior to 20% and 2% respectively with compared to a reference standard.

3.4 | Method validation

Validation recommendations of the Society of Hair Testing were followed (although not standards), including precision, matrix effect, determination of LOD and LOQ, linearity and interferences [12]. A seven-point calibration curve (n = 3) was obtained by spiking blank hair at 5, 10, 20, 50, 200, 500 and 2000 pg/mg. The limit of quantification (LOQ) was defined as the first point of calibration. The limit of detection (LOD) was the lowest concentration of analyte that could be detected with signal to noise ratio superior than 3. The repeatability, matrix effect and interferences were evaluated. For the repeatability, two calibration points at 10 and 500 pg/mg were evaluated 6 times. With respect to matrix effect, 15 different hair blank specimens were extracted and 50 µL of standard solution of furosemide (10 ng/ml) was added at the end of extraction. These specimens were compared to 50 µl of standard solution of furosemide in MeOH (10 ng/ml). For the evaluation of interferences, 10 true negative hair samples collected among laboratory staff were analyzed.

4 | RESULTS AND DISCUSSION

The acidic extraction was chosen based on the Deventer study, in which furosemide was extracted under acidic conditions in urine [13].

Linearity was observed for furosemide concentrations ranging from 5 to 2000 pg/mg (r^2 > 0.997) with a LOQ of 5 pg/mg (precision at 18.7%). The LOD was 3 pg/mg with acceptable ion ratios. QC samples (10 pg/mg and 500 pg/mg), analyzed in duplicate in six independent experimental assays were used for determination a coefficient of variation for precision. These CVs were lower than 20% (15.9% for QC at 10 pg/mg and 5.6% for QC at 500 pg/mg). The matrix effect was found acceptable, being lower than 20%. 10 true negative hair samples collected do not show a positive response for furosemide.

The result of the analysis of the decedent's hair showed a positive response for furosemide at 225 pg/mg. The wash solution was analyzed and was found negative for furosemide. Figure 1 shows the chromatogram obtained after the extraction of the 4 cm hair. This hair concentration was difficult to interpret as there is no information and no data about furosemide testing in hair reported in the scientific literature. In order to interpret this concentration, the authors decided to follow the strategy already described for HCT [10] and a controlled study was carried out in two parts.

Tables 1 and 2 present the results of these two studies, including dosage, hair color, sex, duration of treatment, and concentration of furosemide. All samples from the first part of the study (single dosage; subjects 1-4) produced a negative response with a LOD at

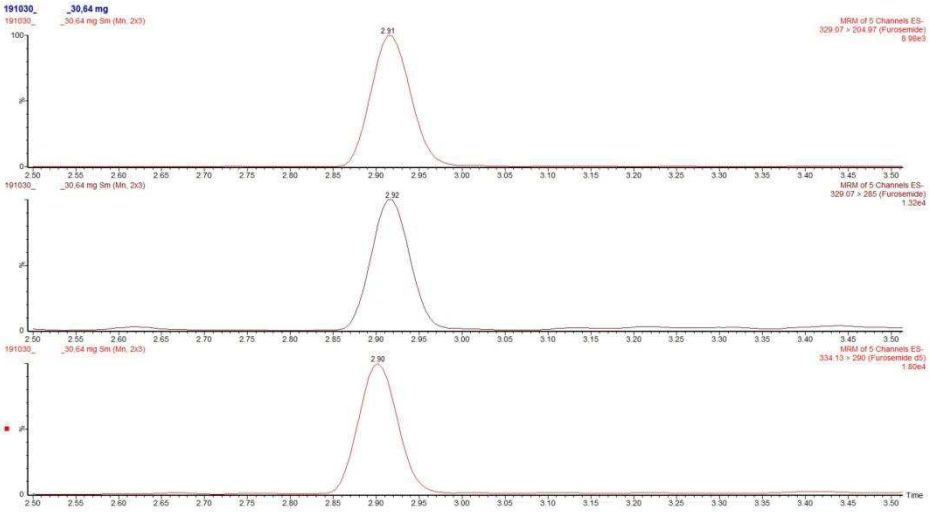


FIGURE 1 Chromatogram of hair case (from top to bottom: 2 transitions of furosemide, 1 transition of furosemide-D5). Hair concentration was 225 pg/mg

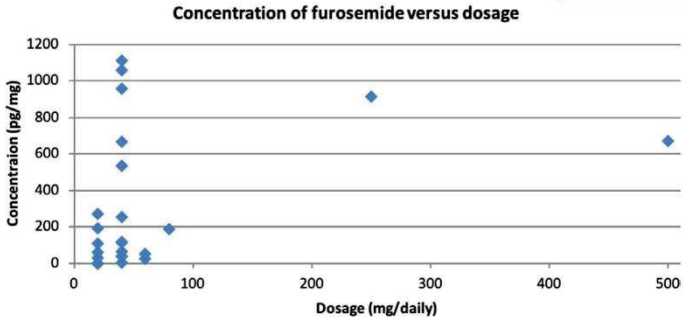


FIGURE 2 Concentration of furosemide in hair versus dosage

3 pg/mg. A single dose 20 mg of furosemide does not seem to give a detectable response using this method.

The second part made it possible to evaluate the concentrations of furosemide in the hair of the patients (subjects 4-28) under daily treatment of the drug. With the exception of patient 9, all subjects were positive for furosemide. The negative finding in patient 9 may be due to the cosmetic treatment of their hair. Furosemide concentrations ranged from 5 to 1110 pg/mg. The authors observed a dispersion (Figure 2) in concentrations when associated with treatment dosage. For example, a treatment of 20, 40 and 60 mg/day can give similar concentrations in hair. Furosemide seems to not deposit uniformly in hair. It can be explained by the lack of binding of the drug to the negative charge of melanin at hair's physiological pH of 5 [5]. Moreover, there does not seem to be a correlation between hair color and hair concentration. Others have reported no correlation between hair color (pigmented and unpigmented) and hair concentration for acidic molecules such as phenobarbital [14]. In addition, there was no apparent variation due to sex. For women, the concentrations ranged from 32 to 1110 pg/mg while men ranged from 5 to 1060 pg/mg. Welch's unequal variances t-test was performed and results indicated no difference between the two groups (male and female) with *P*-value at 0.34.

Moreover, for two patients under daily treatment, chest hair specimens were also collected in addition to head hair (specimens 16 and 22). In the case of there is no head hair, the analysis of other hair such as chest hair might be of interest. With respect to the comparison between chest hair and head hair, the authors cannot provide any conclusion because in one case the concentration of furosemide was higher in chest hair than in head hair and for the second case the results show the opposite situation.

Thanks to this study, the authors were able to better interpret the findings from the analysis of the decedent's hair. The concentration found in the hair (225 pg/mg) seems to be in accordance with repeated exposures to furosemide. However, it was not possible to determine neither the dosage nor the frequency of consumption.

Finally, it has been reported by various authors [15–18] that sweat can contaminate the hair and could therefore cause an artificial increase in the measured concentration. This was not considered important by the authors for several reasons: 1. a decontamination procedure was used; 2. the wash solution was negative for furosemide; and 3. acid drugs are seldom excreted in sweat [19].

5 | CONCLUSION

This article reports the first identification and quantification of a loop diuretic, furosemide, in hair. In this case, analysis of hair enabled the authors to conclude repetitive drug use by the decedent without providing an estimate of the dosage and its frequency. Some diuretics are difficult to interpret in hair specimens, particularly because they are weakly incorporated in hair due to their acidic function. However, hair analysis for diuretics seems to enable the discrimination between a single dose and repeated exposures, which can find application in doping control.

ORCID

Laurie Gheddar https://orcid.org/0000-0002-7804-3946
Jean-Sébastien Raul https://orcid.org/0000-0002-9991-3795
Pascal Kintz https://orcid.org/0000-0002-4174-5362

REFERENCES

1. Freni F, Bianco S, Vignali C, Groppi A, Moretti M, Osculati AMM, et al. A multi-analyte LC-MS/MS method for screening and quantification of 16 synthetic cathinones in hair: Application to post-mortem cases. Forensic Sci Int. 2019;298:115–20. https://doi.org/10.1016/j.forsciint.2019.02.036.
2. Kuwayama K, Nariai M, Miyaguchi H, Iwata YT, Kanamori T, Tsujikawa K, et al. Estimation of day of death using micro-segmental hair analysis based on drug use history: A case of lidocaine use as a marker. Int J Legal Med. 2019;133(1):117–22. https://doi.org/10.1007/s00414-018-1939-9.
3. Günther KN, Johansen SS, Nielsen MKK, Wicktor P, Banner J, Linnet K. Post-mortem quetiapine concentrations in hair segments of psychiatric patients – correlation between hair concentration, dose and concentration in blood. Forensic Sci Int. 2018;285:58–64. https://doi.org/10.1016/j.forsciint.2018.01.020.
4. Ramírez Fernández MdM, Wille SMR, Jankowski D, Hill V, Samyn N. Development of an UPLC-MS/MS method for the analysis of 16 synthetic opioids in segmented hair, and evaluation of the polydrug

history in fentanyl analogue users. Forensic Sci Int. 2020;307:110–37. https://doi.org/10.1016/j.forsciint.2019.110137.
5. Khajuria H, Nayak BP, Badiye A. Toxicological hair analysis: Pre-analytical, analytical and interpretive aspects. Med Sci Law. 2018;58(3):137–46. https://doi.org/10.1177/0025802418768305.
6. Favretto D, Tucci M, Monaldi A, Ferrara SD, Miolo G. A study on photodegradation of methadone, EDDP, and other drugs of abuse in hair exposed to controlled UVB radiation. Drug Test Anal. 2014;6(Suppl 1):78–84. https://doi.org/10.1002/dta.1607.
7. Van Elsué N, Yegles M. Influence of cosmetic hair treatments on cannabinoids in hair: Bleaching, perming and permanent coloring. Forensic Sci Int. 2019;297:270–6. https://doi.org/10.1016/j.forsciint.2019.02.030.
8. Eisenbeiss L, Binz TM, Baumgartner MR, Steuer AE, Kraemer T. A possible new oxidation marker for hair adulteration: Detection of PTeCA (1H-pyrrole-2,3,4,5-tetracarboxylic acid) in bleached hair. Drug Test Anal. 2020;12(2):230–8. https://doi.org/10.1002/dta.2713.
9. Ameline A, Kintz P, Blettner C, Bayle E, Raul JS. Identification of 25I-NBOMe in two intoxications cases with severe hallucinations. Tox Anal Clin. 2017;29:117–22. https://doi.org/10.1016/j.toxac.2016.11.004.
10. Gheddar L, Raul JS, Kintz P. First identification of a diuretic, hydrochlorothiazide, in hair: Application to a doping case and interpretation of the results. Drug Test Anal. 2019;11(1):157–61. https://doi.org/10.1002/dta.2445.
11. Favretto D, Snenghi R, Pertile R, El Mazloum R, Tucci M, Visentin S, et al. Hair analysis to discriminate voluntary doping vs inadvertent ingestion of the aromatase inhibitor letrozole. Drug Test Anal. 2019;11(6):762–71. https://doi.org/10.1002/dta.2555.
12. Cooper GAA, Kronstrand R, Kintz P. Society of hair testing guidelines for drug testing in hair. Forensic Sci Int. 2012;218:20–4. https://doi.org/10.1016/j.forsciint.2011.10.024.
13. Deventer K, Delbeke T, Roels K, Van Eenoo P. Screening for 18 diuretics and probenecid in doping analysis by liquid chromatography-tandem mass spectrometry. Biomed Chromatogr. 2002;16:529–35. https://doi.org/10.1002/bmc.201.
14. Gygi SP, Wilkins DG, Rollins DE. A comparison of phenobarbital and codeine incorporation into pigmented and nonpigmented rat hair. J Pharm Sci. 1997;86(2):209–14. https://doi.org/10.1021/js960268h.
15. Kintz P. Value of hair analysis in post-mortem toxicology. Forensic Sci Int. 2004;142(2–3):127–34. https://doi.org/10.1016/j.forsciint.2004.02027.
16. Kintz P. Segmental hair analysis can demonstrate external contamination in post-mortem cases. Forensic Sci Int. 2012;215(1–3):73–6. https://doi.org/10.1016/j.forsciint.2011.01.041.
17. Couper FJ, McIntyre IM, Drummer OH. Detection of antidepressant and antipsychotic drugs in post-mortem human scalp hair. J Forensic Sci. 1995;40(1):87–90.
18. Wiart JF, Hakim F, Andry A, Eiden C, Drevin G, Lelievre B, et al. Pitfalls of toxicological investigations in hair, bones, and nails in extensively decomposed bodies: illustration with two cases. Int J Legal Med. 2020;134:1339–44. https://doi.org/10.1007/s00414-020-02267-3.
19. Kintz P, Tracqui A, Jamey C, Mangin P. Detection of codeine and phenobarbital in sweat collected with a sweat patch. J Anal Toxicol. 1996;20(3):197–201. https://doi.org/10.1093/jat/20.3.197.

How to cite this article: Gheddar L, Batt MO, Raul JS, Kintz P. Identification of furosemide in hair in a post-mortem case by UHPLC-MS/MS with guidance on interpretation. *J Forensic Sci.* 2020;00:1–6. https://doi.org/10.1111/1556-4029.14585

Conclusion

Les substances de la performance et notamment les agents anabolisants, qui sont les produits les plus appréciés, représentent une véritable menace pour la santé publique en France et à travers le monde.

En effet, l'utilisation des produits de la performance ne concerne plus uniquement les sportifs de haut niveau mais également les amateurs qui souhaitent améliorer leur apparence physique.

Au-delà du caractère dopant lié à ces molécules, il existe un réel besoin analytique dans le domaine clinique et médico-légal.

En effet, ces produits provoquent, à haut dosage et sur le long terme, des problèmes de santé physique et psychique qui ne sont pas toujours connus ou maitrisés.

L'émergence des nouveaux agents anabolisants, les SARMs, représente un autre problème de santé publique puisqu'il est encouragé par une distribution facilitée par Internet. L'usage de ces substances peut avoir des conséquences néfastes sur la carrière des sportifs mais surtout sur la santé des consommateurs comme décrit dans ce manuscrit.

Face à l'émergence de nouveaux produits anabolisants, il est important de mettre en place des méthodes analytiques ciblées dans un laboratoire de toxicologie.

L'analyse des phanères en comparaison avec l'analyse des matrices plus traditionnelles a permis de mettre en évidence des consommations chroniques d'agents anabolisants et de diurétiques, et de dévoiler des problèmes de contamination environnementale ou des problèmes de contamination dû à la consommation de suppléments alimentaires adultérés par des agents anabolisants.

L'analyse des phanères a certaines limites qui peuvent être en partie résolues en utilisant d'autres poils du corps, des ongles mais également en réalisant de la segmentation.

L'objectif principal de ce travail était de caractériser la consommation de trois familles de produits dans les phanères. En 2018, il n'existait pas de méthode analytique dédiée à l'identification de ces substances à Strasbourg. Au fur et à mesure de la mise en place de ces méthodes au sein du laboratoire de toxicologie, les demandes d'analyse pour ces substances ont augmenté et le laboratoire a reçu de multiples demandes d'analyse en France et à l'étranger.

La recherche des produits de la performance dans des cas de décès, de violence non expliquée ou dans des cas de dopage avec suspicion de contamination a intéressé les médecins légistes dans la recherche de la cause de la mort, les cliniciens dans la recherche d'une cause d'intoxication ou d'un

problème d'hypogonadisme ou encore les sportifs dans la recherche d'une possible source de contamination.
Les SARMs sont les nouveaux produits en vogue et sont mis à l'avant de la scène avec des résultats anormaux pour l'ostarine et le S23 lors des Jeux Olympiques de Tokyo en 2021.
Il est donc important de continuer à incrémenter la librairie MS des substances de la performance dès lors qu'un nouveau produit est commercialisé ou détecté sur le marché noir.

Ces trois années d'activité et de recherche ont permis la rédaction de 22 publications nationales et internationales.
La singularité de mes recherches m'a permis de travailler en collaboration avec de nombreuses équipes en France : l'IML de Marseille, le laboratoire ToxGen à Bordeaux, le centre d'addictovigilance de Bordeaux, le laboratoire Barla à Nice, le CHU de Garches, le CH de Marmande et l'Université de Toulouse.

Articles publiés ou en cours d'évaluation

Article 1 : **L. Gheddar**, A. Ameline, J-S. Raul, P. Kintz. Designer anabolic steroids: a challenge for toxicologists. *Toxicol Anal Clin*. 2019;31:293-297.

Article 2 : N. Fabresse, **L. Gheddar**, P. Kintz, A. Knapp, I. A. Larabi, J-C Alvarez. Analysis of pharmaceutical products and dietary supplements seized from the black market among bodybuilders. *Forensic Sci Int*. 2021;doi: 10.1016/j.forsciint.2021.110771.

Article 3 : P. Kintz, **L. Gheddar**, A. Ameline, N. Arbouche, J-S. Raul. Hair testing for doping agents. What is known and what remains to do. *Drug Test Anal*. 2020;12:316-322.

Article 4 : **L. Gheddar**, J-S. Raul, P. Kintz. Testing for stanozolol, using UHPLC-MS/MS and confirmation by UPLC-q-TOF-MS, in hair specimens collected from five different anatomical regions. *J Anal Toxicol*. 2020;44(8):834-839.

Article 5 : P. Kintz, **L. Gheddar**, J-S. Raul. Simultaneous testing for anabolic steroids in human hair specimens collected from various anatomic location has several advantages when compared with the standard head hair analysis. *Drug Test Anal*. 2021;13(7):1444-1451.

Article 6 : P. Kintz, **L. Gheddar**, J-S. Raul. Testing for anabolic steroids in human nail clippings. *J Forensic Sciences*. 2021;66(4):1577-1582.

Article 7 : P. Kintz, **L. Gheddar**, A. Blanchot, A. Ameline, J-S. Raul. In a case of death involving steroids, hair testing is more informative than blood or urine testing. *J Anal Toxicol*. 2021;45(8):829-834.

Article 8 : **L. Gheddar**, A-L. Pélissier, J. Desfeux, F. Niort, J-S. Raul, P. Kintz. Testing for trenbolone, an anabolic steroid, in biological fluids and head hair in a postmortem case. *J Anal Toxicol*. 2021.

Article 9 : F. Aknouche, **L. Gheddar**, A. Kernalléguen, C. Maruejouls, P. Kintz. Anabolic steroids and extreme violence : a case of murder after chronic intake and under acute influence of metandienone and trenbolone. *Int J Legal Med.* 2021;135(4):1449-1453.

Article 10 : P. Kintz, **L. Gheddar.** Evidence of use of drostanolone, an anabolic steroid, at the time the subject committed a murder: place of hair analysis. *Toxicol Anal Clin*. 2021;33:22-225.

Article 11 : **L. Gheddar**, J-S. Raul, P. Kintz. Forensic investigations in a case of aggressive behavior of three dogs : identification of dietary supplements contamination by metandienone and confirmation by hair tests. *Forensic Sci Int: Animals and Environments*. 2021; doi:10.1016/j.fsiae.2021.100022.

Article 12 : P. Kintz, **L. Gheddar**, A. Ameline, V. Dumestre-Toulet, M. Verschoore, J. Comte, J-S. Raul. Complete post-mortem investigations in a death involving clenbuterol after long-term abuse. *J Anal Toxicol*. 2019;43(8):660-665.

Article 13 : **L. Gheddar**, J-S. Raul, P. Kintz. Development and validation of SARMs and metabolic modulators screening in hair using UHPLC-MS/MS : application to a doping case and first identification of S23 in authentic human hair. Soumis le 16 juillet 2021 dans *J Chrom B.*

Article 14 : P. Kintz, **L. Gheddar**, A. Ameline, J-S. Raul. Perspective in evaluating selective androgen receptor modulators in human hair: a short communication. *Ther Drug Monit*. 2021;43:298-300.

Article 15 : P. Kintz, **L. Gheddar**, A. Ameline, J-S. Raul. Identification of S22 (ostarine) in human nails and hair using LC-HRMS. Application to two authentic cases. *Drug Test Anal*. 2020;12:1508-1513.

Article 16 : P. Kintz, A. Ameline, **L. Gheddar**, J-S. Raul. Testing for GW501516 (cardarine) in human hair using LC-MS/MS and confirmation by LC-HRMS. *Drug Test Anal*. 2020;12(7):980-986.

Article 17 : P. Kintz, **L. Gheddar**, C. Paradis, M. Chinellato, A. Ameline, J-S. Raul, M. Oliva-Labadie. PPAR-δ and SARM abuse : clinical, analytical and biological data in a case involving a poisonous combination of GW1516 (cardarine) and MK2866 (ostarine). *Toxics*. 2021;9(10):251. doi:10.3390/toxics9100251

Article 18 : A. Ameline, **L. Gheddar**, J-S. Raul, P. Kintz. In vitro characterization of S-23 metabolites produced by human liver microsomes, and subsequent application to urine in a controlled study: first data about this SARM. Soumis le 9 juillet 2021 dans *Drug Test Anal.*

Article 19 : **L. Gheddar**, J-S. Raul, P. Kintz. First identification of a diuretic, hydrochlorothiazide, in hair: application to a doping case and interpretation of the results. *Drug Test Anal*. 2019;11:157-161.

Article 20 : **L. Gheddar**, J-S. Raul, P. Kintz. Recherche d'hydrochlorothiazide dans les phanères après deux contrôles antidopage. *Toxicol Anal Clin*. 2018;30:268-272.

Article 21 : P. Kintz, **L. Gheddar**, A. Ameline, J-S. Raul. Hair testing for acetazolamide as an evidence of the use of a contaminated dietary supplement. *Drug Test Anal*. 2021;1584-1588.

Article 22 : **L. Gheddar**, M-O. Batt, J-S. Raul, P. Kintz. Identification of furosemide in hair in a post-mortem case by UHPLC-MS/MS with guidance on interpretation. *J Forensic Sci*. 2021;66(1) :272-277.

Bibliographie

AFLD. Rapport d'activité 2019.

AFLD. Rapport d'activité 2020.

AMA. Anti-Doping Testing figures report. AMA. 2019.

AMA. Liste des substances et méthodes interdites. 2021.

Ameline A. Aspects analytiques, cliniques et médico-judiciaires des nouvelles substances psychoactives. 2019.

Anderson PD, Bokor G. Forensic aspects of drug-induced violence. *J Pharm Pract*. 2012;25:41–49.

Birger M, Swartz M, Cohen D, Alesh Y, Grishpan C, Kooteir M. Aggression: the testosterone-serotonin link. *Isr Med Assoc*. 2003;5:653–658.

CAS AMA. 2009/A/1930 WADA v. ITF and Gasquet. AMA.

Christiansen AR, Lipshultz LI, Hotaling JM, Pastuszak AW. Selective androgen receptor modulators: the future of androgen therapy? *Transl Androl Urol*. 2020;9:135-148.

Dalton JT, Mukherjee A, Zhu Z, Kirkovsky L, Miller DD. Discovery of nonsteroidal androgens. *Biochem Biophys Res Commun*. 1998;224:1-4.

Dufayet L, Gorgiard C, Vayssette F, Barbet JP, Hoizey G, Ludes B. Death of an apprentice bodybuilder following 2,4-dinitrophenol and clenbuterol intake. *Int J Legal Med*. 2020;134:1003–1006.

Friedman O, Arad E, Ben Amotz O. Body Builder's Nightmare: Black Market Steroid Injection Gone Wrong: a Case Report. *Plast Reconstr Surg Glob Open*. 2016;4(9):e1040.

Gaillard Y, Vayssete F, Balland A, Pépin G. Gas chromatography mass spectrometric determination of anabolic steroids and their esters in hair. Application in doping control and meat quality control. *J Chrom B*. 1999;735:189-205.

Gaillard Y, Vayssette F, Pépin G. Compared interest between hair analysis and urinalysis in doping controls. Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists. *Forensic Sci Int* 2000;107:361-379.

Ganson KT, Cadet TJ. Exploring anabolic-androgenic steroid use and teen dating violence among adolescent males. *Subst Use Misuse*. 2019;54(5):779-786.

Geyer H, Parr MK, Koehler K, Mareck U, Schänzer W, Thevis M. Nutritional supplements cross-contaminated and faked with doping substances, *J Mass Spectrom*. 2008;48:892-902.

Hernández-Guerra AI, Tapia J, Menéndez-Quintanal LM, Lucena JS. Sudden cardiac death in anabolic androgenic steroids abuse: case report and literature review. *Forensic Sci Res.*2019;4(3):267-273.

Khoodoruth MAS, Khan AA. Anabolic steroids-induced delirium. *Medicine*.2020;99(33):e21639.

Kindlundh AMS, Lindblom J, Bergström L, Nyberg F. The anabolic-androgenic steroid nandrolone induces alterations in the density of serotoninergic HHT1B and 5HT2 receptors in the male rat brain. *Neuroscience*. 2003;119:113–120.

Kronstrand R, Scott K. Drug Incorporation into Hair. In: Kintz P, ed. Analytical and Practical Aspects of Drug Testing in Hair. Forensic science series. Boca Raton, FL: Taylor and Francis; 2007:1-19.

Krug O, Thomas A, Walpurgis K, Piper T, Sigmund G, Schänzer W, Laussmann T, Thevis M. Identification of black-market products and potential doping agents in Germany 2010-2013. *Eur J Clin Pharmacol.* 2014;70(11): 1303-1311.

Laurent S. Antihypertensive drugs. *Pharmacological Research*. 2017;124:116-125.

Leaney AE, Beck P, Biddle S, Brown S, Grace PB, Hudson SC, Mawson DH. Analysis of supplements available to UK consumers purporting to contain selective androgen receptor modulators. *Drug Test Anal*. 2021;13(1):122-127.

Odoardi S, Mestria S, Biosa G, Valentini V, Federici S, Strano Rossi S. An overview on performance and image enhancing drugs (PIEDs) confiscated in Italy in the period 2017-2019. *Clin Toxicol*. 2021;59(1):47-52.

Trenton AJ, Currier GW. Behavioural manifestations of anabolic steroid use. *CNS Drugs*. 2005;19:571-595.

Van Amsterdam J, Opperhuizen A, Hartgens F. Adverse health effect of anabolic-androgenic steroids. *Regul Toxicol Pharmacol*. 2010;57:117–123

Weber C, Krug O, Kamber M, Thevis M. Qualitative and Semiquantitative Analysis of Doping Products Seized at the Swiss Border. *Subst Use Misuse*. 017;52(6):742-753.

Thevis M, Schänzer W. Detection of SARMs in doping control analysis. *Mol Cell Endocrinol*. 2018;464:34-35.

WADA. 2019. Stakeholder Notice regarding meat contamination. WADA.

Zhang X, Sui Z. Deciphering the selective androgen receptor modulators paradigm. *Expert Opin Drug Discov*. 2013;8(2):191-218.

Université de Strasbourg

École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

Laurie GHEDDAR

Détection des substances de la performance dans les phanères : approches analytiques et application à des cas de dopage, des dossiers cliniques et des expertises médico-légales

Résumé

Du fait de leur accès facilité par Internet, de nombreux produits sont détournés par les sportifs de haut niveau mais également par les amateurs (culturistes, par exemple) pour améliorer leur performance physique, esthétique et/ou mentale. Dans le cadre de cette thèse, je me suis intéressée aux substances de la performance suivantes, qui sont interdites en permanence par l'Agence Mondiale Anti-dopage (AMA) : les agents anabolisants classés S1 - les modulateurs sélectifs des récepteurs des androgènes (SARMs) S1.2 - les modulateurs hormonaux et métaboliques S4 et enfin la classe S5 qui comprend les diurétiques et les agents masquants. L'abus de ces substances peut conduire à des effets secondaires de type psychiatrique, de l'agressivité et à des complications pour la santé pouvant mener jusqu'au décès. Il est donc important de savoir les identifier dans un laboratoire de toxicologie médico-légale. Afin de comprendre si les résultats anormaux (dopage) ou positifs (médico-légal) observés sont dus à des contaminations environnementales, à des malversations ou à des pratiques dopantes caractérisées, l'analyse des phanères (cheveux, poils) a été choisie. L'objectif principal de cette thèse a été d'établir des critères d'interprétation pour expliquer les résultats dans les phanères.

La première partie du travail a été consacrée à la mise en place d'une stratégie analytique et au développement et à la validation de trois méthodes d'analyses robustes et sensibles dans les cheveux à l'aide de systèmes de chromatographie liquide/gazeuse couplée à des détecteurs de spectrométrie de masse en tandem. A ce jour, les librairies de spectres MS comportent 20 stéroïdes anabolisants et 10 esters de testostérone, nandrolone, drostanolone et boldenone, 9 SARMs et 2 modulateurs métaboliques associés à la consommation de SARMs et enfin 9 diurétiques. Les méthodes MS sont mises à jour dès lors qu'un nouveau produit est commercialisé.

Après la mise en place des méthodes analytiques, ma thèse a porté sur la caractérisation de l'incorporation de ces substances dans les cheveux, les poils et/ou les ongles. Pour ce faire, les méthodes ont été appliquées à des cas de dopage dans des cas de suspicion de contamination, à des affaires médico-légales dans des cas de violence ou de décès lié à l'abus d'agents anabolisants et à des cas cliniques dans le cadre du suivi thérapeutique des patients admis à l'hôpital.

Pour finir, afin d'augmenter la spécificité des méthodes, d'identifier de nouveaux produits et leurs métabolites dans les urines ou d'identifier des produits inconnus avec précision, il a été nécessaire d'utiliser deux outils supplémentaires : les microsomes hépatiques humains (HLMs) et la spectrométrie de masse haute résolution (HRMS).

Ces travaux ont donné lieu à 22 publications nationales et internationales ainsi qu'à de nombreuses collaborations.

Mots-clés : substances de la performance, chromatographie liquide et gazeuse couplée à la spectrométrie de masse en tandem (UHPLC/GC - MS/MS), cheveux, poils, médecine-légale, dopage

Abstract

Because of their easy access through the Internet, many products are misused by top athletes but also by amateurs (bodybuilders, for example) to improve their physical, aesthetic and/or mental performance. In the framework of this thesis, I was interested in the following performance-enhancing substances, which are permanently banned by the World Anti-Doping Agency (WADA): anabolic agents classified as S1 - selective androgen receptor modulators (SARMs) S1.2 - hormonal and metabolic modulators S4 and finally the S5 class which includes diuretics and masking agents. Abuse of these substances can lead to psychiatric side effects, aggression and health complications that can lead to death. It is therefore important to know how to identify them in a forensic toxicology laboratory. In order to understand whether the adverse analytical findings (doping) or positive (forensic) results observed are due to environmental contamination, malpractice or doping practices, the analysis of keratinous matrices (hair) was chosen. The main objective of this thesis was to establish interpretation criteria to explain the results in hair.

The first part of the work was devoted to the establishment of an analytical strategy and the development and validation of three robust and sensitive analytical methods in hair using liquid chromatography/gas systems coupled with tandem mass spectrometry detectors. To date, the MS spectra libraries include 20 anabolic steroids and 10 esters of testosterone, nandrolone, drostanolone and boldenone, 9 SARMs and 2 metabolic modulators associated with SARM consumption and 9 diuretics. The MS methods are updated as soon as a new product is marketed.

After setting up the analytical methods, my thesis focused on the characterization of the incorporation of these substances in hair and/or nails. To do so, the methods were applied to doping cases in cases of suspected contamination, to forensic in cases of violence or death related to the abuse of anabolic agents and to clinical purposes in the context of therapeutic follow-up of patients admitted to hospital.

Finally, in order to increase the specificity of the methods, to identify new products and their metabolites in urine or to identify unknown products with precision, it was necessary to use two additional tools: human liver microsomes (HLMs) and high resolution mass spectrometry (HRMS).

This work has resulted in 22 national and international publications and numerous collaborations.