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Aspects analytiques, cliniques et médico-judiciaires liés à l'identification des molécules à pouvoir hypoglycémiant dans les intoxications par antidiabétique (s)

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Avant-propos

« You too can be a Toxicologist in two easy lessons; each of 10 years » A. Lehman

Tel était mon défi le jour où j'ai décidé de commencer ma première leçon de toxicologie avec le professeur Pascal Kintz.

Les travaux rapportés dans cette thèse sont le fruit de trois ans de travail dans le laboratoire de toxicologie de l'Institut de médecine légale de Strasbourg. Bien que la date officielle de début du projet soit novembre 2019, ce parcours de formation commencera presque deux ans plus tôt lors d'un stage dans le cadre du projet « Erasmus for traineeship ». Ce stage me sera essentiel pour approfondir mes connaissances en toxicologie médico-légale, tant sur le plan analytique que théorique.

C'est dans cette optique que j'ai été accueillie à l'IML de Strasbourg, à qui je suis reconnaissante pour la formation et l'enrichissement personnel.

L'objectif de cette thèse était de créer des méthodes analytiques pour l'identification et le dosage des antidiabétiques oraux dans le sang et les cheveux, et notamment de l'insuline. La difficulté était évidente en raison de la faible familiarité du laboratoire avec l'analyse des protéines à l'aide de spectromètres de masse classiques ou à haute résolution.

Au-delà des difficultés sur le plan scientifique, cette thèse était un double défi. Étant donné que je suis de nationalité italienne, le français n'est pas ma langue maternelle.
Écrire les résultats de ma recherche et apprendre les subtilités d’une langue qui n’est pas la mienne, n'était pas facile.
Pour cette raison, je peux affirmer que c’était un honneur et une fierté d’avoir pu dépasser la difficulté de rédiger une thèse de sciences en français.

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Liste des abréviations

AAPCC : American Association of Poison Control Centers – Association américaine des centre de control antipoison

ADN : Acide désoxyriboucléique

AMP : adénosine monophosphate

AMPk : AMP-activated protein kinase/ Protéine kinase active par l'AMP

ATP: Adénosine triphosphate

CID: collision-induced dissociation

CV: coefficient de variation

DDP-4: dipeptidyl peptidase 4

EC: energie de collision

EDTA: acide étylènediaminetétraacétique

EI : Impact électronique

ESI: electrospray ionisation

eV: electron volt

FDA: Food and Drug Administration

GC: Chromatographie en phase gazeuse

g/L: gramme par litre

GLP-1: glucagon-like peptide-1

HRMS: High Resolution Mass Spectrometry- Spectrométrie de masse Haute Résolution

INSR: Insulin Receptor

LC: Chromatographie Liquide

M1: 21(A)-Gly-human insulin

MALA: Metformin associated lactic acidosis/ Acidose lactique associée à la metformine

MBTFA : N-Methyl-bis(trifluoroacetamide)

Mg/L : milligramme par litre

MRM : multiple reaction monitoring / surveillance de réaction multiple

µL: microlitre

MS : spectrométrie de masse

MS/MS : spectrométrie de masse en tandem

NaF: fluorure de sodium

Ng/mL: nanogramme par millilitre

PBS : phosphate buffered saline/ solution saline tamponnée au phosphate

Pg/mg : picogramme par milligramme

Q-TOF : Quatrupole Time of Flight- quadrupole à temps de vol

RYGB : Roux-en-Y Gastric Bypass

SGLT-2 : Sodium-glucose cotransporter-2 inhibitors/ Inhibiteurs du cotransporteur-2 sodium-glucose

SI : standard interne

SNC : système nerveux central

SPE : extraction en phase solide

TDM : Therapeutic Drug Monitoring/ Surveillance thérapeutique des médicaments

T_R : temps de retention

UHPLC : Chromatographie liquide ultra haute performance

UV : ultraviolet

$ZnSO_4$: sulfate de zinc

Introduction

Les antidiabétiques sont des médicaments utilisés pour traiter l'une des maladies les plus répandues dans le monde : le diabète.

Malgré l'objectif thérapeutique de ces médicaments, leur mauvaise utilisation a été décrite à des nombreuses reprises. Le plus grand risque associé à leur utilisation inappropriée est l'hypoglycémie. L'hypoglycémie est une baisse du taux de glucose dans le sang qui peut, lorsqu'elle est particulièrement grave, provoquer des effets neuroglycopéniques pouvant entraîner la mort. Le problème concernant les antidiabétiques est qu'ils ne sont pas seulement utilisés par la population diabétique, mais aussi par des personnes ne présentant aucun trouble métabolique. L'utilisation des antidiabétiques est en fait détournée à des fins de suicide et de meurtre, de dopage (en ce qui concerne l'insuline) ou encore afin de provoquer des hypoglycémies factices, notamment dans le contexte du syndrome de Münchhausen et Münchhausen par procuration (pathologie psychiatrique caractérisée par une tendance à provoquer délibérément une pathologie afin d'attirer l'attention) *(Première Partie- 1.1)*. L'incapacité des laboratoires de toxicologie à identifier ces molécules a conduit au fil du temps à une sous-estimation des homicides et des suicides impliquant ces substances et, dans les cas des hypoglycémies factices, à des pancréatectomies non nécessaires.

Au début de ma thèse, il existait, au laboratoire de toxicologie de Strasbourg, une seule méthode pour le dosage de la metformine dans le sang par chromatographie liquide couplée à la spectrométrie de masse en tandem (dont les défauts seront décrits dans ce manuscrit) et une méthode pour le dosage des sulfamides hypoglycémiants dans le sang et les cheveux par LC-MS/MS. Au cours de cette thèse de trois ans, j'ai développé des méthodes analytiques intégrant trois familles supplémentaires d'agents antidiabétiques oraux (glinides, gliptines et gliflozines) dans le sang et les cheveux mais également une méthode pour l'identification et le dosage de l'insuline et de ses analogues synthétiques dans le sang humain en utilisant la chromatographie liquide couplée à la spectrométrie de masse à haute résolution *(Première partie – 1.2, 1.3)*.

Aujourd'hui, le laboratoire de toxicologie de Strasbourg est capable d'identifier et de quantifier 17 antidiabétiques oraux dans toutes les matrices biologiques et, pour la première fois, l'insuline humaine et 5 de ses analogues synthétiques dans le sang. Ces méthodes sont désormais appliquées aux cas médico-légaux et à des demandes hospitalières. La majorité des cas observés au cours de ces trois années sont présentés sous forme d'articles publiés ou en cours d'évaluation.

Première Partie - Contexte

1.1. Antidiabétiques et Syndrome de Münchhausen

Le syndrome de Münchhausen, connu aussi sous le nom de « pathomimie » et « trouble factice », est un trouble psychiatrique caractérisé par le besoin de simuler une maladie, voire de se blesser délibérément, afin d'attirer l'attention des autres. Comme le célèbre baron von Münchhausen (d'où la maladie emprunte le nom) , les personnes atteintes racontent des histoires à la fois dramatiques et mensongères.

Une forme très courante de cette affection est le syndrome de Münchhausen par procuration.

Dans cette forme, le sujet provoque délibérément une pathologie sur une personne à sa charge (souvent un parent avec son enfant) pour attirer l'attention sur lui-même en tant que responsable de la personne blessée.

L'hypoglycémie factice est considérée comme une manifestation du syndrome de Münchhausen.

L'incapacité à identifier une hypoglycémie factice peut conduire à une laparotomie ou à une pancréatectomie inutile.

Une hypoglycémie factice peut, en effet, ressembler cliniquement à un insulinome, et par conséquent toute différenciation entre une hyperinsulinémie endogène et exogène peut être difficile.

L'absence de diagnostic correct peut entraîner une intervention chirurgicale inutile en cas de suspicion d'adénome pancréatique insulino-producteur [1,2].

Il n'y a pas beaucoup de cas dans la littérature d'hypoglycémie factice due à l'utilisation d'antidiabétiques. Cependant, l'absence de publications ne doit pas entraîner la négligence et la sous-évaluation du risque.

L'hypoglycémie induite par les antidiabétiques doit toujours être prise en compte dans le diagnostic différentiel de l'insulinome.

L'exclusion des pathologies susceptibles de provoquer une hyperinsulinémie peut être réalisée en analysant le sang, l'urine et les cheveux du patient (pour mettre en évidence une exposition passée) afin de détecter les antidiabétiques.

Pour cette raison, il est essentiel qu'un laboratoire de toxicologie dispose de méthodes permettant d'identifier la plupart des médicaments antidiabétiques disponibles sur le marché.

1.2. Insuline : sauveuse ou tueuse ?

Le diabète est l'une des maladies chroniques les plus fréquentes dans le monde. En 2020, environ 3,5 millions de personnes en France étaient traitées par des antidiabétiques, ce qui représente 5,3% de la population [3]. En raison de l'incidence croissante du diabète, le traitement par insuline a également augmenté.

Le traitement à l'insuline est recommandé pour la plupart des patients diabétiques de type I et certains diabétiques de type II.

Malgré les bienfaits apportés par l'insulinothérapie, celle-ci n'est pas exempte de risques.

Les surdosages d'insuline, qu'elles soient accidentelles ou intentionnelles, sont potentiellement mortelles en raison de l'altération du métabolisme du système nerveux central (SNC) induite par l'hypoglycémie, qui peut entraîner un dysfonctionnement cérébral, un œdème pulmonaire et qui, si elle n'est pas traitée à temps et prolongée, peut conduire au coma et à la mort neurologique [4].

Avec l'augmentation de la prévalence du diabète, les cas de mésusage et de surdosage d'insuline sont également en augmentation. Depuis 1922, date à laquelle l'insuline a été isolée pour la première fois du pancréas, diverses utilisations non thérapeutiques de cette hormone ont été décrites, allant de son détournement à des fins de dopage dans le sport (en particulier le culturisme) à son utilisation à des fins criminelles, de meurtre et/ou de suicide.

Dans le contexte du dopage, l'insuline est utilisée par les athlètes car, chez les bodybuilders par exemple, elle agit aux côtés de stéroïdes anabolisants comme la testostérone ou l'hormone de croissance humaine pour consolider le tissu musculaire. Les stéroïdes favorisent la croissance du tissu musculaire, tandis que l'insuline empêche la dégradation. En outre, l'insuline renforce également l'endurance des coureurs de demi-fond et d'autres athlètes sur piste en leur permettant de charger leurs muscles en "carburant" glycogène avant et entre les épreuves. Dans ce but, les athlètes doivent prendre de l'insuline et du glucose simultanément pendant quelques heures, en les perfusant à l'aide d'une technique appelée "clamp hyperinsulinique" [5].

La tentation pour les sportifs est évidente. L'insuline disparaît rapidement de l'organisme (demi-vie 4 minutes). Même si elle était détectée, jusqu'au début des années 2000, il n'y avait aucun moyen de la distinguer l'insuline endogène. Un certain nombre de cas frappants ont amené les spécialistes de la lutte contre le dopage à se préoccuper de cette question et à prendre des mesures pour mettre au point des méthodes d'analyse spécifiques et sensibles adaptées au problème. Un exemple est le cas du cycliste italien Marco Pantani, gagnant du Tour de France en 1998 et suspendu pour quatre ans en raison de la découverte de seringues à insuline dans sa chambre d'hôtel en 2002 lors du Tour d'Italie [6].

L'intérêt pour l'analyse de l'insuline par spectrométrie de masse à haute résolution dans le contexte du dopage a donné lieu à une publication scientifique ***[Article 1].***

Il y a encore 20 ans, les antidiabétiques les plus identifiés après intoxication étaient les biguanides et les sulfonylurées. Cependant, dans le rapport 2020 de l'American Association of Poison Control Centers (AAPCC), les cas d'insuline ont été multipliés par 3, dépassant désormais les intoxications aux sulfonylurées [7].
Par rapport aux cas décrits au 20ème siècle pour lesquels moins de 5% des cas étaient des cas de suicide, au cours des 20 dernières années, 90% des surdosages d'insuline étaient des tentatives de suicide**.** Depuis 1927, date à laquelle la première tentative de suicide a été signalée, de nombreux cas de suicide ou de tentatives de suicide ont été rapportés [8].
Il n'est pas surprenant que l'insuline soit utilisée à des fins suicidaires puisque les patients diabétiques ont un risque beaucoup plus élevé de développer une dépression que le reste de la population [9]. Toutefois, ce nombre semble encore sous-estimé en raison de l'incapacité des laboratoires à identifier et à distinguer l'insuline humaine des insulines synthétiques et en raison de l'instabilité chimique de l'insuline en particulier dans les échantillons *postmortem*. Les phénomènes de dégradation se produisant en *postmortem*, en particulier l'hémolyse, contribuent à la dégradation de l'insuline.
Ce phénomène rend ces échantillons inutilisables par les méthodes immunologiques.
En effet, au fil du temps, les méthodes analytiques couramment utilisées pour mesurer l'insuline ont été les techniques de dosage radio-immunologique et immunoenzymatique. Ces méthodes présentent certains inconvénients, notamment en ce qui concerne leur spécificité, et en particulier des réactions croisées avec d'autres molécules telles que les analogues de l'insuline humaine, le peptide C, la proinsuline et les métabolites de l'insuline.

En raison des changements pathologiques insaisissables, du manque de preuves morphologiques et de l'absence de méthode analytique spécifique utilisée en routine dans les laboratoires de toxicologie médico-légale, il est difficile de diagnostiquer une intoxication à l'insuline.
En médecine légale, la plupart des médecins légistes utilisaient et utilisent encore des mesures indirectes de l'insulinémie *antemortem,* tel que le calcul du rapport peptide C:insuline. Ce calcul est basé sur le principe que l'insuline et le peptide C sont sécrétés en quantités identiques par le pancréas, de sorte que, physiologiquement, leur rapport chez une personne saine devrait être d'environ 1. Un rapport supérieur à 1 démontrant l'administration exogène d'insuline. Les autres paramètres utilisés sont le dosage du glucose dans l'humeur vitrée en utilisant la formule de Traub (pour remonter à la glycémie au moment du décès) et le dosage des corps cétoniques [10].
La discussion des problématiques liées à l'analyse de l'insuline a donné lieu à une publication scientifique ***[Article 2].***

En médecine légale, le délai entre le prélèvement de l'échantillon et l'analyse est souvent très long, et les concentrations identifiées sont donc souvent très faibles. En outre, de nombreux analogues de l'insuline sont disponibles sur le marché, tous ayant une structure et un poids moléculaire très similaires. Par

conséquent, des méthodes très spécifiques et sensibles sont nécessaires. Dans la littérature, la plupart des méthodes de spectrométrie de masse haute résolution (HRMS) pour l'identification des analogues de l'insuline n'ont pas été appliquées à des cas réels d'intoxication et à des cas *postmortem*.

Ce sont pour ces raisons que j'ai décidé de développer une méthode d'identification et de discrimination de l'insuline humaine de ses 5 de ses analogues synthétiques en utilisant comme technologie la chromatographie liquide couplée à la spectrométrie de masse à haute résolution (UHPLC-Q-TOF). Le développement de la méthode sera décrit dans la partie 4.

Ce type de technologie permet de discriminer les insulines malgré leurs caractéristiques analytiques très similaires (en termes de temps de rétention et de spectre), offrant ainsi la possibilité de quantifier également les traces d'insuline et de les identifier simultanément dans un seul test. En outre, il est possible d'analyser tout type de matrice, ce qui est très intéressant en médecine légale où la matrice sanguine n'est pas toujours disponible.

Article 1: Interest of HRMS systems in analytical toxicology: Focus on doping products

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GENERAL REVIEW

Interest of HRMS systems in analytical toxicology: Focus on doping products

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Summary Performance and image enhancing drugs are misused by top athletes, young people and amateurs like bodybuilders. Their misuses lead to adverse analytical findings in sport but also physical and mental health risks. Anti-doping laboratories must set up analytical strategies and develop precise, robust and sensitive methods to detect all analytes prohibited by the World Anti-Doping Agency (WADA). In recent years, high resolution mass spectrometry (HRMS) systems have emerged and are gradually replacing more traditional systems for screening and quantification. The targeted compounds in this review are the drugs prohibited by the WADA in-and out-of competition and glucocorticoids. Advanced technologies were applied for anti-doping, forensic and clinical purposes. The contribution of HRMS in the analysis of doping products allows a retrospective analysis to detect new metabolites in order to increase the detection windows and to identify new synthetic products. These systems are also used for new applications like study of metabolomics, characterisation of metabolites and applications in alternative matrices (hair, dried blood spots). Moreover, the misuses of these substances lead the consumers to purchase on the black market.

Abbreviations

AAFs	Adverse analytical findings
AAS	Anabolic androgenic steroids

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ACTH	Adrenocorticotropic hormone
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AIF	All ion fragmentation
AMPK	Adenosine monophosphate-activated protein kinase
APB	Athlete's biological passport
AAS	Anabolic androgenic steroids
AAFs	Adverse analytical findings
CERA	Continuous erythropoietin receptor activator
CID	Collision-induced dissociation
CRH	Corticotropin releasing hormone
DART	Direct analysis in real time-high resolution mass spectrometry
DBS	Dried blood spots
DHEA	Dehydroepiandrosterone
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ESAs	Erythropoiesis-stimulating agents
ESI	Electrospray ionization
EPO	Erythropoietin
FSH	Follicle stimulating hormone
FWHM	Full width at half maximum
GC	Gas chromatography
GHRH	Growth hormone-releasing hormone
GHRPs	Growth hormone releasing peptides
GHS	Growth hormone secretagogues
GnRH	Gonadotropin-Releasing Hormon
HCD	High—energy C-trap dissociation
hCG	Human chorionic gonadotropin
HES	Hydroxyethyl starch
hGH	Human growth hormone
HLM	Human liver microsomes
HKM	Human kidney microsomes
HRAM	High resolution accurate mass spectrometry
HRMS	High resolution mass spectrometry
IAC	Immunoaffinity cartridges
IGF- I	Insulin-like growth factor I
LC	Liquid chromatography
LH	Luteinising hormone
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MRPL	Minimum required performance level
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSIA	Mass spectrometry immunoassay
NCE	Normalized collision energy
NMR	Nuclear magnetic resonance
P—III -NP	Procollagen III amino-terminal propeptide
PPAR-δ	Peroxisome proliferator-activated receptors
PRM	Parallel reaction monitoring
Q-Orbitrap	Quadrupole-orbitrap mass spectrometry
Q-TOF	Quadrupole time-of-flight mass spectrometry
QuEChERS	Quick, Easy, Cheap, Efficient, Rugged and Safe
rHuEPO	Recombinant Eryhtropoietin
rhGH	Recombinant human growth hormone
RIA	Radioimmunoassay
rLH	Recombinant luteinizing hormone
SARMs	Selective androgen modulator receptors
SERMs	Selective estrogen receptor modulators
SPE	Solid-phase extraction
TSH	Thyroid stimulating hormone
UV	Ultraviolet
WADA	World Anti-Doping Agency

Introduction

In human sport, lots of products are prohibited because of their misuse to enhance physical and/or psychological performance. After the scandal that took place in the world of cycling with the Festina case, the World Anti-Doping Agency (WADA) was created in 1999. WADA is an independent international foundation. It is responsible for promoting, coordinating, and monitoring the fight against doping in sport, the management of scientific research, education, anti-doping development and the supervision of compliance with the World Anti-Doping Code.

The Code is a document that harmonizes doping-related practices and prohibited substances across countries and sports. The prohibited substances are classified by ban period and by family of substances. The first category includes six families that are permanently banned and classified in section S0 up to S5 (non-approved substances, anabolic agents, peptide hormones, beta-2-agonists, hormones, and diuretics). The second includes four families, S6 to S9, which are only prohibited in competition (stimulants, narcotics, cannabinoids, and glucocorticoids). And finally, there is a third category in which beta-blockers are banned in-and out-of-competition or only depending on the sport [1].

Concerning animal sports, all products are prohibited in-and out-of-competition. Moreover, some products are prohibited by the European Union (EU) as growth promoters [2].

Anti-doping laboratories work on urine for screening drugs and blood for testing peptide hormone. In urine, the main analytes present are the transformation products or metabolites. Metabolites are not always commercially available and/or can be very expensive. Moreover, false negative tests are not allowed by WADA. In general, all substances were detected and quantified by liquid or gas chromatography coupled with tandem mass spectrometry (LC-MS/MS or GC-MS/MS). In recent years, high resolution mass spectrometry (HRMS) systems have emerged and are gradually replacing more traditional systems for screening [3]. Today, there are two types of high-resolution technologies widely used: the time-of-flight (TOF) and the orbitrap, launched by ThermoFisher Scientific.

These systems allow precise mass measurements with an accuracy of less than 5 ppm. In addition, they allow full scan acquisitions and avoid the optimization of multiple reaction monitoring transitions required in LC-MS/MS and GC-MS/MS. Finally, it is easy to add new substances in the libraries and it is possible to make retrospective analyses. Furthermore, for some products, there is no information in literature especially for new compounds such as designer steroids. Moreover, in terms of doping, samples are kept for up to 10 years [4].

The misuse of performance drugs can lead to severe health problems. For example, this is the case for anabolic steroids for which their misuse can lead to severe health effects like cardiac diseases. The issues and the interest of

HRMS are also stackable to clinical and forensic laboratories issues for which it is necessary to know the cause of a disease or death.

All these products are therapeutic indications but no clinical approval for all, therefore the authors focused on applications in anti-doping, forensic and clinical toxicology in humans and animals.

Method

The authors review the state of the art in the use of HRMS systems for doping products analysis. In this study, products that are prohibited only in-competition (i.e., stimulants), are not included because they will be the subject of another bibliographic review excluding glucocorticoids. Therefore, the authors are interested in the analysis of doping substances by HRMS systems and more particularly in those permanently prohibited by the WADA and glucocorticoids banned only in competition. For each family, we sought to know what existed for their analysis in the doping, clinical and forensic fields, and what place HRMS occupied. Therefore, we used open access articles on Pubmed, Web of Science or Science Direct.

Actual applications in anti-doping, forensic and clinical toxicology

Screening for anabolic steroids

Anabolic androgenic steroids (AAS) are a class of steroidal hormones developed from a natural and endogenous human hormone: testosterone. Testosterone is secreted by the gonads, the testes in males and the ovaries in females, in smaller quantities. Initially, they were developed for therapeutic purposes to stimulate bone growth, increase muscle mass, and induce male puberty in cases of hypogonadism. Because of their anabolic properties, AAS are used for doping purposes. Indeed, WADA has prohibited these substances and classified them as S1 class. The number of adverse analytical findings (AAFs) is more important for this class than the other class. In 2019, anabolic agents represented 44% (1825 on 4180) of all AAFs in all sports [5]. Moreover, their easy access through the internet has attracted a larger public. Indeed, amateurs also use these products for aesthetic purposes. The misuse of these substances can lead to health problems such as cardiac arrest but also mental problems such as the increasing of violence. Therefore, the interest to identify them is multiple. S1 class includes endogenous and exogenous AAS but also other anabolic agents. These substances are cited in the non-exhaustive list of the Code [1].

The authors have reviewed the literature related to high resolution for these three groups of substances in the fields of human and animal doping, clinical and forensic. The applications described in this paragraph are only applications in biological fluids (blood, urine), and meat.

Exogenous AAS

In sport, top athletes misuse AAS to increase muscle mass, decrease recuperation time or to increase their aggressiveness in fight sport, i.e., boxing. To fight these misuse, anti-doping controls in urine are set up in-an out-of-competition period. Anti-doping laboratories must follow two important rules. The first is to identify all exogenous AAS (including metandienone, trenbolone, testosterone esters, steroid esters..). The prohibited list of WADA is updated every year. The second is to be able to identify them at low concentrations in the urine. These concentrations, called minimum required performance level (MRPL) are described in the WADA Technical Document—TD2019MRPL [6]. For exogenous AAS, MRPL is fixed at 5 ng/mL in urine for all AAS excluding dehydrochlormethyltestosterone, metandienone, 17(-methyltestosterone and stanozolol for which MRPL is fixed at 2 ng/mL.

Mass spectrometry is the technique of choice for the analysis of these small molecules. The triple quadrupole systems were chosen and used by several laboratories for decades. Nevertheless, these techniques have a big disadvantage. Indeed, it is a targeted approach that can only include a limited number of compounds to safeguard a good sensitivity. Therefore, laboratories are turning to HRMS to be able to add new substances in libraries and do retrospective analysis. Retrospective analysis showed its strength after the 2008 Beijing Olympics. Moreover, HRMS enables continuous scan and to detect non-target compounds compared with tandem MS methods.

Many studies in scientific literature describe the screening of doping products namely exogenous AAS in urine. Indeed, there are several articles that mention the development of screening methods for performance substances by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) [7,8,10] and gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) [8,9,11]. Concerning sample preparation, methods such as solid phase extraction (SPE) [9], dilute-and-shoot [7] and liquid-liquid extraction (LLE) [8,9,11] are widely used.

Novel approaches are also developed to enhance the specificity and sensitivity. For example, He et al. analysed 13 steroids, including trenbolone, by liquid chromatography coupled to quadrupole-orbitrap mass spectrometry (LC-Q-Orbitrap) coupled with parallel reaction monitoring (PRM). This method allowed to reach limits of detection (LOD) lower than 25 times the MRPL required by the WADA and showed that the use of this coupling allowed increasing the specificity of the method [12]. Raro et al. compared two analytical methods: liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-Q-TOF) and LC-Q-Orbitrap to study metabolomics related to the misuse of testosterone cypionate (T cypionate). Two methods gave different mass characteristics but the same conclusion and the same marker of T cypionate: 1-cyclopentenoylglycine [13].

Concerning GC-methods, the derivatization step is needed. Chemical modification with TMS is the most used derivatization and is known to improve sensitivity and selectivity. In another study performed by Cha et al. a new method was presented to analyse TMS derivatives of AAS by gas chromatography coupled to linear trap quadrupole-Orbitrap (GC-LTQ-Orbitrap). This study was applied to spiked urine and showed good sensitivity and selectivity. It could be applied to the simultaneous detection of AAS [14].

Otherwise, due to the short half-life of most AAS, a great interest has been shown by anti-doping laboratories to the

research of long-term metabolites in urine. For that, several laboratories developed different strategies.

The first study is about stanozolol. Stanozolol remains the most widely used AAS by athletes. It is therefore important for laboratories to increase the specificity and the detection window. In this study, Göschl et al. present an online SPE method coupled with LC-Q-Orbitrap. This method allows the identification of glucuronide metabolites of stanozolol until 28 days without derivatization step [15].

In another study, it has been shown that longer detection times can be achieved by focusing on the sulfated metabolites of AAS rather than the gluco-conjugates. The authors presented an analytical method capable of identifying unhydrolyzed sulfated metabolites and precursor AAS with GC-Q-TOF [16]. Another strategy consists of the use of the hydrogen isotope ratio mass spectrometry and LC or GC - high resolution/high accuracy mass spectrometry. This method was applied for the identification of metandienone and trenbolone metabolites [17,18].

Another issue is the identification of the new synthetic steroids. Some authors have developed strategies to identify designer steroids without reference material. Nielen et al. described an androgenic bioactivity/LC-Q-TOF mixed method to test for synthetic steroids in urine [19].

The use of AAS is also prohibited in animal sports and is closely monitored, especially in horse racing. Furthermore, the use of growth-promoting compounds (such as AAS that increase muscle mass) has been strictly prohibited in the EU since 1986 [2]. Several authors described the identification of AAS by LC-HRMS. Methods were applied in several matrices such as equine testis, bovine bile, urine and equine plasma [20—23].

On the other hand, long-term abuse of anabolic agents can lead to adverse health effects. Physical health problems have been noted in the literature: necrosis due to injections of anabolic esters [24], cardiovascular problems and even death [25]. Because of the hazardous nature of these products, forensic and clinical laboratories must test for these drugs. Screening for the analysis of AAS in forensic context was described. For example, Fabresse et al. developed a method in whole blood by LC-Q-Orbitrap to test for 11 AAS with limit of quantification (LOQ) at 5 ng/mL. Method was applied to two forensic cases in blood and urine [26].

Endogenous AAS

Endogenous AAS are derived from testosterone and are physiologically present in the human body. In view of the difficulties associated with the determination of these compounds, WADA has advised anti-doping laboratories to use the ratio testosterone/epitestosterone (T/E). T/E ratio is evaluated to assess testosterone misuse (T/E > 4) as dosage of testosterone is not sufficient. However, there are factors that influence these values such as inter-individual variation, so anti-doping laboratories have introduced the steroid profile assay [27]. The analysis of these substances (testosterone, epitestosterone, androsterone, etiocholanolone, dehydroepiandrosterone (DHEA)..) allows to establish the athlete's biological passport (APB) and to follow the fluctuation of their value throughout their careers. Identification and quantification of endogenous AAS is also an indirect approach to detect exogenous AAS use. Indeed, it has been shown that the administration of exogenous AAS can suppress the excretion of endogenous AAS [28]. Moreover, these substances are also consumed exogenously. The analysis of the steroid profile is therefore important for the follow-up of the APB, to detect new products such as designer steroids and endogenous AAS. The screening and quantification of endogenous substances in urine has been described in GC-HRMS [29,30] and LC-Q-Orbitrap [31].

Abushareeda et al. presented in 2017 a comparison between GC-Q-TOF and GC-MS/MS. They showed that methods of analysis were similar in terms of LOD [29]. In a second study of the same group two types of GC-HRMS are evaluated and compared: Q-Orbitrap and Q-TOF systems [30]. The Q-Orbitrap generally produces higher resolution spectra at a lower scan rate and higher dynamic range. This study showed very good results for both systems (low LOQ, below MRPL). Nevertheless, the Q-Orbitrap presented better mass accuracies due to its higher resolution and less matrix effect [29]. The use of full scan HRMS allows to decrease the background noise coming from the urine matrix, to increase the resolution power and the mass accuracy.

While anti-doping laboratories perform routine analyses on urine, serum could be an alternative because it is less prone to manipulation or contamination. The use of serum has been described by Elmongy et al. using LC-Q-Orbitrap for which LOQ ranged from 0.006 and 7.904 ng/mL for non-conjugated, and gluco-and sulfato-conjugated endogenous AAS [32]. Ponzetto et al. also described a quantitative method in serum for 5 endogenous AAS using LC-LTQ-Orbitrap with targeted selected ion monitoring (t-SIM) mode and presented lower LOQ ranging from 50 to 1000 pg/mL [27].

The analysis of these substances also has clinical relevance. Indeed, the measurement of steroid hormones including endogenous AAS can help in the diagnosis of endocrine disorders (hirsutism, infertility..) [33]. For example, Kaabia et al. have developed a comprehensive steroid profile in urine using SPE and LLE steps followed by analysis with LC-Q-Orbitrap with LOQ at 0.01 ng/mL [31].

In addition, an alteration in endogenous AAS (DHEA, testosterone, androstenedione) levels may be caused by exposure to environmental pollutants such as dioxin, which appears to cause endocrine disruption. In this respect endogenous AAS could be analysed to indirectly test exposure to dioxin.

Two studies have described analysis in human serum and breast milk for testing testosterone and DHEA by GC-HRMS [34,35]. In the first study described by Dong et al. in 2020 [34], the authors showed that the perinatal exposure to dioxins results in a change in steroidogenesis. The results of their study suggested that an increase of dioxins in breast milk was associated with a reduction in testosterone levels in boys' serum samples and progesterone levels in girls.

Screening for other anabolic steroids

Class S1 also includes other anabolic agents classified as S1.2 by the WADA [1]. The following molecules are included in this class: clenbuterol, tibolone, zeranol, zilpaterol and selective androgen modulator receptors (SARMs). MRPL is fixed at 2 ng/mL for all compounds of this class excluding clenbuterol for which MRPL is at 5 ng/mL [6]. Tibolone and zilpaterol

were added to the prohibited list in 2005 and SARMs in 2008. Clenbuterol is a ®-2 agonist and will be discussed in section 2.3.

SARMs are the new anabolic steroids and are considered as designer steroids. They are derivatives of AAS and of bicalutamide, an anti-androgen. Due to their anabolic properties, they are misused by top athletes and amateurs to enhance physical performance (fat burning, increase muscle mass). Anti-doping laboratories have developed analytical methods for testing SARMs in urine. Stacchini et al. presented a method to test for SARMs in urine. The big challenge was to analyse all SARMs simultaneously because there are many different structures [36]. In another study, Thevis et al. presented the search of mass characteristics for YK-11, a steroidal SARM; HRMS is used as a tool of elucidation of mass characterisation [37]. Other publications related to SARMs and HRMS concern the research and characterisation of metabolites *in vivo* and *in vitro*. This part will be the subject of the following paragraph (section 3.1.).

Only one article was published for testing zilpaterol in a screening for β-agonists (including clenbuterol). Method was applied in feed and animal body fluid and tissues with LC-Q-Orbitrap but no zilpaterol was found in biological or feed samples [38].

Tibolone is a synthetic steroid which mimics the activity of estrogen and progesterone. Only Son et al. have presented a study about the pharmacokinetic of tibolone and have shown that the use of GC-HRMS enabled to detect 3α-hydroxytibolone and 3β-hydroxytibolone, collected at 15 and 24h after oral administration which was not detected by GC-MS [39]. In the literature, there is no application in HRMS for zilpaterol.

Screening for peptides hormones, growth factors, related substances, and mimetics

In recent years, peptide hormones have gained an important role in pharmaceutical research. Although these drugs have been developed for specific therapeutic uses, some of them can be used to improve performance in both human and animal sport. Hence, the list of substances banned by the WADA nowadays also includes substances of higher molecular weight described under the section S2: ''Peptides hormones, growth factors and related substances'' [1].

Advances in molecular biology, particularly DNA recombination techniques, have led to a misuse of peptide hormones and related substances. This has challenged doping control laboratories, which have found themselves developing methods to identify and quantify peptides that may be structurally identical to their endogenous counterparts or differ slightly in their peptide sequence or glycosylation pattern.

These substances may be either of natural origin, when extracted and purified from human tissues or fluids, or produced by DNA recombination techniques.

Additional difficulties are related to the identification of peptide hormones whose release is stimulated by other substances, and which often have a very short half-life that greatly reduces the time to detect them in body fluids [40,41].

Another issue is the misuse of peptides purchased on the black market. As these peptides were originally synthesized for therapeutic purposes, their use by healthy individuals may lead to adverse effects. Some of these preparations are not clinically approved and others are simply of poor quality [42].

To enforce the ban on these substances in the athlete's world and given the health risk they pose, it was necessary to implement identification and quantification procedures using sensitive methods which can identify proteins in picomolar concentrations with unequivocal specificity.

While the analysis of low molecular weight compounds has been successfully carried out using methods based on mass spectrometry, progress is starting to be made for peptides [41].

However, the identification and quantification of peptides is in most cases still carried out by means of immunological tests developed primarily for clinical purposes [43,44]. These tests differ in the instrumentation used, the specificity of the antibodies and the quality of the standards.

These variations lead to a high variability in the results of the tests, which triggers the need to develop uniform analytical procedures.

Several methods for the analysis of various matrices such as plasma, serum and urine have been developed to identify and quantify numerous drugs of peptide nature. These substances represent a considerable challenge for doping control laboratories with regard to the ability to distinguish the synthetic substance from the natural analogue as well as the ability to provide the analytical sensitivity required to reveal pharmacologically relevant concentrations in control samples.

HRMS allows peptides and proteins with high peptide sequence homology to be distinguished by its ability to measure very small mass differences.

This section of the paper reviews the HRMS methodologies used for the identification of peptide hormones and their releasing factors described in section S2 of the WADA Prohibited Substances List (Table 1).

Erythropoietins and agents affecting erythropoiesis

The first category in section S2 concerns erythropoietins and agents affecting erythropoiesis.

Erythropoietin (EPO) is a glycoprotein hormone that regulates the production of red blood cells, whose release is stimulated by a reduction in oxygen in the blood. Since the 1980s, the development of synthetic recombinant EPO (rHuEPO) for therapeutic purposes has allowed the treatment of various types of anaemia as well as renal and tumour diseases. Because of their ability to increase oxygen transport in the blood and thus increase muscle oxygenation, they have been misused by athletes, especially to boost their physical endurance.

The high similarity between human and equine EPO (84% identical at the protein level) has also led to the use of these substances in horse doping [45].

Detection methods for these substances are mainly based on immunological methods such as enzyme-linked immunosorbent assay (ELISA), electrophoresis and western blotting [46—49].

Table 1 Summary of methods' characteristics for peptide and protein analysis by HRMS.

Peptides	Matrix	LOD/LOQ	Sample preparation	MS instrument	Reference
ESAs					
rHuEPO	Plasma	500 pg/mL	Immunopurification	MS/MS + Q-Orbitrap	[50]
Sotatercept	Serum	10 ng/mL	Immunopurification	MS/MS + Q-Orbitrap	[51]
Peginesatide	Whole blood	1 ng/mL	Precipitation/digestion	MS/MS + Q-Orbitrap	[53]
Gnrh					
Buserelin, Deslorelin	Urine	10 pg/mL 12.5 pg/mL	Precipitation/SPE WCX	MS/MS + Q-Orbitrap	[61]
Gonadorelin	Urine	25 pg/mL		MS/MS + Q-Orbitrap	[62]
Goserelin		10 pg/mL	Dilute and shoot		
Leuprorelin		10 pg/mL			
Nafarelin		50 pg/mL			
Triptorelin		10 pg/mL			
Buserelin		0.125 pg/mL			
Desloretin		0.125 pg/mL			
Goserelin		0.125 pg/mL			
Nafarelin		0.25 pg/mL			
Triptorelin		0.25 pg/mL			
Corticotrophins					
CRF	Plasma	0.5 ng/mL	SPE MCX	Full MS + TOF	[78]
CRH	Plasma	200 pg/mL	Immunopurification	MS/MS + Q-Orbitrap	[63]
GH					
rhGH	Plasma	2.5 ng/mL	Precipitation/SPE C4 SPE C4	MS/MS + Q-Orbitrap	[65]
GHRH					
Sermorelin,	Urine	0.2 ng/mL	Immunopurification	MS/MS+ Q-Orbitrap	[67]
Tesamorelin, CJC-1295	Blood/urine	< 5 pg/mL/ 0.2 ng/mL	Ultrafiltration/ Immunopurification	MS/MS + Q-Orbitrap	[71]
Semorelin, CJC-1293, CJC-1295					
Tesamorelin					
GHRPs					
Alexamorelin	Blood- DBS	1 ng/mL	SPE	MS/MS + Q-Orbitrap	[167]
GHRP-1		10 ng/mL			
GHRP-2		2 ng/mL			
GHRP-3		5 ng/mL			
GHRP-4		2 ng/mL			
GHRP-5		2 ng/mL			
GHRP-6		2 ng/mL			
Hexarelin		10 ng/mL			
GHS					
Ipamorelin	Urine	0.05 ng/mL	Dilute-and-shoot	MS/MS + Q-Orbitrap	[62]
Anamorelin		0.125 ng/mL			
Growth Factors and modulators					
IGF-1	Blood	50 ng/mL	SPE C18	MS/MS + Q-orbitrap	[74]
IGF-1	Serum	15 ng/mL	Ultracentrifugation	MS/MS + Q-Orbitrap	[76]

As the use of these substances implies an increase in certain blood parameters, such as hematocrit, erythrocyte count and hemoglobin, the dosage of the latter is used to indirectly trace the administration of an erythropoiesis-stimulating agent.

Nevertheless, the methods need high sensitivity and specificity. For this purpose, several methods using HRMS have been described and applied to both humans and animals [45,50—54].

In 2017 Joré et al. [45] described a metabolomic approach to prove CERA (Continuous erythropoietin receptor activator) intake in horses 74 days after administration.

The authors' hypothesis was that most ESAs (Erythropoiesis-stimulating agents) have an effect on the metabolome, and the study of the variations in the metabolome following the intake of ESAs could prove their use. After administration of CERA on horses, the latter was analysed by a LC-Q-TOF, following protein precipitation of

the plasma and filtration and dilution of urine. This technique enabled the detection of CERA up to 31 days after administration, and using urine and plasma metabolomic fingerprints, it was possible to discriminate between pre- and post-CERA administration samples up to 74 days (43 days longer than with an ELISA method). A new generation of ESAs is represented by EPO-mimetic peptides, of which peginesatide is the major representative. This protein has a completely different structure compared to EPO but a high affinity for the EPO receptor.

Since it is structurally different from human EPO, its detection would not be compatible with classical EPO detection tests using anti-EPO antibodies that would not recognize peginesatide [40].

Knowing that conventional doping control assays for EPO will fail in detecting peginesatide, a sensitive assay was developed by Möller et al. [54]. A pegylated EPO-mimetic peptide was synthesised as a model compound and based on the latter, a detection method employing an enzymatic digestion with subtilisin and a mass spectrometric detection of a pentapeptide fragment was developed.

A LOD of 1 ng/mL was accomplished using a LC-Q-Orbitrap instrument with a small volume of plasma (100 μL).

Peptide hormones and their releasing factors

Chorionic gonadotropin (CG) and luteinizing hormone (LH) and their releasing factors in males

In the second category of section S2 of the substances prohibited in sport (for males only), one of the representatives is the human chorionic gonadotropin (hCG).

hCG is a peptide hormone produced during pregnancy by the placenta. Consequently, it is not normally present in healthy subjects, except in certain tumour diseases. Its use for indirect androgenic doping is well known, since like other hormones such as luteinising hormone (LH), it acts on the pituitary gland by inducing the release of testosterone by testicular Leydig cells [41]. hCG is composed of two subunits: alpha and beta. The alpha subunit is common to many hormones such as the thyroid stimulating hormone (TSH) and LH, while the beta subunit is characteristic only of hCG. Having highly homologous primary structures, hCG and LH bind to the same receptor [55].

The recombinant version of the LH hormone (rLH) has recently come available on the market, but it is not investigated in the anti-doping world because injections of high doses of rLH results in negligible levels of LH. Unlike the latter, hCG has a longer half-life and a single dose of its recombinant form causes a high stimulation of testosterone levels in blood and urine [41].

hCG is commonly determined in urine by immunoassays, but recently, methods involving tandem mass spectrometry analysis following an immunopurification step have also been used [56,57].

The post-translational glycosylations which exist in the recombinant version of the LH hormone (rhCG) results in the presence of residues which are not present in endogenous hCG, making it useful to test an exogenous administration of hCG. [41].

WADA requires a specific test in which intact hCG is detected. Recently, a method based on the analysis of hCG glycoforms at the intact level by nanoLC- Q-TOF was presented [55].

An improvement of this method has recently been described by Al Matari et al. [58] which allowed for the first time the identification of 33 glycoforms of hCG beta at the intact level and 42 glycoforms of the alpha subunit. This method involves a pre-concentration step on the trapping column to increase the injected volume and an acidification of the mobile phase to promote ionisation.

The developed method also allows its use for the semi-quantification of these isoforms. The LOQ, determined as a function of the overall concentration of rhCG, has been defined at 12.5 μg/mL for the alpha subunit and 25 μg/mL for the beta subunit and is proposed by the authors for rapid quality control to detect and compare the glycoforms present in recombinant synthetic hCG.

The secretion of gonadotropins such as LH and the follicle stimulating hormone (FSH) is regulated by GnRH (Gonadotropin-Releasing Hormone), a decapeptide secreted by the hypothalamus which controls the secretion of gonadotropins by the pituitary gland. Synthetic GnRH agonists are now used in the form of long-acting depot injections to treat prostate cancer.

Although the long-acting form would be deleterious to athletic performance, administration for short periods triggers a temporary increase in serum of LH and testosterone levels that persists for 5-10 days [41].

The dosage of GnRH analogues is performed by means of immunological tests, but these are not applicable for anti-doping detection due to their lack of specificity [41]. Given the low concentrations to be found in complex biological matrices, sensitive and specific methods are required.

Several mass spectrometry-based methods have been reported for the identification of GnRH and its agonists [59,60].

In 2020, a method is reported [61] that allows the detection of 40 bioactive peptides including GnRH analogues at low pg/mL level in horse urine using LC-Q-Orbitrap. Analytes are extracted from urine by mixed-mode cation exchange SPE cartridges. In fact, most methods use SPE technology as sample clean-up, as the polar nature of these peptides does not allow for LLE. The assay allowed for LODs between 5 and 10 pg/mL.

A second approach is reported by Judák et al. in 2017 [62], which involves a method based on simple dilute-and-shoot of urine prior to analysis by a LC-Q-Orbitrap system. As there is no pre-clean-up, nor initial sample concentration, the ability to identify small concentrations depends on the efficiency of ionization. The addition of DMSO reagent to the mobile phase enhances peptide ionization and allows applying a dilute-and-shoot strategy, making this method advantageous in time and cost-saving by the omission of the sample preparation step.

The applicability of the method was demonstrated by the analysis of urine from patients treated with desmopressin and leuprolide, the presence of which was demonstrated in all the urines analysed.

Corticotropins and their releasing factors

In elite sport ''Corticotropins and their releasing factors'' are also banned by the WADA.

Corticotropin releasing hormone (CRH) is a peptide hormone with a molecular weight of approximately 4.7 kDa composed of 41 amino acids. This hormone is released by the hypothalamus as an adaptive response to stress. CRH in turn promotes gene transcription and the synthesis of the precursor of the adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal gland to produce glucocorticoids such as cortisone and cortisone [63].

CRH and analogues are commonly analysed by immunological analysis, in particular radio-immunological analysis (RIA).

In a 2017 study [63], a method for identifying CRH in plasma and serum using a nanoLC-Q-Orbitrap was presented. Sample preparation involved immunopurification followed by analysis in HRMS.

The accomplished LOD was 200 pg/mL and allowed for detection in plasma of 3 patients after intravenous administration of 100 μg of CRH finding concentrations between 7.6 and 18.9 ng/mL.

Growth Hormone (GH), its fragments and releasing factors

One of the first-generation peptide hormones that has received the most attention in terms of efforts by anti-doping authorities is the human growth hormone (hGH).

hGH is a polypeptide hormone consisting of 190 amino acids and secreted by the pituitary gland. It is present in two isoforms: a 20 kDa isoform and a 22 kDa isoform.

The 22 kDa isoform is the most abundant and is responsible for most of hGH biological activity. The development of molecular biology technologies has led to large-scale production of recombinant growth hormone (rhGH) to treat mainly hGH deficiencies in humans. Because of its properties as a promoter of muscle and bone growth and its ability to accelerate healing processes, it is used not only as a doping substance in human and animal sports, but also to increase meat production in farms.

Consequently, various analytical methods have been developed in order to demonstrate short- and long-term rhGH abuse. The identification of rhGH is carried out by means of two methods. The first is an immunological method which measures the two main isoforms of hGH. As the synthetic version presents only the 22 kDa isoform, exogenous administration of hGH would lead to an increase in the ratio of the two isoforms. The second approach is based on the dosage of two biomarkers: insulin-like growth factor I (IGF-I) and procollagen III amino-terminal propeptide (P-III-NP).

However, the increase in these markers proves manipulation of the hypothalamic-pituitary axis regardless of the substance used [41,64].

Over the last decade, various methods have been developed in MS and HRMS [64—66].

In 2016, a method has been described [65] in which three recombinant GHs of different species (human, porcine and equine) are simultaneously identified using a SPE method following precipitation with ammonium sulphate and proteolysis by trypsin. The proteotypic peptides obtained were then analysed by a nanoLC-Q-Orbitrap. The fully validated method resulted in LODs ranging from 0.5 to 1.25 ng/mL.

Some authors consider that in order to highlight the long-term effect of a hormone treatment, it is necessary to evaluate the metabolic changes associated with the administration of a given hormone. In some studies, a metabolomic approach is used to measure the global biological effect following GH treatment. Traditionally, nuclear magnetic resonance (NMR) has been used for this purpose, but recent advances in mass spectrometry have contributed to the expansion of its use. HRMS allows detection of low concentrations and accurate mass measurement for efficient subsequent structural elucidation.

HRMS methods have been developed and applied to animal matrices mainly to highlight metabolome changes after hGH administration [64,66].

The release of GH is regulated by the growth hormone-releasing hormone (GHRH). This hypothalamic hormone of 44 amino acids has an enhancing effect on the physical performance of athletes and is therefore included in the S2 section of substances prohibited by WADA along with its analogues.

The problems associated with its analysis relate to its instability in matrices such as blood and urine and the very low concentrations at which it is present (around pg/mL).

LC-MS is the preferred technique for the analysis of GHRH analogues in doping control but requires concentration and immunopurification steps of the sample [67—72].

In a recently published study [67], GHRH analogues are analysed after immunopurification of human urine using magnetic beads and subsequent analysis with a LC-Q-Orbitrap system. The method provides a LOD of 200 pg/mL.

In 2015, Knoop et al. [68] developed a method involving immunoaffinity purification using multichannel pipettes equipped with protein A/G mass spectrometric immunoassay (MSIA)-Tips and conditioned with polyclonal anti-GHRH antibodies and subsequent LC-Q-Orbitrap analysis for the identification of 4 GHRH analogues (sermorelin, CJC-1293, CJC-1295 and tesamorelin).

The method not only provided a better LOD than the previous method (< 50 pg/mL), but also allowed the identification of intact GHRH analogues up to 4 hours after intravenous administration.

Other substances included in the same section are the ghrelin receptor agonists to which the growth hormone secretagogues (GHS) and growth hormone releasing peptides (GHRPs) belong. These substances were mainly developed for the treatment of growth hormone deficiency [73]. Because they stimulate GH secretion, they are considerably abused in sport, as evidenced by the 26 AAFs reported in 2019 for GHRPs and GHS by the WADA [5] and for this reason there is a need to develop and improve analytical methods for these substances.

The identification and quantification of these substances is carried out by anti-doping laboratories using both matrices such as blood and urine and alternative matrices such as dried blood spots (DBS) as a preliminary analysis. Subsequently, either 'dilute-and-shoot' approaches or purification and concentration using SPE prior to mass spectrometry analysis are used.

In order to improve the detection of this type of substances by offering a wider detection window, particular attention has been given to the detection of metabolites. This part is discussed in section 3.1.

Growth factors and growth factor modulators

Due to the short half-life of hGH, its measurement after exogenous administration results in a narrow detection window. For this reason, WADA has implemented tests for hGH biomarkers to increase the detection window.

IGF-I is the primary mediator of the effects of GH. An increase in IGF-I corresponds to an increase in GH levels. Its detection in serum is carried out in anti-doping laboratories, due to its direct use either as an anabolic or as a marker of GH use, since serum concentrations remain elevated for several days after hGH intake, and in the clinical field for the follow-up of patients with hypothalamic-pituitary growth hormone axis disorders. The measurement of IGF-I is based both on immunological approaches and more often on mass spectrometry [41]. However, immunological methods require rather demanding standardisation between different anti-doping laboratories, thus mass spectrometric methods are preferred. The latter can use two types of approach, the bottom-up, approved by WADA, and the top-down. The bottom-up approach involves enzymatic digestion using trypsin and quantification of the peptides obtained. However, this approach is very laborious, time-consuming and induces high analytical variability between laboratories [74]. Due to the relatively low molecular weight (7.6 kDa) and the improved performance of the recent mass spectrometers, tryptic digestion has become an unnecessary step. For this reason, most laboratories prefer the top-down approach where the protein is found intact. Several top-down approaches have been developed in different matrices [74—76]. Recently, Coppieters et al. [74] developed a method for the quantification of intact IGF-I in serum prior to analysis by LC-Q-Orbitrap.

Detection of intact protein in serum prior to mass spectrometry analysis requires sample preparation, in this case by precipitation combined with SPE. This type of preparation minimises the workload and provides a method that can be used for anti-doping or clinical purposes to handle the large sample numbers.

Considering that IGF-I is found in the bloodstream at fairly high concentrations (> 100 ng/mL), the method developed provides an interesting LOQ of 50 ng/mL.

Since peptide drugs reach low concentrations in blood and urine, information about their metabolism may increase their window of detection.

However, there are some peptide hormones such as TB-500 that are not approved and for which there are no excretion studies in humans. TB-500 is a peptide isolated in 1981 from the thymus with an anabolic effect, as it regulates actin synthesis in muscle. It has often been used in racehorses where it has been shown to increase physical performance, but it has not been tested on humans [40]. As WADA prohibits any type of substance that affects muscle growth, this substance is nevertheless included in the S2 section of prohibited substances. For this type of product, blood tests are the only proof of intake. Several methods have been developed for this purpose in low resolution mass spectrometry and some also in HRMS. Notably, Esposito et al. [77] developed a HRMS method involving extraction by precipitation followed by SPE for blood and SPE only for urine. HRMS was used only for the identification of the intact molecule, and later also for the identification of 5 metabolites produced by TB-500 following incubation with HLM [78].

Screening for β-2 agonist

β_2-adrenergic agonists are among the oldest therapeutic agents most prescribed for the treatment of asthma but also for numerous respiratory diseases such as the chronic obstructive pulmonary disease due to their bronchodilator activity. Their action is mediated by binding β_2-adrenergic receptors which are present in airway smooth muscle. β_2-adrenergic agonists are derivatives of the phenyl-β-ethanol amines with different substituents in the aromatic ring or in the terminal amine group.

They exist in two pharmaceutical classes: short-acting-β_2-agonists and long-acting-β_2-agonists. β_2-agonists belong to a class of drugs that is widely abused both as a therapeutic agent and for their possible anabolic effects for doping purposes [79].

WADA has prohibited the use of this class of drugs and set MRPL for β_2-agonists at 20 ng/mL in urine, except for formoterol and salbutamol for which a finding of their presence is considered an AAF if their concentration exceeds 1000 ng/mL for salbutamol and 40 ng/mL for formoterol [1,80].

In 2019, WADA reported 153 cases of AAFs for β_2-agonists, with terbutaline accounting for 52% of its group and being the most abused [5].

Possible anabolic effects in humans are linked to their ability to cause fatty mass loss through acceleration of the fatty acid degradation process and inhibition of protein degradation, which would contribute to muscle mass gain.

Due to their ability to redistribute nutrients, these drugs are used illicitly in the livestock industry to reduce fat mass and increase muscle mass in farm animals such as pigs and ruminants [81].

Recent studies have also shown an increased risk of unintentional doping following the consumption of meat contaminated mainly with clenbuterol [82].

In addition, β_2-agonists have several adverse effects when consumed orally, in particular irritation of the throat, cardiovascular effects measurable in terms of increased blood pressure and pulse.

In order to control the illicit use of these drugs, several methods have been developed for the identification and quantification.

Concerning sample preparation, methods such as SPE and purification using immunoaffinity cartridges (IAC) are quite efficient and widely used.

Regarding analytical methods, RIA and enzymatic tests have been developed and are still used today. Different types of biosensors have also been developed as an alternative approach in livestock meat.

These sensors usually contain an antibody that interacts with the analyte. The binding is then measured optically and converted into an electronic signal. This method allows multiple residues to be identified in several samples simultaneously.

However, the sensitivity of these tests is lacking and is often not compatible with the very low levels required in urine and tissue.

For this purpose, various methods have been developed in GC or LC/MS often with high-resolution configurations that have been found to be the most powerful approaches to measure β_2-agonists [79].

Recently, HRMS has received more attention for their sensitivity and ng/mL quantification capability.

This type of technology has proven to be very powerful for screening and quantifying trace amounts of β_2-agonists in some livestock meats and fluids to ensure food safety and public health [81,83—85].

Using one of the latest HRMS technologies (Q-Orbitrap), Li et al. [81] achieved a LOD of 0.02-1.2 ng/mL and 0.07-12.85 μg/kg for solids.

Although the use of β_2-agonists is banned in some countries such as China and Europe, they are still abused by some breeders, illegally.

Guo et al. [83] in 2015 developed a method using the same technology to detect β_2-agonist residues in some farm meat samples which tested positive for salbutamol, clenbuterol, ractopamine and chlorprenaline. The method exhibited a LOD in the range of 0.0033-0.01 g/kg.

Given the short half-life of these drugs, some studies have exploited HRMS technology for the determination of metabolites and degradation products [86,87].

Georgakopoulos et al. [86] performed a study of reproterol excretion in equine urine. Following administration of 20 mg of reproterol in a horse, only the main metabolite is detected in the urine but not the parent drug. Using gas chromatography coupled to a reverse geometry double focusing mass spectrometer (GC-HRMS), the presence of the metabolite was confirmed after accurate measurement of diagnostic ions.

Screening for hormone and metabolic modulators

According to the WADA list of the prohibited substances in sports, the hormone section is composed of aromatase inhibitors, anti-estrogens and selective estrogen receptor modulators (SERMs) and agents preventing activin receptor IIB activation. With metabolic modulators (adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptors (PPAR-δ), insulins, and insulin-mimetics, meldonium and trimetazidine) they constitute the S4 class of prohibited substances [1].

Hormone

Hormones count numerous compounds with various masses, properties, and metabolism. HRMS has been used in various fields for different goals. Kollmeier et al. reported LC-Q-TOF use for structural confirmation of fragments ions of formestane and its fragment ion pathway elucidation [88].

In biological matrices, HRMS had demonstrated its interest to formally identify compounds, as in case of agents preventing activin receptor IIB activation, for which differentiation from endogenous antibodies consists of a detection challenge. In a first analytical step, this kind of compounds have to be reduced to be observed as their molecular weight is too high. In case of follistatin, bimagrumab and domagrozumab, tryptic digestion was required before analysis [89—91]. Then, using LC-HRMS or nanoLC-HRMS, two or three peptides related to the hormone were monitored to formally identify the compounds. In case of bimagrumab, addition of ion mobility as orthogonal separation technique has facilitated unambiguous detection of the hormone. For a more sensitive detection and to facilitate isolation of compound from serum, combination of immunoaffinity purification using antigen coated magnetic beads, tryptic digestion and detection by HRMS was performed.

With the accurate mass data, HRMS was also used in metabolism studies. Mainly in urine, as it is the major matrix used in doping control, metabolism studies were conducted for several compounds with HRMS such as tamoxifen [92,93], toremifene [92,93], cyclofenil [94], clomiphene [93,95], arimistane [96]. Most of these studies were performed in human matrices and some in animals such as horses [94]. Dahmane et al. reported identification of tamoxifen metabolites in serum in 20 clinical samples [97]. Instrumentations used were Q-Orbitrap [92] or Q-TOF [93,95,96] coupled to liquid [92,93,95] or gas [96] chromatography. In all cases, spectrometric accurate information was obtained, offering the possibility to identify new metabolites, which could be potential biomarkers for monitoring compound administration in matrices.

To highlight the presence of hormones in real biological samples, HRMS were already largely used for its high sensitivity. First, in a qualitative way as reported by Ameline et al. who used LC-Q-TOF as confirmation technique to highlight presence of letrozole in hair samples (LOD 1 pg/mg) [98]. Thus, in a quantitative way, as Favretto et al. who demonstrated that LC- LTQ-Orbitrap was recommended to detect weak concentrations of the same compound in hair samples (LOD 5 pg/mg) and to discriminate a chronic use to an occasional administration [99]. In both cases, samples were analysed in the context of doping. Fabresse et al. reported a validated LC-Q-Orbitrap method for numerous compounds applied to whole blood and hair samples [26]. Tamoxifen LOD of 1 ng/mL and 10 pg/mg respectively in blood and hair allowed to highlight it in two forensic *post-mortem* cases. Deaths were not related to tamoxifen, but doping products were expected and confirmed thanks to HRMS.

Metabolic modulators

AMPK activators and PPAR-δ agonists activate the transcriptional receptors which are involved in processes enhancing aerobic capacity and increasing mitochondrial genesis. Administration of this kind of substances could improve physical performances and endurance. Thus, these compounds are monitored by doping labs.

The endogenous substance 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is an AMPK activator. This polar compound was studied by HRMS after dilute-and-shoot injection whatever the biological matrix plasma [100] or urine [100,101]. As AICAR is an endogenous substance, a reliable quantification is required to distinguish between endogenous concentrations and illicit application. Görgens et al. validated LOD inferior to 10 ng/mL in urine using LC-Q-Orbitrap and a HILIC column [101], whereas Kwok et al. reported LOD of 2.1 ng/mL and 13.7 ng/mL in plasma and urine, respectively, using LC-Q-Orbitrap [100].

Further applications of this family of substances concern the study of *in vitro* and *in vivo* metabolism which will be described in section 3.1.

Insulins

Human insulin is an endogenous anabolic hormone essential to regulate carbohydrate metabolism, leading to regulation of blood glucose level. Insulin therapy is required for type 1 and many type 2 diabetic patients. Two types of insulin analogues were synthesised according to onset and half-life: the rapid-acting analogues such as aspart, glulisine and lispro; and the long-acting analogues such as glargine, detemir and degludec. In addition to use in a medical context, insulins were misused in years following their commercialization as in doping to provide energy reserves as glycogen and to decelerate catabolism [102,103]. Therefore, use of insulins in sports has been forbidden since 1999 [103]. The MRPL is 50 pg/mL in human urine [6]. Moreover, insulins have also been used for suicidal or criminal purposes. Identification and quantification of insulins are complex in biological matrices. In doping the most analysed matrix is urine. However, as insulins are reabsorbed at 99% by proximal tubular cells, a small amount of protein is urinary excreted. In case of blood analysis (doping/clinical/forensic context), insulins undergo a rapid degradation in presence of hemolysis which requires a specific sample management and leads to low concentrations.

To formally identify insulins, the use of mass spectrometry is required as only few amino acids modifications exist between human insulin and their analogues. Last years, development of insulin analysis by mass spectrometry has extended, particularly in doping. Recently, reviews of literature dedicated to insulins presented the used technologies to analyse these compounds: quadrupole, orbitrap or TOF [102,104]. The limitation of TOF systems is their limited resolution generally around 40,000 FWHM, whereas Orbitrap systems could rise resolution until > 400,000 FWHM. This point is interesting as to distinguish glulisine form aspart a minimum resolution of 400,000 FWHM is required [102]. Without this resolution, distinction could be done by comparison of product ions after fragmentation. In the same way, as the difference between lispro and human insulin is only a switch between two aminoacids, they share the same molecular mass. Thus, no distinction in full-scan or SIM mode is possible and fragmentation must be performed to distinguish the first from the second. The product ion m/z 217.118, corresponding to fragment B (y2), is specific to lispro.

In doping, the most recent methods reach very low concentrations of insulins. Thomas et al. proposed insulins quantification in plasma using an LC-Q-Orbitrap method with LODs between 0.2 to 0.5 ng/mL [105] or using an LC-Q-TOF method presenting LODs between 0.1 to 0.8 ng/mL [106]. As long-acting insulins detemir and degludec have a different structure with a fatty acid group acylated to their peptic chain, LODs are generally more important for these two insulins. In urine samples, LODs of 10 pg/mL were validated for rapid-acting insulins by coupling nanoLC with a Q-Orbitrap [107] or an LTQ-Orbitrap [108]. To reach these concentrations several milliliters of samples were extracted. Concerning forensic context, a LOD was validated at 1.0 ng/mL for rapid-acting insulins (2.5 ng/mL and 10 ng/mL for degludec and detemir, respectively) in *postmortem* blood (250 μL) by LC-Q-Orbitrap [109]. Using the same system, a qualitative method was validated in *postmortem* tissues with a LOD between 25 to 80 ng/g according to insulins and matrices (kidney, liver, muscle, injection site) [110]. Insulins were also quantified in vitreous humor (500 μL) by an LC-Q-Orbitrap method with LOD of 0.2 ng/mL [111].

Using HRMS coupled with sample pretreatment such as immunoaffinity or immunopurification, LOD were considerably diminished. Thus, insulins are ever better detected in all contexts. Finally, HRMS offers the possibility to some authors to study insulin metabolism particularly in urine samples in doping [112].

Insulin mimetics

Among metabolic modulators, some compounds are considered as insulin mimetics as they mimic the action of insulins by activation of tyrosine kinase domain of the insulin-receptor or by inhibition of the protein tyrosine phosphatases [113]. Some of them were synthesised from plants or fungal extract such as the benzoquinone derivative L-783,281 [113] or generated from screening of phage display peptide libraries such as s597 [114]. These compounds are not authorized in sports because they have properties enhancing athlete's performances. Globally, HRMS have been used to identify structure of these compounds and to monitor their presence in samples in the laboratory as they are potential doping agents [114].

Meldonium

Meldonium was synthesised in the 1970s as a growth-promoting agent for animals. Later, the drug was approved for its medical indications such as its anti-ischemic properties [115]. It has been then misused in sports for its positive effect on the endurance performance of athletes, its enhancement of rehabilitation after exercise, its protection against stress and its activation of central nervous system functions [115]. Meldonium has been included in the WADA list of prohibited substances since 2016. MRPL is 200 ng/mL in human urine [6]. As meldonium is a highly polar compound its extraction from biological samples simultaneously with other major doping agents presenting different properties is a real challenge. However, its excretion in urine under unchanged form makes it an ideal compound for ''dilute-and-shoot'' analytical process. Thus, recently, a screening method of meldonium and over 300 doping agents was published. To detect meldonium at a satisfactory concentration, 10 μL of fresh urine was added to an SPE (C18) extract before injection on a LC-Q-Orbitrap, resulting in a LOD of 100 ng/mL [116]. In 2015, Görgens et al. reported a simple dilution of urine sample before injection on LC-MS/MS (initial testing assay) and on LC-Q-Orbitrap (confirmatory analysis). The use of HRMS allowed validation of a LOD of 10 ng/mL from 270 μL of urine sample, compared to 200 ng/mL with LC-MS/MS [115]. The disadvantage of the simple dilution is the presence of endogenous interferences which could interfere with analysis and lead to false positives as experienced by Kim et al. with acetylcholine [117]. However, product ions of meldonium can be separated in HRMS. Indeed, positive mode is used to analyse meldonium

and the monitored ions are the precursor ion m/z 147.1128 [115,116] and the most abundant product ion m/z 58.0654 [115,117]. A LC-Q-Orbitrap method was also used to determine urinary detection windows of meldonium for doping control purposes after single or multiple oral administration [118].

Trimetazidine

Trimetazidine is a coronary vasodilator and was prescribed from 1963 for the treatment of angina pectoris [125]. It has been then misused in sports for its mechanism of action, which imply a decrease on demand for oxygen when glucose is used instead of free fatty acids [119]. Thus, trimetazidine has been included in the WADA list of prohibited substances since 2014. To perform its analysis several types of urine sample pre-treatment were reported: a simple dilution [120] or a direct injection after addition of internal standard and centrifugation [119] or a SPE extraction from 5 mL of sample [121]. Sigmund et al. used an LTQ-Orbitrap method to measure accurate mass of trimetazidine and its urinary metabolites. The method was applied to four real samples from athletes and the authors obtained elemental composition and structure information on main trimetazidine metabolites in urine [119]. They observed that the most abundant signal was generated from the unchanged drug rather than its metabolites. Apart following trimetazidine intake, the compound could be detected after lomerizine administration. This substance is permitted in sports. Thus, in doping laboratories it is essential to distinguish lomerizine from trimetazidine intake.

Screening for diuretics and masking agents

Masking agents modify urine excretion or induce hemodilution and thus, could be used in sports to falsify the results of doping controls. The substance class of masking agents comprises primarily diuretics but also includes probenecid and plasma expanders, as well as substances with a similar chemical structure or biological effect.

Diuretics family is composed of substances which increase urine flow and volume and decrease body mass [122]. These characteristics have for consequence to dilute residues of potential sport doping agents; thus, diuretics are included in the WADA list of prohibited substances in sports (S5 class) [1]. The MRPL is 250 ng/mL for this class of substances [6]. Giancotti et al. tried to elucidate the fragmentation pattern of diuretics by using Q-Orbitrap. In this class of substances, all compounds have not the same chemical properties and acid-base behavior, as amiloride which contains no acid function conversely to ethacrynic acid [123]. These differences required specific method adjustments. Indeed, intensity of amiloride was better with a positive ionization whereas intensity of ethacrynic acid, furosemide and bendroflumethiazide was better with negative ionization. This particularity was also described by Peters et al. [122]. Diuretics analyses have been developed by using HRMS in plasma [124], DBS [125], and urine [126], as well as in human [123,125] and animal [124,126] matrices. Wasfi et al. studied pharmacokinetic and metabolism of furosemide in camels after intravenous administration, as this substance is prohibited in camel racing. None of the expected metabolites were detected in plasma suggesting a possible urine excretion as unchanged or metabolized with a small extent [124]. Metabolism of bumetanide was also studied by using LC-Q-TOF in urine of rat and only 6 metabolites were identified [126].

Excepted plasma expanders and diuretics, desmopressin is to our best knowledge the masking agent the most studied by HRMS. Desmopressin induces haemodilution by decreasing haematocrit and haemoglobin concentration [127]. During numerous years, desmopressin was detected by immunoassay. Even if the risk of cross-reaction with its endogenous analog, vasopressin, was weak, it became mandatory to formally identify compounds in doping control. Thus, several methods were developed in HRMS to analyse specifically desmopressin in urine [125,128—130] and in plasma [131]. LODs ranged from 50 pg/mL [128] to 500 pg/mL [130]. In order to formally identify desmopressin and to discriminate its detection from those of vasopressin, it was required to perform analysis on the reduced peptide which allowed to target specific ions [128]. With desmopressin analysis by using Q-TOF, it was demonstrated that HRMS was a reliable technique for large molecules such as peptides quantification [132].

Plasma expanders significantly increase plasmatic volume. They prevent dehydration and decrease hematocrit values to conceal erythropoietin abuse [133]. Hydroxyethyl starch (HES) and dextran are the major representative compounds of this class of substances and have been the most studied by using HRMS to this day. HRMS methods have been developed for this type of substances because with other detection techniques it was impossible in matrix to discriminate oligosaccharides and glucose from endogenous compounds. For example, in diabetic patients, concentration of glucose is often abnormally high [134]. HRMS allowed to formally distinguish the different polysaccharides. These large substances were easily fragmented and produced protonated charged fragments [135]. Nevertheless, due to their high molecular weight, these compounds produce numerous precursor ions in the source. To overcome this issue, a strategy based on in-source collision-induced dissociation (CID) has been developed [133,135]. This technique leads to specific fragments in source that could be used to formally identify substances. More recently, Wang et al. observed that using the maximum energy of 100 eV in ionization source, HES and dextran induced large fragments (> m/z 1000) implying a weak intensity. The authors developed a technique coupling all ion fragmentation (AIF) and in quadrupole fragmentation to achieve high fragmentation efficiency (HCD). Obtained fragments were lighter (< m/z 1100) and peak intensity was 100 times higher. By coupling in-source CID at 100 eV and HCD fragmentation (NCE 40%), Wang et al. improved intensity of plasma expanders detection to reach a LOD of 3 μg/mL for dextran in rat plasma [136].

Screening for glucocorticoids (prohibited only in competition)

Glucocorticoids are a group of low molecular weight lipophilic drugs belonging to the steroid hormone class, a

category of hormones produced by the adrenal cortex under stress conditions.

These substances have an analgesic and anti-inflammatory effect and are therefore used to treat inflammatory diseases such as arthritis, asthma or rhinitis. However, in some areas they may be misused in livestock feed as growth promoters or as doping substances in the world of sport to improve athletic performance.

Thus, glucocorticoids promote gluconeogenesis by stimulating the breakdown of fatty acids and proteins and suppress the feeling of tiredness and pain [137].

These drugs have a wide range of adverse effects on the cardiovascular system, bone tissue and central nervous system, and therefore uncontrolled use of these drugs could be harmful to human health. In addition, some hormone-regulated tissues, such as the breast, ovaries and prostate, may respond with abnormal cell proliferation leading to hyperplasia [138].

Glucocorticoids are tested by a wide variety of analytical methods including immunological methods such as RIA, but also methods such as GC and LC - MS or UV.

Although immunoassays are sensitive and characterised by ease and speed of use, their disadvantages are their lack of specificity and the risk of cross-reactions with precursors or metabolites.

As confirmatory methods, GC and LC - MS are preferred. However, GC is not optimal for measuring glucocorticoids in complex biological matrices due to the lack of suitable derivative agents [139].

Due to their sensitivity and selectivity, LC-MS is considered the gold standard for the analysis of glucocorticoids in complex biological matrices.

However, glucocorticoids have to be researched in trace amounts in biological matrices due to their short half-life. For this reason, in recent years HRMS is gaining interest in research laboratories and laboratories performing routine analyses.

Herrero et al. in 2014 [140] compared triple quadrupole mass spectrometry and orbitrap for the determination of 9 glucocorticoids in complex matrices such as wastewater and obtained a good correlation between the two methods. However, HRMS prevented false results due to full-scan acquisition, so that interferences from the matrix can be identified and evaluated. Parameters such as LOD and LOQ proved to be similar. For some analytes, LOD and LOQ were even better using the HRMS. The methods developed provided a LOD of 5 pg/mL and a LOQ of 20 ng/mL for most of the substances researched.

Because of their abuse in the field of sport, WADA has published a list of substances belonging to this class that are prohibited [1].

Due to their short half-life, finding these substances in blood and urine samples is very difficult and if detected they are found in very low concentrations.

For this purpose, various methods have been developed for the identification and dosage of these substances in serum, plasma and urine samples using high-resolution analysers [141—145].

The preferred extraction method for serum samples is generally LLE using MTBE as extraction solvent. Some authors using Q-TOF as detection systems have obtained LOQs of 5 ng/mL for molecules such as prednisone and prednisolone [142], while others using Q-Orbitrap have achieved LOQs of less than 1 ng/mL for substances such as cortisone, corticosterone and cortisol [141].

The misuse of these substances in farmed meat has raised several fears about the presence of steroid hormones in foods of animal origin.

To ensure the safety and health of consumers, it has been necessary to develop methods capable of identifying both these substances and their degradation products in the meat of various animals consumed by humans.

For the qualitative and quantitative determination of these substances, GC and LC-MS are the most widely used. However, due to the difficulties associated with the derivatization reaction, LC has become more popular for the analysis of these compounds.

The need to search for traces has led in recent years to the expanded use of HRMS, which not only allows retrospective data analysis but also the identification of residues of these substances at very low concentrations.

Lopez et al. [138] developed a method based on a LC-Q-Orbitrap for the determination of steroid hormones and their metabolites in three types of animal meat. Using the QuEChERS extraction method, good results and recovery were obtained. The authors achieved LOQs ranging from 1.0 to 2.0 μg/kg. To prove the suitability of the method, it was applied to samples of farm animal meat to see if there was any misuse of these substances. Two steroids were found in two different meat samples. The development of sensitive methods provides information on the misuse of these substances and since most glucocorticoids have many fragments in common, this can be used to identify new compounds not present in the databases.

In fact, the ban on the use of glucocorticoids for non-medical purposes has led to the search for counterfeit products on the black market that pose a risk to public health [146,147].

Another important analytical challenge in the anti-doping field is the efficient separation of isomers which are often the cause of legal arguments.

In this respect, HRMS allows the discrimination of each isomer at very low concentrations from complex biological matrices [148—150].

Karatt et al. [148] have discussed the issue of three glucocorticoids: dexamethasone, betamethasone and paramethasone. The first two are mainly used to treat metabolic and inflammatory diseases, while the last one is used to treat asthmatic diseases and allergies. The similarity of these molecules also results in similar behaviour at chromatographic level, which is the reason for the difficulty in separating the isomers using a classical chromatographic system.

In the study, they were able to distinguish these isomers on the fragmentation pattern of each isomer, which represents the fingerprint of a molecule, and the ratios between the various ions formed, using a LC-Q-Orbitrap.

The fragmentation pattern they proposed was then applied to complex matrices such as blood and horse urine, treated with LLE for the first one and SPE for the second.

The authors have demonstrated that they can distinguish these co-eluting isomers even in samples in which all three are present at very low concentrations.

Innovative approaches

In vitro and *in vivo* drug metabolism studies

The increasing variety of HRMS techniques based on soft ionization and TOF single/triple-stage or hybrid MS-instruments, gives precious information to propose or assign structures to supposedly generated metabolites. In recent years, an increasing number of research projects have been carried out focusing on the structural elucidation of presumed metabolites of doping substances using HRMS. Considering the huge amount of data produced by these contributions, only an overview of these studies is presented below. The analytical workflows used in the reviewed studies were summarized in Table 2.

In a research focused on the detection and characterization of stanozolol metabolites in human urine, Pozo et al. reported the application of a LC-Q-TOF method to mice and human excretion studies. A total of 19 stanozolol metabolites were detected and a possible structure for each metabolite has been proposed on the basis of the product-ion spectra with accurate TOF mass measurements [151]. Another excretion study based on the urine analysis from four volunteers receiving the aromatase inhibitor exemestane was carried out to detect new exemestane metabolites in human urine by GC-HRMS, after derivatization with methoxyamine and MSTFA/TMS-imidazole [152]. The metabolism of the stimulant benfluorex was investigated in 2010 by Thevis et al. LC-Q-Orbitrap experiments were carried out for the characterisation of four metabolites detected in urine samples coming from a patient treated with a single oral dose of 300 mg benfluorex [153].

In vitro models simulating human, or animal metabolic pathways were used to obtain information on drug metabolism in the early stages of clinical studies. These methodologies have been applied to the so-called new designer drugs under clinical approval.

For example, the metabolism of the drug candidate S107 (a substance tested for the treatment of cardiac arrhythmia that improves cardiac and skeletal muscle functions) was investigated using *in vitro* models, and numerous generated phase I and II metabolites were characterised by LC-LTQ-Orbitrap experiments [154]. Another class of non-approved drugs with an increasing appeal in the sports doping community is SARMs. Arylpropionamide-derived SARMs were investigated using human liver microsomal (HLM) preparations and the structures of the metabolites were identified by HRMS [155,156]. *In vitro* generated metabolites were confirmed by *in vivo* experiments, and several metabolites were selected as target analytes for sports drug testing [157]. Similarly, the metabolism of LGD-4033 (Ligandrol), a SARM with a pyrrolidinyl-benzonitrile core, was extensively studied by in vitro metabolism experiments employing HLM and Cunninghamella elegans (*C.elegans*) preparations, as well as electrochemical metabolism simulations [158]. Five mono and bis-hydroxylated metabolites were identified and structurally confirmed by LC-HRMS experiments. In following studies, some of these metabolites were detected and characterised by LC-HRMS in human and equine urine samples, after the controlled administration of LGD-4033 [159,160]. The *in vivo* metabolism of the steroidal SARM YK-11 and the anabolic steroids methylstenbolone and trenbolone was investigated by Piper et al. by analysing post-administration urine specimens. Several metabolites, relevant to sports drug testing, were characterised using GC-HRMS (as trimethylsilyl derivatives) and/or LC-HRMS instruments [18,161,162]. Moreover, GC-HRMS and GC-MS/MS techniques were recently employed for detecting novel A-Ring reduced metabolites of the anabolic steroids methyltestosterone and metandienone [163].

Regarding the development of analytical methods able to detect small peptides for anti-doping purposes, the *in vitro* metabolism of GnRH was studied by HRMS [61,164]. Ten synthetic analogs of GnRH were investigated using human kidney microsomes (HKM), and the structures of the metabolites were identified by LC-Q-Orbitrap [164]. In the same study, the *in vitro* obtained metabolites were added into UPLC-MS/MS method for GnRH analogs determination, and the presence of one of these was confirmed in the real urine sample.

Several in vitro studies have been carried out on HLM to investigate in vitro metabolism of GHS and GHRPs, and various methods have been developed for this purpose, particularly using HRMS [62,73,165—167].

Guan et al. [166] describe an approach using liquid chromatography coupled to a quadrupole- orbitrap mass spectrometer, in which they highlight the interest of using a HILIC column instead of classical reversed-phase chromatography columns due to the high hydrophilicity of metabolites that are poorly retained in this type of column. For the same purpose Lange et al. [167] performed an *in vitro* study for metabolites of 3 ghrelin mimetics and identified 76 metabolites.

SR-9009 is a synthetic AMPK activator. HRMS was used to study its metabolism in urine [168,169]. Cutler et al. identified *in vitro* SR-9009 metabolites in horse plasma and urine. The metabolites were confirmed by *in vivo* analysis of the same matrices in real samples after SR-9009 administration. The authors described HRMS as an important tool to metabolism investigation [169].

PPAR-δ agonists potentially used in sports are synthetic compounds such as adiporon and 112254 for which *in vitro* and *in vivo* metabolism was studied in rat urine. Characterisation of metabolites was possible thanks to HRMS use [170]. The most studied PPAR-δ agonist by HRMS is GW1516 or GW501516. Its detection by MS is possible with a positive [171,172] and a negative mode [173]. However, Ishii et al. reported that addition of 10 mmol/L of glycerin in acetonitrile to the final extract could facilitate yield of $[M-H]^-$. Then, using the negative ionization mode with this modification, MS intensities were 10-fold higher than those obtained in the positive mode [173]. The two major identified metabolites of GW1516 are the sulfoxide and the sulfone. In addition, metabolism study by HRMS allowed to identify seven other metabolites in horse plasma and urine [174].

Okano et al. developed an LC-Q-TOF method to determine lomerizine metabolites structures in urine. They concluded that trimetazidine was only detected in *in vivo*

Table 2 Overview of metabolism studies on doping agents using HRMS.

Compounds	Prohibited class	Sample	Pretreatment	MS-instrument	Ionization mode	Reference
Stanozolol	S1	Mice urine Human urine	Enzymatic hydrolysis LLE (pH 9.2)	LC-QTOF	ESI±	[151]
Exemestane	S4	Human urine	Enzymatic hydrolysis LLE (pH 10) Derivatization (TMS)	GC-QTOF	EI	[152]
Benfluorex	S6	Human urine	Dilute and shoot	LC-Q-Orbitrap	ESI+	[153]
S-107	S0	HLM and S-9	Enzymatic hydrolysis SPE (Oasis HLB)	LC-LTQ-Orbitrap	ESI+	[154]
4 Aryl-Propionamide derived SARMs	S1	HLM and S-9	LLE (acidic pH)	LC-LTQ-Orbitrap	ESI-	[155]
Ostarine Andarine	S1	HLM and S-9 Dog urine	LLE (pH 4.5-5.0) SPE (PAD-1)	LC-Q-Orbitrap	ESI-	[156]
Andarine	S1	Human urine	Enzymatic hydrolysis SPE (PAD-1)	LC-LTQ-Orbitrap	ESI-	[157]
LGD-4033 (Ligandrol)	S1	HLM *C. Elegans* suspension	Dilute and shoot	LC-Q-Orbitrap	ESI-	[158]
LGD-4033 (Ligandrol)	S1	Human urine	Enzymatic hydrolysis Dilute and shoot	LC-QTOF	ESI-	[159]
LGD-4033 (Ligandrol)	S1	ELM Equine urine	Enzymatic hydrolysis LLE (pH 6.8)	LC-LTQ-Orbitrap	ESI-	[160]
YK-11	S1	Human urine	Enzymatic hydrolysis SPE (C18) + LLE (pH 7-10) HPLC Clean-up Derivatization (TMS)	GC-QTOF GC-Q-Orbitrap	EI	[161]
Methylstenbolone	S1	Human urine	Enzymatic hydrolysis SPE (C18) + LLE (pH 7-10) HPLC Clean-up Derivatization (TMS)	GC-QTOF GC-Q-Orbitrap	EI	[162]
Trenbolone	S1	Human urine	Enzymatic hydrolysis SPE (C18) + LLE (pH 7-10) HPLC Clean-up Derivatization (TMS) for GC	LC-Q-Orbitrap GC-QTOF GC-Q-Orbitrap	ESI+ EI	[18]
Methyltestosterone Metandienone	S1	Human urine	Enzymatic hydrolysis LLE (Basic pH) Derivatization (TMS)	GC-QTOF	EI	[163]
10 GnRH	S2	HKM Human urine	SPE (Oasis WCX)	LC-Q-Orbitrap	ESI+	[164]
27 bioactive peptides	S2	Equine plasma	SPE (anion exchange)	HILIC-Q-Orbitrap	ESI+	[166]
Capromorelin Macimorelin Tabimorelin	S2	Rat urine Human serum HLM	Dilute and shoot/SPE (urine) Protein precipitation (serum, HLM)	LC-Q-Orbitrap	APCI+	[167]
SR-9009 SR-9011	S4	HLM	Dilute and shoot	LC-Q-Orbitrap	ESI±	[168]

Table 2 (*Continued*)

Compounds	Prohibited class	Sample	Pretreatment	MS-instrument	Ionization mode	Reference
AdipoRon 112254	S4	HLM and S-9 Rat urine	Protein precipitation and evaporation (HLM, S-9) Direct injection (urine)	LC-Q-Orbitrap	ESI+	[170]
GW1516	S4	HLM	Protein precipitation ± LLE	LC-Q-Orbitrap	ESI+	[172]
Lomerizine/ Trimetazidine	S4	HLM Human urine	SPE	LC-QTOF	ESI+	[121]

HLM: Human Liver Microsomes; HKM: Human Kidney Microsomes; ELM: Equine Liver Microsomes; LLE: Liquid-Liquid Extraction; SPE: Solid-Phase Extraction; S-9: S9 Fraction; ESI: Electrospray Ionization; EI: Electron Ionization; TMS: Trimethyl-silylation; LTQ: Linear Trap Quadrupole; QTOF: Quadrupole time-of-flight.

samples [121]. More recently, using a LC-Q-Orbitrap the same team developed a very sensitive method to detect lomerizine, trimetazidine and another specific metabolite of lomerizine (M6) in human urine. From 0.5 mL, LOD and LOQ of trimetazidine were assessed at 0.02 ng/mL and 0.05 ng/mL, respectively. Their previous method without high resolution could not reach these limits. The proof of concept was performed on ten real doping cases, concluding that after lomerizine intake trimetazidine is systematically detected in association with the M6 [120].

Alternative matrices: hair and dried blood spots (DBS)

The study of alternative specimens (e.g., oral fluid, hair, exhaled breath, DBS, nails) has gained significant relevance in forensic and clinical chemistry, including sports drug testing. In the last years, among the alternative matrices, hair and DBS have received particular attention in doping analyses, showing advantages but also exhibiting limitations if compared to the currently employed routine doping control samples consisting principally of urine and blood.

Supporting urine and blood analysis, hair testing demonstrated to provide complementary information in selected cases of AAFs with banned substances, differentiating the misuse of the drug from a single inadvertent and/or accidental intake [175]. On the other hand, the speed and ease of sampling of DBS, together with its low invasiveness, intrusiveness, risk of adulteration, and low transportation and storage costs, make the DBS attractive for the application in sports drug testing [176]. Therefore, several analytical methods were recently developed for the detection of doping agents in hair and DBS, including methods based on HRMS detection. Today, HRMS able to scan all masses in a predefined m/z mass range for the duration of the analysis at high resolution without loss of sensitivity are present on the market. This makes it possible to search a very large number of compounds simultaneously and allows for retrospective analysis of compounds that were not originally included in the method.

Hair

One of the first applications of HRMS for the analysis of anabolic steroids in hair samples was presented by Strano-Rossi et al. in 2013 [177]. The procedure for the detection of 25 among anabolic steroids and their esters consisted of an overnight methanolic extraction of the hair sample (30 mg) by sonication, followed by a LC-HRMS screening using a Q-Orbitrap mass spectrometer operating in full scan mode (m/z 110—800) in APCI under positive ionization mode, with nominal resolving power of 100,000 FWHM. In case of screening positive results, a confirmation method based on in-source CID was developed, acquiring ions from 70 to 500 m/z with a resolving power of 50,000 FWHM, and obtaining the accurate masses of both precursor and fragment ions. The characteristic fragmentation of the analytes, the exact masses of the precursor and fragment ions (mass error within ± 1 ppm) together with the characteristic isotopic cluster allowed the correct identification of the analytes. Moreover, the method was validated for quantitative purposes with LODs values in the interval 0.01-0.05 ng/mg and a LOQ of 0.5 ng/mg for all analytes. The method was successfully applied to a real-life case, resulting in the identification of testosterone undecanoate (measured accurate mass of 457.3678; mass error < 0.5 ppm) in the hair of a suspect at a 5 ng/mg concentration.

A similar study on the detection and quantification of 11 anabolic steroids in the blood (androstenedione, dihydrotestosterone, boldenone, epitestosterone, mesterolone, methandienone, nandrolone, stanozolol, norandrostenedione, testosterone, trenbolone) and eight more in hair samples (nandrolone phenylpropionate, nandrolone decanoate, testosterone propionate, testosterone benzoate, testosterone cypionate, testosterone decanoate, testosterone phenylpropionate, testosterone undecanoate) using a Q-Orbitrap mass spectrometer was published by Fabresse et al. in 2017 [26]. Again, the ionization was carried out with an APCI ion source operating in positive ionization mode, and the protonated molecular ions of the steroids were identified with exact mass (mass error < 3 ppm for all analytes) and retention time. Data were acquired in full

scan mode over a mass range of 100—800 m/z at the maximal resolving power of 140,000 FWHM. The method was also validated for the quantification of the steroids in different matrices. LODs values in the hair matrix were in the interval 2-20 pg/mg and LOQs were between 10 and 100 pg/mg, with the higher LOD/LOQ values obtained for the esters. The method allowed the detection and quantification of testosterone (18 pg/mg), trenbolone (10 pg/mg), and nandrolone (23 pg/mg) in two *postmortem* hair samples.

Favretto et al. published an HRMS method for the detection and quantification of the aromatase inhibitor letrozole in hair [99]. The hair analysis was performed on an LC-LTQ-Orbitrap system with the scope of discriminating voluntary doping vs. inadvertent ingestion of letrozole in a real case. The detection was carried out with the mass spectrometer operating in positive ESI mode and the mass acquisition in the range of m/z 80—600 using a target mass resolution of 60,000 FWHM and a scan time of 0.65 sec. The detection of the analytes (letrozole and its main metabolite bis(4-cyanophenyl)methanol) was based on accurate mass measurements of the protonated ions (286.1087 and 235.086), the correspondence between the observed isotopic pattern and the calculated one, and the relative retention time. Starting from 10 mg of hair sample, values of 5 pg/mg and 15 pg/mg were calculated for LOD and LOQ, respectively, for letrozole. The sensitivity of this method made possible the detection of letrozole in head hair, after a single intake of 0.62-2.5 mg of letrozole, at a 16 to 60 pg/mg concentration. No presence of letrozole metabolite was revealed in the real samples.

An analogous study concerning the detection of letrozole in hair samples by LC-MS/MS and LC-HRMS was recently published by Ameline et al. [98]. LC-Q-TOF technique was employed as a confirmatory method after the LC-MS/MS analysis. Letrozole was extracted from hair (20 mg) after 1 h methanolic extraction at room temperature and solvent evaporation. The HRMS system consisted of a Q-TOF mass spectrometer operating in ESI positive ion mode. The data were acquired in full-scan mode from m/z 50 to 1000, at 6 eV, while for MS/MS fragmentation of target ions, collision energy ranging from 10 to 40 V was applied. Letrozole was correctly identified with a mass error less than 5 ppm from its exact mass. The combination of two independent methods for the detection of a substance (LC-MS/MS and LC-HRMS) is a requested requirement by the doping authorities to consider a valid hair test, as WADA accredited laboratories do the same for the confirmation of any adverse analytical findings.

Following this approach, several doping real-case studies regarding the application of the LC-HRMS as a confirmation method after the LC/MS-MS detection in the hair of stanozolol [178], ostarine [179] and cardarine [171] were presented by Kintz et al. In all studies, the HRMS detection was based on a Q-TOF instrument. Conditions for the TOF spectrometer scan mode were as follows: scan range m/z 50-1000 at 6 eV (low energy) and m/z 50-1000 with a collision energy ramp from 10-40 eV (high energy). Stanozolol was detected after that 30 mg of decontaminated hair were incubated in 1 mL of 1 M NaOH for 10 min at 95 °C and extracted with diethyl ether. The presence of stanozolol (73 pg/mg) was confirmed in real head hair samples detecting the mass at m/z 329.2580, with a mass error of −2.2 ppm, when compared to a reference standard (m/z 329.2588). The presence of the SARM ostarine (S22 or MK-2866) was confirmed in real hair samples after incubation of 50 mg of the matrix in a pH 9.5 buffer, followed by extraction with organic solvents. The detection was carried out with the mass spectrometer operating in negative ESI mode. The correct identification of the analyte was achieved by monitoring the exact masses of the deprotonated molecule at m/z 388.0909 together with three diagnostic ions at m/z 269.0536, 118.0294, and 185.0323. The LOD was estimated at 0.5 pg/mg. The presence of ostarine was confirmed in real hair samples at concentrations between 12 and 168 pg/mg. Moreover, in the same context, the method was successfully applied for the detection of ostarine in real nails samples at concentrations of 111 and 61 pg/mg, in fingernails and toenails, respectively.

Likewise, the PPAR-δ agonist cardarine (also known as GW501516 or GW1516) was identified by LC-MS/MS and LC-Q-TOF in two 2-cm hair segments of a frequent user of the drug at a concentration of 32 and 22 pg/mg, after methanol incubation of 20 mg of hair with ultrasound for 1 hour [171].

Due to their capabilities to increase physical and endurance performances, PPAR-δ agonists were considered to have a high potential for use also in animal doping, including horse racing doping [173,174]. In this context, a study about the detection of cardarine and its metabolites in equine hair by LC-HRMS, after a controlled administration, was recently published by Ishii et al. [174]. The pulverized equine hair samples (200 mg) were extracted with methanol, followed by further purification, and the extracts were analysed using a Q-Orbitrap instrument equipped with a heated ESI source operating in both positive and negative ion mode. In order to reveal also the presence of cardarine metabolites, full MS experiments were carried out in the scan range m/z 50—750 with a nominal resolving power of 60,000 FWHM. In case of positive results, a further parallel reaction monitoring protocol based on in-source CID was developed, acquiring product ion spectra from the following precursor ions: m/z 452.0607 (cardarine), m/z 468.0557 (cardarine sulfoxide), and m/z 484.0506 (cardarine sulfone), plus other three precursor ions from minor metabolites. The method was fully validated and the estimated LODs and limits of confirmation (LOCs) for the three target analytes were 0.02 pg/mg and 0.03 pg/mg, respectively. After a single nasoesophageal dose of cardarine (150 mg) administered to a thoroughbred female horse (mare), the hair samples collected from 2 weeks post-administration turned out positive to cardarine and its metabolites, proving that an administration of a single low dose of cardarine can be detected by hair analysis.

In racehorse or greyhound doping the administration of AAS is often carried out by intramuscular injection of esterified forms, to allow a slow release resulting in extended half-life and prolonged effect. Therefore, the ability to detect intact esterified forms of endogenous steroids in hair is appealing as it can provide unequivocal proof of their exogenous origin. For this scope, Choi et al. developed a comprehensive LC-Q-Orbitrap method for the detection of 72 AAS and/or their esters in equine hair [180], as an update of a previous work on the detection of 48 AAS [181]. In order to allow more target analytes to be covered in a single injection, the Q-Orbitrap mass spectrometer operated

in targeted MS/MS mode, making use of the idling time during parallel the MS acquisition for filling of several precursor ions into the collisional cell for the next experiment. Starting from 50 mg of equine hair sample, and employing a combined LLE/SPE extraction procedure, LODs values were estimated between 0.25 and 80 pg/mg. The method was then applied for the analysis of two real samples coming from horse racing doping controls, allowing the detection of anabolic steroids at ppb levels, namely boldione (2 pg/mg), 4-androstenedione, and testosterone propionate (3 pg/mg).

A similar LC-HRMS approach for the detection of testosterone esters in dog hair following LLE combined with SPE clean up with Bond-Elut amino cartridges was proposed by Devi et al. [182]. In order to enhance the sensitivity of the method, testosterone esters were added with methoxyamine hydrochloride to form the methyloxime derivatives. The method was validated for the detection and quantification of the propionate, phenylpropionate, isocaproate, decanoate, and enanthate esters of testosterone in Greyhound hair. The LODs for the three testosterone esters, phenylpropionate, isocaproate, and decanoate was 0.05 pg/mg and the LOD for the propionate was 0.025 pg/mg while for the testosterone enanthate was 0.5 pg/mg. The Q-Orbitrap mass spectrometer was operated in heated electrospray ionization positive mode with an ion spray voltage of 3000 V. The acquisition was carried out in targeted MS/MS mode with a resolution of 35,000 FWHM. The method was applied to hair samples collected from male greyhounds before and after a single administration of a mixture of testosterone esters (3-4 mg/kg). Concentrations of testosterone esters in the interval 0.03-0.60 pg/mg were measured in dogs' hair from 35 to 91 days post-administration.

β_2-agonists such as clenbuterol can be detected in blood and urine for a relatively short period after administration. In order to improve the detection window for these substances and to know the history of consumption, some studies have been carried out on the analysis of hair matrices [183,184].

Schlupp et al. [183] administered 0.8 μg of clenbuterol to horses for 10 days. Up to 360 days after administration, mane and tail hair were analysed in addition to blood and urine. After 30 days, clenbuterol was no longer detectable in either blood or urine, while already on the 5th day, clenbuterol was detected in the hair. A method was developed using GC-Q-Orbitrap following extraction of the analyte by ultrasounds followed by SPE, which provided an LOD of 0.2 pg/mg and resulted in concentrations ranging from a minimum of 0.5 pg/mg to a maximum of 21 pg/mg.

In a study by Machnik et al. [184], better results were obtained by analysing hair collected from pregnant women under Spiropent (clenbuterol-HCl) treatment. The hair samples were digested by incubation in KOH at 70 °C, after LLE, purification by IAC, the sample was derivatized with MSTFA. Using a double focusing mass spectrometer with reversed geometry, a LOD of 0.8 ng/ng was obtained and concentrations in the range of 2 to 236 ng/g were found in the hair of the analysed women.

Dried Blood Spots (DBS)

One of the first approaches for the determination of prohibited drugs in DBS for doping controls was presented by Thomas et al. in 2012 [185]. In this study, the MS detection was carried out combining a quadrupole mass filter, an HCD cell and an ion trap analyser and acquiring the single product ion mass spectra for previously selected precursors of 26 model compounds (THC, THC-COOH, methylhexaneamine, methylphenidate, cocaine, nikethamide, MDMA, MDA, strychnine, mesocarb, salbutamol, formoterol, clenbuterol, methandienone, stanozolol, bisoprolol, propranolol, metoprolol, anastrozole, clomiphene, exemestane, dexamethasone, budesonide, andarine, SARM S1, and hydrochlorothiazide), at a resolution of 17,500 FWHM. The method also included a full scan data acquisition in positive and negative mode at resolving powers of 70,000 FWHM each, making possible a non-targeted analysis for retrospective data evaluation. Starting from 20 μL of blood sample spotted on Sartorius TFN DBS card and following a double LLE with methanol/tert-butyl-methyl-ether/acetone, LODs in the range 0.05-0.5 ng/mL were obtained, making the method suitable for an initial testing procedure for prohibited substances in doping control analysis.

A multi-target approach for the screening of eight AAS esters (nandrolone phenylpropionate, trenbolone enanthate, testosterone acetate, testosterone cypionate, testosterone isocaproate, testosterone phenylpropionate, testosterone decanoate, and testosterone undecanoate) plus nandrolone in DBS was developed by Tretzel et al. [186]. Again, the identification and determination of the target compounds was conducted by a Q-Orbitrap operating in targeted MS/MS mode. From 20 μL of blood on a Whatman DBS sampling card, LODs in the ppb range (0.5 ng/mL for nandrolone phenylpropionate and 0.1 ng/mL for the other analytes) were accomplished by the preparation of the methyloxime derivatives of the target compounds, as the analysis of the derivatized analytes showed a 10-fold increase of the peak area ratios if compared to the not derivatized 3-oxo-steroids. The method was then applied on DBS specimens collected after a single oral dose of 80 mg of testosterone undecanoate. The positivity of authentic samples in the time period 2-8 h post-administration substantiates the suitability of the DBS sampling technique for the analysis of intact anabolic steroids, allowing an unequivocal proof of the administration of conjugates of exogenous testosterone and its derivatives.

Another study proving the suitability of the DBS sampling coupled to HRMS detection for the analysis of anabolic steroids esters was recently proposed by Solheim et al. [187]. For the extraction, 20 μL of blood were spotted on the DBS sampling cards (FTA® DMPK-C), and 1 mL of a mixture of ACN:MeOH (50:50 v/v) was added to the punched spot. After filtration and derivatization with 2-hydrazino pyridine, the extracts were analysed by LC-HRMS/MS. The nanoflow LC system was combined with PRM using a Q-Orbitrap mass spectrometer. The resolution of the PRM experiments was set to 35,000 FWH, monitoring the protonated precursor ions in a m/z 2 mass window. The method was validated and the LODs were estimated between 50 and 200 pg/mL. The method was successfully applied on DBS samples from men receiving two intramuscular injections of a mixture of testosterone esters, some of which were detected for at least five days in all subjects receiving testosterone ester injections.

Regarding other anabolic agents, stanozolol, dehydrochloromethyltestosterone (DHCMT, oral Turinabol), and clenbuterol were detected in DBS by LC-HRMS/MS with an LOD of 0.1, 2.5, and 0.03 ng/mL, respectively [188,189]. To qualify the developed methods for doping control purposes, authentic DBS samples were analysed after a single oral dose of 5 mg stanozolol, 5 mg DHCMT, or 80 μg clenbuterol. The analysis of stanozolol and DHCMT in DBS resulted in peak levels of approximately 10 and 11 ng/mL in blood, respectively. Measurable concentrations of the drugs occurred within 3 days after the application. Similarly, clenbuterol was detected in DBS samples up to 24 h to 72 h after administration. These results showed that DBS could be a useful supplemental sample matrix for anabolic agents also in out-of-competition testing.

In light of the considerable blood concentrations of some therapeutic proteins, several studies on the detection of peptide hormones and growth factors in DBS by LC-HRMS were published in the last decade. In 2012, Moller et al. demonstrated that peginesatide can be analysed from DBS samples with a LOD of 10 ng/mL [190]. The method was based on the extraction, proteolytic digestion and cation exchange purification of DBS followed by a LC-HRMS/MS analysis monitoring the precursor ion at m/z 584.31 related to the target peptide GPIT (1-nal) resulting from the enzymatic digestion. Similarly, Lange et al. proposed the detection of the stimulating protein sotatercept and bimagrumab (myostatine inhibitor) from DBS samples by protocols like those applied to serum, with LODs between 250 and 500 ng/mL [191]. DBS (20 μL of blood) were extracted into an aqueous buffer solution supported by ultrasonication, and immunoaffinity purification was carried out by magnetic nanoparticles equipped with either immobilized Protein-G (initial testing procedure) or immobilized activin A (confirmation procedure). LC-HRMS/MS analysis, conducted on a Q-Orbitrap mass spectrometer operating in both full scan and targeted MS/MS mode, enabled the detection of the target analytes in spiked as well as authentic post-administration samples.

Another example demonstrating the suitability of the LC-HRMS technique for the analysis of higher molecular mass drugs, like insulin and its synthetic or animal analogues (human, lispro, aspart, glulisine, glargine, detemir, degludec, porcine and bovine insulin) in DBS was presented by Thomas et al. [192]. Starting from blood spots of 20 μL, the extraction protocol consisted of a double LLE with a mixture of acetonitrile/3% acetic acid (60/40, v/v) followed by a SPE and immunoaffinity purifications. Mass spectrometric detection was accomplished using either an ion mobility/Q-TOF or a Q-Orbitrap-equipped instrument, in full MS and MS/MS mode or targeted MS/MS mode, respectively. The assay validation demonstrated adequate sensitivity (LOD of 0.5 ng/mL for most insulins: 1.5 ng/mL for insulin detemir) allowing the detection of normal non-fasting levels of human insulin and eight synthetic analogs in DBS. Moreover, authentic DBS collected from a type-I diabetic individual using insulin Aspart were successfully analysed providing proof-of concept data.

A simple and effective procedure based on a fast methanolic extraction and a LC-HRMS detection of the growth hormone secretagogue ipamorelin in DBS was recently proposed by Gerace et al. [193]. MS and MS/MS data were collected by a Q-TOF mass spectrometer operating in the positive ESI mode. A double data-independent acquisition procedure for MS acquisition was carried out, namely:

- TOF-MS scan (m/z 230-800);
- TOF-MS/MS scan, monitoring all the fragments in a mass range 50-720 m/z.

The mass spectrometer acquired a full scan and MS/MS data at a resolving power of 35,000 FWHM. The positive electrospray ionization of ipamorelin resulted in the predominant doubly protonated ion at m/z 356.7001. To increase the sensitivity of the method, CID experiments of the precursor ion were performed, giving fragment ions derived from the y-series at m/z 146.1292 (y1), 490.2820 (y3), 627.3396 (y4), and the b-series at 223.1191 (b2), 420.2041 (b3) and 567.2720. Starting from 40 μL of blood, LOD and LOQ values, calculated using the Hubaux-Vos algorithm, were 2.5 ng/mL and 5.0 ng/mL, respectively.

Lange et al. developed a comprehensive LC-HRMS method for the quantitative detection of 46 low molecular mass (< 2 kDa) peptide/non-peptide drugs and drug candidates, after a fully automated DBS sample preparation [194]. For the sample extraction, a robotic multi-purpose sampler was used for the automatic sampling, purification (SPE and strong-cation exchange cartridges) and concentration of the DBS extracts. The MS was carried out on a Q-Orbitrap analysis comprising alternating full scan MS experiments (m/z 300 - 1500) and targeted MS/MS mode with an inclusion list of 53 precursor ions. The lower molecular mass peptides < 2 kDa were predominantly observed as doubly-charged molecules; LODs were in the range 0.5-20 ng/L. This procedure proved effective by testing GHRP-2 and GHRP-6 post-administration study samples.

Meldonium (classified in the section Hormone and Metabolic Modulators) was quantitatively detected in DBS by LC-HRMS [195]. Meldonium was tested in urine, plasma, dried plasma spots (DPS), and DBS samples, in the context of a controlled administration pilot study, by a validated LC-HR-MS/MS method with a LOD of 20 ng/mL.

The study revealed that the concentrations of meldonium in DBS were 30-times higher than those detected in DPS samples, showing that meldonium may be incorporated into erythrocytes and gradually released to plasma [195].

Black market

The desire to improve physical and mental performance has given rise to an illegal traffic on the Internet of food and dietetic supplements that can be supplemented with AAS, metabolic modulators or even peptide hormones. It is therefore necessary to analyse them to know the products in circulation. Several studies describe the identification of dietary supplements by HRMS.

Four studies in the literature describe the identification of performance products in dietary supplements over a long period.

In France, 75 pharmaceutical products were seized and analysed by LC-Q-Orbitrap over a 3 year-period from 2016 to 2019, but quantified by LC-MS/MS. The following products were identified: AAS (72%), PDE5 (tadalafil, sildenafil),

2-mimetic, peptide hormones, SERMs, aromatase inhibitors, diuretics [196].

The following analytical strategy was developed and applied in Switzerland and Germany. The identification was done by LC-Q-TOF then on GC-Q-TOF and then by nanoLC-HRMS, but the quantification was done by LC-MS/MS [42,197].

In Switzerland, 1190 supplements were analysed following this strategy and 75% were identified as AAS, 12% as peptide hormones, 9% as hormones and metabolic modulators. Anastrozole, aromatase inhibitor product, presenting a higher product quality compared to other seizures [197].

In Germany, 337 supplements were analysed following the same strategy. 288 were identified as AAS and 56 were other performance products. It is interesting to note that in this study, 55% of the AAS were not correctly labelled [42].

In Italy, a similar study was conducted from 2017 to 2019. Here the samples were identified and quantified by LC-Q-Orbitrap [198].

For some new compounds, like designer steroids, it is necessary to use multiple analytical tools simultaneously to accurately identify the compound, namely the liquid chromatography-high resolution accurate mass-mass spectrometry (LC-HRAM-MS). This was the case for the identification of two designer steroids, androsta-3,5-diene-7,17-dione and 2'-chlorotestosterone, in dietary supplements [199,200]. First, LC-MS screening allowed the detection of unknown compounds with steroid characteristics. Then, thanks to the simultaneous use of HRMS and NMR methods, unknown compounds have been identified.

Thevis et al. have identified thanks to LC-HRMS and comparison with reference material, S4 (Andarine), SARM, in oil solution purchased as tea extract [201]. More recefntly, Leaney et al. have analysed 20 SARMs marketed online by LC-HRMS. 60% of products were adulterated with other SARMs [202].

HRMS is very useful for testing large molecules as hormones and peptides. The hormone GHRP-2 has been identified by LC-HRMS offered as an over-the-counter food supplement [203]. The use of a LC-Q-Orbitrap allows the identification not-conjugated CJC-1295 in a sealed powder. Despite the absence of reference standards, the authors were able to characterise the peptide using HRMS, combining the mass spectrum with knowledge of its structure [72].

Another new rapid and versatile approach was presented by Doué et al., allowing the identification of 21 anabolic steroid esters in supplements by direct analysis in real time-high resolution mass spectrometry (DART) [204].

Finally, in a recent study, Chen et al. have elucidated two new corticoids (betamethasone dibutyrate and betamethasone tributyrate) thanks to the determination of exact mass using LC-Q-Orbitrap mass spectrometer [205].

To conclude, one molecule per family of dopants mentioned in this review has been identified in dietary supplements by HRMS. Nevertheless, it seems that AAS remain the most identified substances and the most popular by consumers.

Conclusions

Within the last decade, advances in mass spectrometry have promoted the introduction of more and more sensitive detectors and particularly HRMS. This technology counts several benefits as accurate measurements ($m/z < 5$ ppm), simultaneous untargeted screening with effective targeted accurate masses acquisition and time saving assay with possibility of retrospective data analysis. In the context of doping products analysis, HRMS has demonstrated its usefulness by formally identifying compounds from endogenous analogs and by easily distinguishing peptides and proteins with high sequence homology. With the constant evolution of prohibited substances list, another positive point of HRMS is its ability to easily add new compounds in spectral libraries and to search a very large number of compounds simultaneously. Moreover, structural elucidation of peptides and fragmentation patterns of various compounds is facilitated with these new spectrometers. With recent advances, both quantitative and qualitative analysis of doping agents and their metabolites could be performed on a single run. Methods coupling untargeted and targeted acquisition allow to enhance sensitivity without loss of spectral information. Globally, the same LODs have been observed compared to tandem mass spectrometry but the crucial point of HRMS is its higher dynamic range of detection. With their increasing sensitivity, actual high resolution mass spectrometers discriminate some isomers at very low concentrations from complex biological matrices such as urine, blood, DBS or hair samples. Moreover, the use of full scan HRMS allows to decrease the background noise coming from the matrix, reducing sample preparation steps without losing specificity. Thereby, HRMS is more and more used for metabolism studies in urine or to determine urinary detection windows of numerous compounds. Alone or in association with other techniques such as tandem mass spectrometry, HRMS is nowadays a method of choice for both research and routine analyses.

Disclosure of interest

The authors declare that they have no competing interest

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Article 2: Identification de l'insuline dans le domaine médico-légale : problématiques analytiques

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Identification de l'insuline dans le domaine médico-légal : problématiques analytiques

RÉSUMÉ

L'insuline est le médicament polypeptidique le plus utilisé pour traiter le diabète. Son utilisation dans un cadre non thérapeutique a souvent été décrite, allant de la consommation comme substance dopante dans le milieu sportif à l'utilisation à des fins suicidaires ou criminelles, et également dans le cadre des troubles factices anciennement appelés syndrome de Münchhausen. De plus, des versions illégales de ce médicament sont disponibles sur le marché noir et ont été responsables de nombreux accidents dans le passé. La recherche de l'insuline et de ses analogues synthétiques dans les échantillons biologiques *post-mortem* est très complexe. L'instabilité de l'insuline dans le sang total, les conditions pré-analytiques particulières, les poids moléculaires élevés et l'absence de méthode analytique suffisamment spécifique, ont amené la plupart des laboratoires de toxicologie à ne pas rechercher l'insuline dans ces échantillons biologiques en routine. Par conséquent, le nombre d'intoxication et de décès causé par l'administration exogène d'insuline est très certainement sous-estimé. Cet article décrit deux cas d'administration d'insuline exogène dans un contexte criminel de tentative de meurtre suivie d'une tentative de suicide. La présence d'insuline asparte dans les deux échantillons de sang (5,7 et 2,4 ng/mL) a fourni des preuves concrètes de l'administration d'insuline exogène. Il semble essentiel, pour la santé publique et pour l'interprétation médico-légale, de développer des méthodes sensibles et spécifiques permettant d'identifier, de discriminer et de quantifier l'insuline humaine et ses analogues, pour une utilisation en routine.

MOTS-CLÉS : Insuline - Immunoanalyse - Spectrométrie de masse - Suicide - Homicide - Médecine légale

Identification of insulin in forensic: analytical issues

SUMMARY

Insulin is the most widely used polypeptide drug to treat diabetes. Its misuse has often been described, from consumption as a doping substance in sport to use for suicidal or criminal purposes, and also in the context of factitious disorders formerly known as Munchausen syndrome. Additionally, illegal versions of this drug are available on the black market and have been responsible for many accidents in the past. The detection of insulin and its synthetic analogues in biological samples is very complex. The instability of insulin in the blood, the special pre-analytical conditions, the high molecular weights and the absence of sufficiently specific analytical method, have led most toxicology laboratories to not test for insulin in biological samples in routine. Therefore, the number of poisoning and deaths caused by exogenous administration of insulin is most certainly underestimated. This article describes two cases of exogenous insulin administration in a criminal context of attempted murder followed by attempted suicide. The presence of aspart insulin in both blood samples (5.7 and 2.4 ng/mL) provided concrete evidence for the administration of exogenous insulin. It seems essential, for public health and for forensic interpretation, to develop sensitive and specific methods to identify, discriminate and quantify human insulin and its analogues, for routine use.

KEYWORDS : *Insulin - Immunoanalysis - Mass spectrometry - Suicide - Homicide - Forensic*

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I - INTRODUCTION

Bien que la découverte et la synthèse des insulines synthétiques aient permis de sauver de nombreuses vies au fil des années, leur mauvaise utilisation a été décrite à de nombreuses reprises. En 1922, l'insuline a été isolée et testée pour la première fois chez l'Homme. Depuis, diverses utilisations non thérapeutiques de cette molécule ont été décrites, allant de son utilisation à des fins de dopage dans le milieu du sport (1-2) jusqu'à son utilisation à des fins suicidaires, voire criminelles (3-5). Ces mésusages conduisent les utilisateurs à se fournir sur le marché noir, qui n'est soumis à aucune norme stricte de qualité. Ces utilisations détournées représentent de graves risques pour la santé. En effet, de nombreuses hospitalisations ont été signalées en rapport avec l'utilisation d'insuline vendue sur le marché noir (6-7).

Différentes formulations d'analogues de l'insuline ayant des propriétés pharmacocinétiques différentes ont été synthétisées. Les analogues synthétiques de l'insuline peuvent être divisés en deux catégories : les insulines à action rapide (lispro, asparte et glulisine), conçues pour imiter la sécrétion pancréatique rapide et intense d'insuline après un repas, et les insulines à action prolongée (glargine, détémir et dégludec), caractérisées par l'absence de pic plasmatique et une concentration active constante dans le sang sur une longue période. Sur la base du profil pharmacocinétique souhaité, la séquence peptidique initiale de l'insuline a été modifiée par l'ajout, la substitution ou l'inversion d'acides aminés, et le résultat conduit à des analogues de l'insuline ayant des structures et des poids moléculaires toutefois très similaires (8).
Les cas d'intoxication à l'insuline ne sont généralement pas accidentels mais, dans 90 % de cas, il s'agit de tentatives de suicide qui semblent être plus fréquentes chez les personnes ayant des proches diabétiques ou chez les professionnels de la santé. Les réels cas accidentels sont principalement liés à des

erreurs d'administration dans les hôpitaux (3).
Dans le domaine médico-légal, diagnostiquer une intoxication à l'insuline est complexe. La difficulté réside dans l'absence de preuve morphologique, dans les changements pathologiques insaisissables et dans l'absence de test spécifique et fiable capable de distinguer les différents types d'insuline tout en étant suffisamment sensible pour doser de faibles concentrations, soulignant l'importance des éléments anamnestiques et circonstanciels. En raison de l'utilisation d'aiguilles très fines, la recherche du site d'injection est désormais presque impossible. En outre, l'hypoglycémie pouvant être tolérée pendant plusieurs heures avant l'apparition d'un syndrome hypoglycémique ou le décès, selon le type d'insuline utilisé, la concentration sanguine peut être très faible au moment du recueil de l'échantillon (5).
L'analyse toxicologique des analogues de l'insuline est difficile en raison de leur poids moléculaire élevé, de l'instabilité de l'insuline dans le sang total et des complexités liées à la préparation des échantillons et aux tests instrumentaux. La première difficulté commence à la phase pré-analytique et concerne le traitement de l'échantillon. Le sang total est la matrice la plus utilisée pour les analyses toxicologiques. Cependant, en *post-mortem*, le sang est soumis à des phénomènes de dégradation, d'hémolyse et de coagulation qui rendent l'échantillon inadapté aux analyses immunochimiques traditionnelles (9). A ce jour, les méthodes d'analyse les plus utilisées sont les techniques de dosage radio-immunologique (RIA) et immunoenzymatique, qui présentent certains inconvénients, notamment en ce qui concerne leur spécificité. Ces méthodes ne sont pas en mesure de discriminer spécifiquement les différents analogues de l'insuline, ni de mesurer avec précision leur concentration (10). Surtout, elles ne sont validées que sur plasma et sérum non hydrolysé, ce qui les rend peu utilisable sur échantillons *post-mortem*.
La discrimination des analogues de l'insuline et la mise en évidence d'une administration exogène, ayant entrainé le décès, est complexe. Ainsi, le nombre de cas d'intoxication fatale à l'insuline est probablement largement sous-estimé. Ces dernières années, en raison de l'incidence croissante du diabète (11) et de l'utilisation plus large de l'insuline synthétique, les cas d'utilisation inappropriée de cette substance à des fins de dopage ou dans un contexte criminel, mais également dans le cadre de troubles factices appelés autrefois « syndrome de Münchhausen » (12-13) ont augmenté. Malgré l'augmentation de l'utilisation abusive de cette hormone, peu de cas sont décrits dans la littérature. En raison de toutes ces problématiques, la détermination de l'insuline n'est pas réalisée de manière systématique dans tous les laboratoires de toxicologie médico-légale. D'un point de vue interprétatif, la discrimination entre l'insuline endogène et exogène, ainsi que la quantification, semble essentielle. A cet égard, ces dernières années, des progrès ont été réalisés dans la préparation des échantillons, dans l'instrumentation et dans les méthodes de détection des analogues de l'insuline (14-16).
Cet article présente deux cas d'administration exogène d'insuline chez des sujets non diabétiques, qui mettent en évidence l'importance et la nécessité de développer des méthodes analytiques spécifiques et sensibles permettant de distinguer les différents analogues synthétiques de l'insuline et de doser leurs concentrations sanguines, même très faibles.

II - CAS CLINIQUE

Une femme de 48 ans, ayant des antécédents de dépression, appelle les services d'urgence et explique avoir tenté de tuer sa fille de 10 ans en lui injectant de l'insuline asparte (NovoRapid®, Novo nordisk). Au cours de son interrogatoire, elle déclare avoir tenté de se suicider en s'injectant le même type d'insuline, après l'injection à sa fille. L'insuline avait probablement été obtenue grâce à sa profession d'infirmière. La femme déclare avoir regretté son geste et avoir donné quelques morceaux de sucre à l'enfant avant d'appeler les secours. A leur arrivée aux urgences, environ 4 heures après l'injection, les deux sujets présentaient des signes vitaux normaux et la glycémie de la mère était à 0,89 g/L. Deux seringues et un stylo (FIASP FlexTouch®, Novo nordisk) à insuline, vides, ont été retrouvés sur les lieux. Des prélèvements sanguins de l'enfant (tube Vacutainer® (Becton-Dickinson) sur fluorure de Na, bouchon gris) et de la mère (tube Vacutainer® (Becton-Dickinson) sur héparinate de lithium, bouchon vert) ont été réalisés et ont été transférés au laboratoire de toxicologie pour un criblage large. Les échantillons ont été conservés à + 4° C jusqu'à l'analyse, sans centrifugation.

III - ANALYSES TOXICOLOGIQUES ET RÉSULTATS

La recherche de l'insuline dans les échantillons sanguins a été réalisée après précipitation des protéines, en présence d'un étalon interne (insuline bovine) dans un mélange d'acétonitrile et de méthanol, puis par une extraction en phase solide (SPE) en utilisant des cartouches Waters™ OASIS MAX (3 mL, 60 mg). Après élution dans un mélange méthanol/acide acétique (5:1), les analytes ont été séparés par chromatographie liquide sur une colonne CORTECS C_{18+} (Waters™) et détectés par spectrométrie de masse haute résolution sur un système Waters™ Xevo™ G2-XS QTof (LC-HRMS).
Dans les seringues et le stylo, l'insuline a été recherchée après dilution dans un mélange eau/acide acétique (2 %), puis soumis à l'analyse par LC-HRMS. La méthode développée prévoit la recherche de 4 analogues de l'insuline humaine : asparte, lispro, glargine et détémir (en plus de l'insuline humaine et de l'insuline bovine utilisée comme étalon interne). Pour l'insuline asparte, l'ion multichargé $[M+5]^{5+}$ a été mesuré à 1165,94242 (la masse de l'insuline asparte calculée à partir de cet

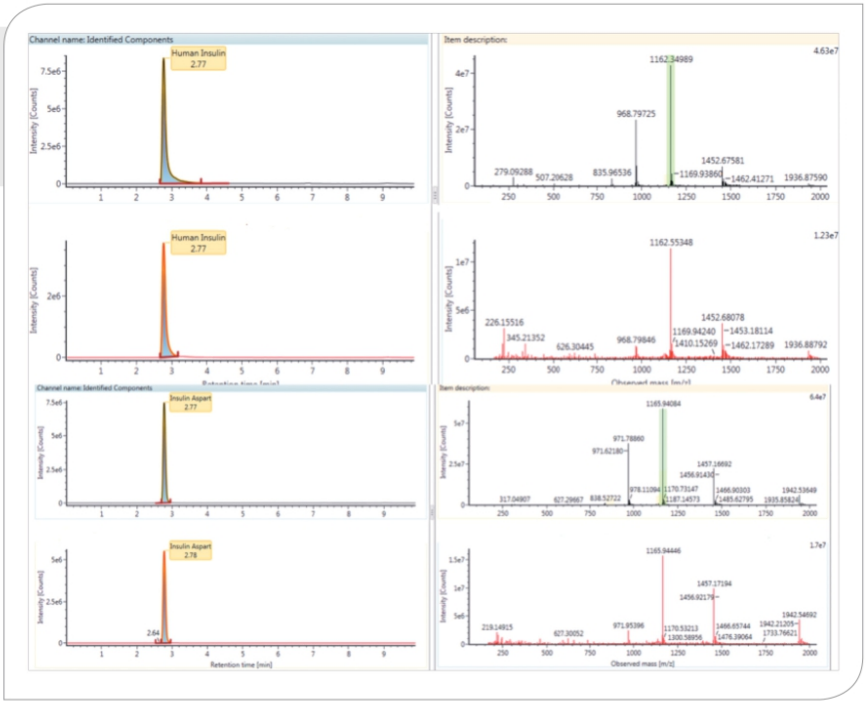

Figure 1
Chromatogrammes et spectres de l'insuline humaine et de l'insuline asparte obtenus en LC-HRMS

ion est de 5824,7121 Da), alors que pour l'insuline humaine et bovine, les ions multichargés $[M+5]^{5+}$ ont été retrouvés respectivement à *m/z* 1162,34172 et 1147,53228. Les ions multichargés $[M+6]^{6+}$ à 971,95242 et 968,79023 ont par ailleurs été utilisés pour discriminer l'insuline asparte de l'insuline humaine (*Figure 1*).

La présence d'insuline asparte a été confirmée dans les deux échantillons sanguins, dans les seringues et dans le stylo par LC-HRMS.

Après identification, la quantification de l'insuline asparte a été réalisée sur un système de chromatographie liquide couplé à un spectromètre de masse en tandem (LC-MS/MS) Waters™ Xevo™ TQS micro. Une courbe d'étalonnage en 6 points a été préparée en utilisant les concentrations suivantes : 0, 1, 5, 10, 50 et 100 ng/mL d'insuline asparte dans le sang. Pour la validation de la méthode, la courbe d'étalonnage en 6 points a révélé une bonne linéarité dans la gamme 0-100 ng/mL avec un coefficient de corrélation allant de 0,9994 à 0,9999 pendant 3 tests. La LOD et la LOQ sont respectivement de 0,5 ng/mL et 1 ng/mL. La répétabilité était de 18,5 et 10,2 % à 1 et 10 ng/mL, respectivement, et la reproductibilité était de 17,2 et 8,3 % à 0,1 et 10 ng/mL, respectivement.

Concernant les échantillons analysés, des concentrations à 2,4 et 5,7 ng/mL ont été mesurées respectivement dans le sang de l'enfant et de la mère (*Figure 2*).

Les analyses complémentaires effectuées lors du criblage toxicologique ont révélé la présence d'éthanol (1,72 g/L), de bromazépam (1,6 mg/L) et de venlafaxine (265 ng/mL) dans le sang de la mère. En revanche, aucun autre xénobiotique d'intérêt toxicologique n'a été trouvé chez l'enfant.

IV - DISCUSSION

Les résultats d'analyse obtenus ont fourni des preuves concrètes de la présence d'un analogue synthétique de l'insuline chez deux sujets non diabétiques. L'administration simultanée de grandes quantités d'éthanol et de bromazépam a probablement entrainé une altération de l'état psychophysique de la mère. De plus, la venlafaxine a été dosée à la limite haute thérapeutique.

Les données de l'enquête ont permis de mettre en évidence une tentative d'homicide suivi d'une tentative de suicide par administration d'insuline asparte. Ces cas sont intéressants car ils mettent en évidence la méthodologie et l'instrumentation disponibles permettant de discriminer les différents analogues de l'insuline et de déterminer des concentrations même faibles. Aujourd'hui, l'idée que l'insuline représente le crime parfait est dévaluée par les avancées analytiques. La mise au point des méthodes actuelles permet de rechercher l'insuline dans des échantillons difficiles tels que les échantillons *post-mortem*. En effet, la problématique est liée aux phénomènes de décomposition qui se produisent après la mort, en particulier l'hémolyse, qui semble jouer un

rôle majeur dans la dégradation de l'insuline (9). Par ailleurs, l'hémolyse ne permet plus d'utiliser des méthodes immunologiques, très sensibles à ce phénomène. Cette dégradation commence dans l'organisme, après la mort, et se poursuit également dans le tube, après le prélèvement de l'échantillon. Pour éviter cette dégradation, il existe des pratiques pré-analytiques qui peuvent être mises en place, telles que la séparation immédiate du sérum par centrifugation et le stockage immédiat à -20 °C (17). Cependant, en médecine légale, ces pratiques ne peuvent que très difficilement être appliquées systématiquement, et ne permettent que très rarement l'obtention d'un sérum non hydrolysé.

En médecine légale, en plus des éléments anamnestiques-circonstanciels, divers indicateurs tels que le glucose dans l'humeur vitrée, le lactate et les corps cétoniques dans le sang, sont utilisés pour retracer la glycémie *ante-mortem* (18-19). En outre, depuis 1967, et encore aujourd'hui, la recherche et le dosage du peptide C, est utilisé pour prouver, à travers le calcul du rapport insuline/peptide C (valeur normale ≈ 1), les administrations d'insuline exogène. Dans ce cas, il faut considérer que, chez les sujets présentant un déficit de production d'insuline (auto-anticorps dirigés contre les cellules ß du pancréas, diabétiques de type 1), le rapport insuline/peptide C peut devenir supérieur à 1, en raison de la demi-vie prolongée de l'insuline (9,20-21).Toutes les mesures décrites ci-dessus restent des mesures indirectes et, de nos jours avec les méthodes analytiques sensibles et spécifiques existantes, il est possible de présenter une vraie preuve d'hypoglycémie induite par l'administration d'insuline exogène par l'identification et le dosage de ses analogues.

Bien que les cas d'hypoglycémie factice, induits par l'injection d'insuline exogène semblent être assez fréquents, la littérature apparait comme limitée. Par conséquent, le nombre total de cas authentiques semble être sous-estimé puisque de nombreux cas sont classés comme hypoglycémies accidentelles, ou sont masqués par l'incapacité du laboratoire à identifier l'insuline et ses analogues synthétiques. Ce n'est que récemment que le nombre de cas rapportés a augmenté, notamment en raison des avancées analytiques.

Identifier l'insuline comme étant la cause de l'hypoglycémie et déterminer le type d'analogue impliqué ne repose, souvent, que sur des constatations anamnestiques et circonstancielles (témoins, note de suicide, présence de seringues à insuline). En médecine légale, les doutes et les ambiguïtés ne peuvent être acceptés et il est donc nécessaire d'identifier et de quantifier le type d'insuline impliqué, afin de pouvoir prouver avec certitude que l'administration d'insuline exogène a contribué à l'intoxication ou au décès. Depuis les années 1960, il est possible d'identifier et de quantifier l'insuline grâce aux méthodes immuno-enzymatiques, en particulier par dosage radio-immunologique (22).

En 2012 (23), a été décrit le cas d'une femme non diabétique qui est décédée suite à un surdosage d'insuline. L'insuline a été déterminée par des tests immunologiques à 194 ng/mL, tandis

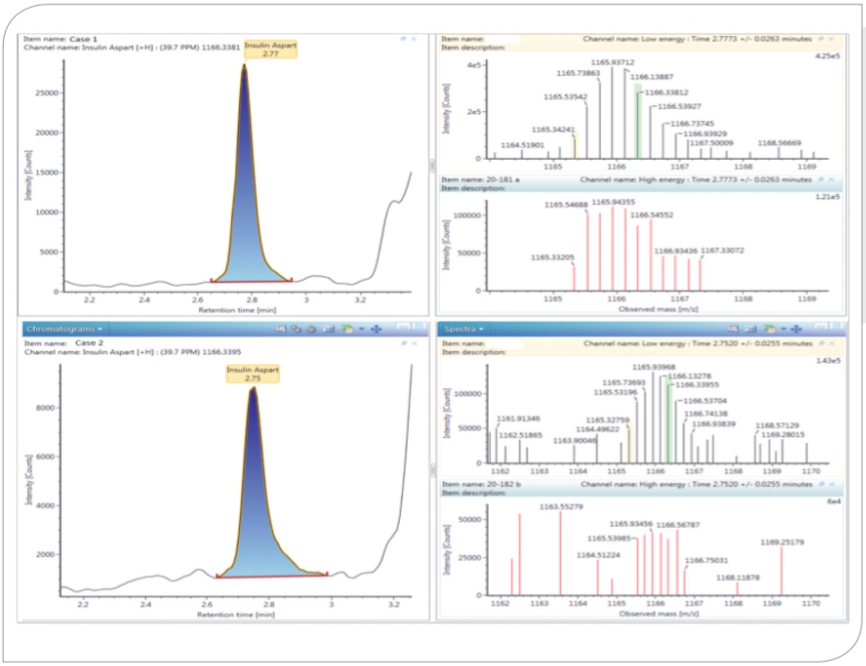

Figure 2
Chromatogrammes obtenus après extraction de l'insuline asparte dans les échantillons de sang de la mère (en haut) et de la fille (en bas) Les concentrations étaient, de haut en bas, de 5,7 ng/mL et de 2,4 ng/mL.

que la concentration de peptide C était dans une fourchette normale (1,7-4,8 ng/mL). Plus récemment, il a été rapporté un cas de tentative de suicide d'un sujet non diabétique qui s'est auto-injecté 600 unités d'insuline asparte (NovoRapid®). Après détection et quantification de l'insuline par des tests immunologiques, l'insuline asparte était mesurée à 62,9 ng/mL, 4 heures après l'injection.

D'un point de vue médico-légal, ces tests ne sont pas adaptés et ne sont pas suffisants, surtout en l'absence d'information anamnestique complémentaire. Ceci est principalement dû au fait que les tests immunologiques ne sont pas capables de fournir des résultats discriminatoires et quantitatifs. La spécificité des anticorps représente l'une des principales limites car ils ne peuvent pas correctement discriminer l'insuline humaine des analogues, des métabolites, des autres insulines d'origine animale (utilisées dans le passé pour le traitement du diabète) et des produits de dégradation de l'insuline (25). La présence d'analogues dans un échantillon peut donc faussement augmenter ou diminuer l'estimation d'insuline humaine (26). Certaines insulines à action prolongée (ex. détémir) sont associées à une concentration sanguine élevée après administration. Si l'insuline prescrite n'est pas connue, les valeurs sériques peuvent être faussement interprétées comme un surdosage (27). En outre, la présence d'anticorps anti-insuline ou de liaison aux protéines plasmatiques (comme pour les insulines détémir et dégludec avec l'albumine) peut compromettre la quantification de l'insuline. En *post-mortem*, ces tests peuvent également présenter des réactions croisées aux produits de dégradation de l'insuline dans le sang total hémolysé (28). Par ailleurs, tous les tests immunologiques ne sont validés que pour du sérum ou du plasma, jamais pour du sang total.

Compte tenu de la haute spécificité requise et des faibles concentrations à mesurer, une méthode analytique telle que la chromatographie liquide couplée à la spectrométrie de masse semble nécessaire.

L'insuline asparte a été identifiée et quantifiée dans deux tentatives de suicide documentées. Dans un cas décrit en 2016 (29), l'insuline asparte a été mesurée par LC-MS/MS à 76,46 ng/mL, 7 heures après l'injection. La même année (26), il a été rapporté un cas de suicide avec de l'insuline asparte, qui a été déterminée par un dosage par chromatographie liquide couplée à la spectrométrie de masse en tandem à 161,8 ng/mL.

L'analyse par spectrométrie de masse, contrairement à l'immunodosage, permet d'identifier l'analyte, en discriminant l'insuline humaine, les analogues, les métabolites et les produits de dégradation, et de réaliser une quantification précise pour une large gamme de matrices biologiques (9-10).

La précipitation dans des solvants tels que le méthanol ou l'acétonitrile, qui est utilisée dans la plupart des protocoles d'extraction, élimine de nombreuses interférences et les liaisons possibles avec les anticorps ou les protéines plasmatiques (15). La spectrométrie de masse ne présente pas de problème de réactivité croisée et offre une meilleure spécificité et une meilleure sensibilité, et se prête à l'identification de plusieurs cibles dans un seul test. Différentes technologies ont été décrites : chromatographie liquide couplée à un analyseur de masse simple (LC-MS) (30) ou à un analyseur de masse en tandem (LC-MS/MS) (14, 31), et, plus récemment à la spectrométrie de masse haute résolution (HRMS) (32). Les analogues de l'insuline ont été développés avec de légères différences structurelles pour obtenir diverses propriétés pharmacocinétiques. Cependant, leurs caractéristiques moléculaires restent très similaires, notamment concernant leurs structures et leurs poids moléculaires. Par conséquent, ces composés présentent des caractéristiques analytiques similaires (temps de rétention et spectre de masse), rendant très difficile la différenciation, même avec les approches les plus modernes. Les analogues, comme l'insuline humaine et l'insuline lispro, ou comme l'insuline asparte et l'insuline glulisine, qui ont le même poids moléculaire, ne peuvent donc être discriminés les uns des autres que par les spectres de masse de leurs produits ioniques, obtenus avec des analyseurs de masse en tandem de haute résolution (14). Un autre avantage est que la spectrométrie de masse peut être réalisée quelle que soit la matrice avec une préparation adéquate de l'échantillon (33). Ceci a un intérêt en toxicologique *post-mortem*, car les échantillons de sang ne sont pas toujours disponibles ou analysables. Compte tenu de tous ces facteurs, la spectrométrie de masse semble être la technologie la plus adaptée à l'analyse de l'insuline, que ce soit dans un contexte clinique ou médico-légal, permettant de fournir une preuve non contestable de décès lié à un surdosage d'insuline.

V - CONCLUSION

L'utilisation de l'insuline dans un contexte médico-légal n'est pas une nouveauté. Les enquêtes sur les cas présumés de décès ou d'intoxication par insuline impliquent l'évaluation de divers facteurs tels que des investigations approfondies sur les lieux, un examen complet du dossier médical et des tests toxicologiques appropriés. La grande instabilité de l'insuline (en particulier dans les échantillons *post-mortem*), sa similarité marquée avec ses analogues disponibles dans le commerce, le délai entre l'autopsie et les analyses toxicologiques, et l'absence de pratique pré-analytique adéquate, ont conduit au fil des années à une sous-estimation des cas d'intoxications volontaires à l'insuline. Comme le démontre le cas décrit, l'utilisation des approches analytiques actuelles (en particulier la spectrométrie de masse en tandem et la spectrométrie de masse haute résolution), permet de surmonter les problèmes analytiques relatifs à ce composé et d'apporter, en association avec la présence de données anamnestiques et circonstancielles, des preuves concrètes de l'administration d'insuline exogène. ■

DÉCLARATION DE LIENS D'INTÉRÊTS

Les auteurs déclarent ne pas avoir de liens d'intérêts.

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1.3. Intérêt de l'analyse des cheveux dans les expositions aux antidiabétiques

L'analyse des cheveux permet d'obtenir des informations historiques sur l'exposition d'un sujet à une substance à la suite d'une utilisation chronique ou, dans certains cas, d'une exposition unique. Les médicaments et les drogues incorporés dans les cheveux restent relativement stables pendant de nombreux mois, voire des années (selon la longueur disponible) , ce qui fait que les cheveux représentent une preuve à long terme de la consommation de drogues, puisqu'ils peuvent refléter la consommation sur une période plus large que celle de l'urine et du sang (quelques heures à quelques jours). Grâce à ces avantages, l'analyse des cheveux est devenue une pratique courante dans les enquêtes médico-légales. Un intérêt médico-légal a été observé dans le contexte des crimes facilités par la drogue car il est possible de différencier, par segmentation, une exposition unique d'une consommation chronique [11].

Le diagnostic de l'hypoglycémie peut être simple chez les sujets diabétiques mais devient un défi chez les sujets qui n'ont pas d'antécédent d'exposition à ces médicaments.
La recherche de ces composés dans les cheveux trouve son intérêt dans les cas d'hypoglycémies inattendues, car cette analyse permet de discriminer les hypoglycémies causées par exposition aux antidiabétiques à d'autres raisons comme par exemple, l'insulinome.
En outre, l'analyse segmentée des cheveux est capable de refléter la consommation d'un médicament sur une période donnée et donc d'être utile pour le suivi thérapeutique des médicaments ou pour les médecins légistes qui recherchent la cause de la mort dans un cas d'empoisonnement suspect.

Dans la littérature, il y a un manque de données disponibles sur l'identification des agents antidiabétiques dans les cheveux. Les rares articles ont démontré l'utilité de l'utilisation de cette matrice.

A l'Institut médico-légal de Strasbourg, un cas de décès en 2006 a démontré l'intérêt de la recherche de ces substances dans les cheveux. Une exposition au glibenclamide (un sulfamide hypoglycémiant) a été mise en évidence chez un individu non-diabétique dont la cause de la mort n'était pas claire et pour lequel l'échantillon de sang prélevé à l'autopsie ne contenait aucune trace de cette substance. L'analyse des cheveux représentait la seule preuve d'une exposition à cet antidiabétique [12].

Il semble très important de comprendre comment les médicaments antidiabétiques sont incorporés dans les cheveux et de pouvoir déterminer quelles concentrations sont observées en cas de traitement thérapeutique et quelles concentrations sont observées en cas de surdosage.

En raison de l'absence de donnée dans la littérature sur ce sujet, j'ai développé une méthode qui intègre un 13 antidiabétiques oraux qui a fait l'objet d'une étude afin d'obtenir des valeurs de référence pour pouvoir interpréter les concentrations trouvées chez les consommateurs d'antidiabétiques.

Deuxième Partie – La Metformine

2.1. Présentation

La metformine est utilisée dans le traitement pharmacologique de pointe du diabète de type 2 et est le médicament le plus couramment prescrit pour cette maladie dans le monde, seul ou en association avec l'insuline ou d'autres traitements hypoglycémiants.

La metformine fait partie de la famille des biguanides. Les biguanides sont dérivés de la galégine ou 2-(3-méthylbut-2-ényl)guanidine, qui était initialement extraite de la plante *Galega officinalis,* utilisée à l'époque médiévale à des fins curatives, car elle était connue pour avoir des effets hypoglycémiants.

La galégine avait toutefois un effet trop toxique, raison pour laquelle la metformine et la phenformine ont été créées, mais n'ont été introduites comme médicaments antidiabétiques que dans les années 1950. La metformine a été synthétisée pour la première fois en 1922 et introduite comme médicament antidiabétique en 1957 à la suite d'études menées par Jean Sterne.

La metformine est un biguanide qui contient, comme son nom l'indique, deux paires de guanidines avec des substitutions supplémentaires.

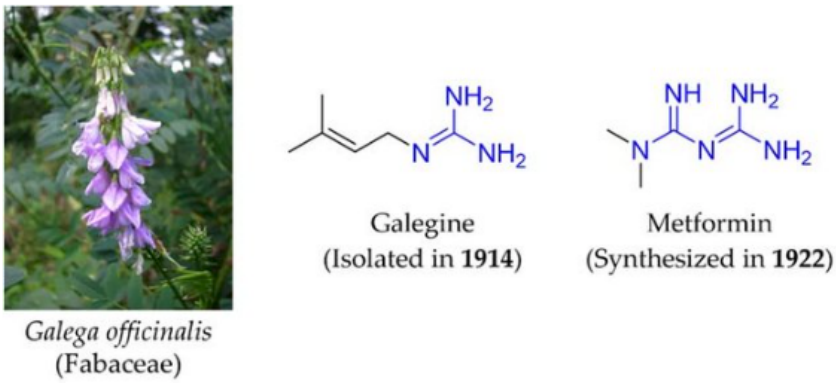

Figure 1. Structures chimiques de la metformine et de la galegine [36]

Après la prise de metformine à libération immédiate, environ 70 % de la dose est absorbée dans l'intestin grêle et excrétée sous forme inchangée dans l'urine.

Après la consommation d'une dose thérapeutique de 1500 mg de metformine, des concentrations plasmatiques maximales de 1,8 à 4,0 mg/l sont atteintes.

Le mécanisme d'action de la metformine n'est pas encore totalement élucidé. On pense actuellement qu'elle réduit la concentration sanguine de glucagon et diminue la glycémie, en réduisant la production de glucose par le foie (en diminuant la gluconéogenèse), en augmentant

sa consommation par les tissus périphériques (en augmentant la glycolyse) et en réduisant l'absorption de glucose par l'intestin.

Le mécanisme qui permet à la metformine d'inhiber la gluconéogenèse hépatique semble être lié à une inhibition du complexe I de la chaîne respiratoire mitochondriale dans les hépatocytes, ce qui entraîne une suppression de la production d'ATP, nécessaire à la production de glucose (6 ATP pour chaque molécule de glucose synthétisée). Cette inhibition entraîne une augmentation du rapport AMP:ATP (indiquant une altération de la stabilité énergétique des cellules) qui active l'AMPk qui, à son tour, active le catabolisme des acides gras et des sucres qui conduira à la formation d'ATP afin de restaurer l'homéostasie énergétique (Figure 2).

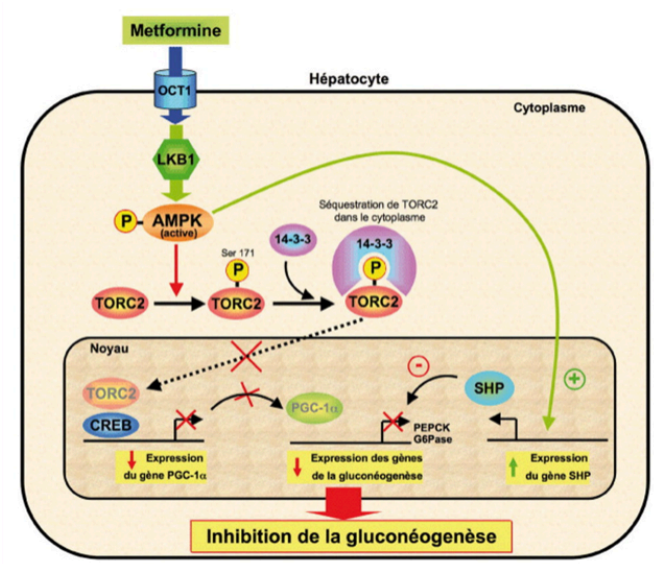

Figure 2. Mécanismes d'inhibition de la gluconéogenèse hépatique par la metformine [37]

Il semblerait également que la metformine agisse dans l'intestin en augmentant le métabolisme anaérobique du glucose dans les entérocytes, ce qui entraîne une réduction de l'absorption du glucose et une augmentation de la libération de lactate vers le foie.

Les effets indésirables de la metformine sont principalement de nature gastro-intestinale : diarrhée, nausée et douleurs abdominales.

Ces effets sont principalement dus à l'accumulation de la metformine dans les entérocytes de l'intestin grêle.

Un autre effet fréquent de la metformine et des biguanides en général, qui a conduit au retrait de la phenformine et même de la metformine aux Etats-Unis jusqu'en 1995, est l'acidose lactique ou MALA (Metformin-associated lactic acidosis). Ce phénomène se produit en raison de l'interférence de la metformine avec la respiration mitochondriale, ce qui stimule le métabolisme anaérobie et entraîne une augmentation de la production de lactate [13,14].

Bien que la metformine soit considérée comme un médicament assez sûr, sa mauvaise utilisation a été décrite dans la littérature.

L'AAPPCC a signalé 9256 cas d'empoisonnement à la metformine en 2020, dont 758 enfants de moins de 5 ans, ce qui la classe deuxième après l'insuline [7].

En raison du risque accru pour les diabétiques de développer un syndrome dépressif, il n'est pas surprenant que les surdosages de metformine soient parfois intentionnels.

Pouvoir identifier et doser la metforminémie est essentiel pour un laboratoire de toxicologie, du fait de son utilisation à des fins médico-légales mais surtout cliniques.

Connaître la concentration de metformine dans le sang d'un patient ayant pris, accidentellement ou volontairement, une surdose de metformine, permet de comprendre les mécanismes de toxicité et de prévoir son évolution en réanimation.

En outre, la surveillance thérapeutique de la metformine est nécessaire pour s'assurer que la concentration de metformine se situe dans la fourchette thérapeutique recommandée. Les études de stabilité de la metformine deviennent une partie importante de la bioanalyse TDM afin de fournir des données précises sur la concentration du médicament pour des ajustements de dose appropriée.

Dans cette thèse, je me suis particulièrement intéressé à l'analyse des cheveux.

En ce qui concerne les antidiabétiques, le monde de la toxicologie s'est peu intéressé à l'analyse de cette matrice, bien que son intérêt soit évident.

Afin de mieux expliquer les événements hypoglycémiques peu clairs, comme dans le contexte du syndrome de Münchhausen ou de Münchhausen par procuration ou dans des cas de soumission chimiques, l'analyse des cheveux semble être très utile en complément du sang et de l'urine, car elle fournit des données rétrospectives sur l'exposition aux drogues.

2.2. Développement d'une méthode d'identification et de quantification dans le sang

Pour la détection et le dosage de la metformine dans les échantillons de sang dans les domaines médico-légal et clinique, une méthode a été développée qui représente une adaptation d'une méthode déjà publiée dans la littérature [15].

La metformine est principalement recherchée par chromatographie liquide couplée à une détection UV, par spectrométrie de masse (LC-MS) ou par spectrométrie de masse en tandem (LC-MS/MS). Cependant, l'analyse de la metformine par chromatographie liquide présente certaines difficultés analytiques, notamment une forte possibilité d'effets matrice. En outre, les extractions liquide-liquide avec un tampon et divers solvants organiques sont difficiles à réaliser en raison de sa polarité élevée, car la metformine a tendance à rester dans la fraction aqueuse. Enfin, lorsqu'on utilise la chromatographie en phase inverse comme méthode de séparation, la metformine n'est pas bien retenue sur la colonne, ce qui se traduit par un temps de rétention très court et par la présence de nombreuses substances co-éluantes [16]. Afin de surmonter ces problèmes et donc empêcher l'élution de la metformine dans le front de solvant et obtenir un bon résultat chromatographique de nombreuses publications ont été publiées décrivant l'utilisation de méthodes chromatographiques telles que l'appariement d'ions en phase inversée ou la chromatographie par échange de cations. Cependant, ces méthodes présentent également certains inconvénients, notamment un manque de sensibilité, l'utilisation d'une préparation longue et complexe de l'échantillon ou l'utilisation d'un grand volume d'échantillon.

Pour obtenir une séparation chromatographique appropriée, la chromatographie en phase gazeuse semble être le meilleur choix.

Seulement 200 µL de sang sont nécessaires pour la recherche de metformine.

Le protocole suivant a été validé pour la metformine. Les transitions utilisées, le temps de rétention (T_R) et la limite de quantification (LQ) et de détection (LD) sont résumés dans le tableau 2.

Protocole d'extraction

L'extraction implique une précipitation des protéines suivie d'une dérivation de l'extrait sec.

Un volume de 0,2 mL de sang total humain est surchargé avec 20 µL de 100 mg/L de metformine-D6 (étalon interne deutéré) et soumis à une précipitation des protéines avec 3 mL d'acétonitrile. L'échantillon est ensuite mélangé au vortex pendant 1 min, puis centrifugé à 3500 rpm pendant 5 minutes. Après centrifugation, le surnageant est transféré dans un autre tube et évaporé à sec sous un courant d'azote à 50 °C. Après séchage, l'échantillon est dérivé avec 40 µL de MBTFA à 80 °C pendant 60 min, puis transféré dans des flacons d'injection.

Un volume de 0,2 µL est ensuite injecté dans le système de chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem (GC-MS/MS) (Agilent 7010B, Santa Clara, USA).

Paramètres du système GC-MS/MS

La séparation GC a été réalisée sur un appareil Agilent GC 8890 en utilisant une colonne Agilent 30 m × 0,25 mm, 0,25 µm HP-5MS UI pour la séparation. Une injection de 0,2 µL en mode split (ratio : 50 :1) avec un flux de 1,2 mL/min d'hélium utilisé comme gaz porteur a été réalisée. Le gradient de température est décrit ci-dessous dans le tableau 1.

	Taux °C/min	**Valeur °C**	**Temps de maintien min**
Initial		100	1
Ramp 1	20	210	0
Ramp 2	40	270	3

Tableau 1. Gradient de température pour l'analyse de la metformine

Le temps total d'exécution était de 11 min. Un détecteur sélectif de masse triple-quadripôle 7010B a été utilisé pour l'analyse des composés. Le spectromètre de masse en tandem a fonctionné en mode d'ionisation électronique (EI) positif et le mode de surveillance des réactions multiples (MRM) a été utilisé pour l'identification de la metformine et de la metformine-D6. Les conditions suivantes se sont avérées optimales pour l'analyse de la metformine et de l'étalon interne : énergie des électrons à 70 eV, température d'entrée frontale à 280 °C, température de la source d'ions à 230 °C et température d'interface à 280° C. Le délai de solvant a été fixé à 4 min.
Les transitions ainsi que les temps de rétention, la LD et la LQ sont décrits dans le tableau 2 ci-dessous.

Analyte	**Transition de quantification**	**Transition de confirmation**	**T_R (min)**	**LD (ng/mL)**	**LQ (ng/mL)**
Metformine	303,0 > 288,2	303,0 > 125,1	4,95	0,1	1
Metformine -d6	309,1 > 291,1	309,1 > 128,0	4,97	/	/

Tableau 2. Transitions, temps de rétention, LD et LQ de la metformine et de la metformine-d6

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

Malgré le rôle important de la metformine dans la réduction de la mortalité et de la morbidité dans la population diabétique, la metformine est associée à un risque accru d'accident vasculaire cérébral et d'acidose lactique qui peut s'avérer fatal. Afin de documenter l'exposition à un médicament, les cheveux sont considérés comme la matrice de choix en complément du sang et de l'urine, car ils fournissent des détails historiques sur l'exposition d'un sujet à un ou plusieurs médicaments. La mesure de la concentration de metformine dans les cheveux peut être importante pour les toxicologues judiciaires qui enquêtent sur un empoisonnement criminel ou dans le contexte d'un syndrome de Münchhausen par procuration. En toxicologie clinique, la surveillance des médicaments à l'aide des cheveux pour documenter l'observance du traitement à la metformine n'a pas encore été décrite. Afin de documenter l'intérêt de l'analyse des cheveux pour la metformine, j'ai développé et validé une méthode utilisant un système GC-MS/MS.
Les échantillons sont analysés par GC-MS/MS en 11 minutes. La méthode a été développée et validée. Les paramètres de la méthode sont décrits dans l'article ci-dessous ***[Article 3].***

Lorsque cette méthode a été créée, il n'existait qu'un seul article [17] dans la littérature rapportant les concentrations de metformine dans les cheveux. Étant donné que les concentrations obtenues dans les cheveux varient selon les laboratoires, il a été nécessaire d'obtenir nos propres valeurs de référence.
De ce fait, la méthode développée a été appliquée à des cheveux authentiques obtenus auprès de 9 patients diabétiques sous traitement quotidien (1-3 g/jour). Les cheveux des 9 patients ont été testés positifs dans la gamme des faibles ng/mg avec des concentrations allant de 0,3 à 3,8 ng/mg. Cette étude a montré que, en comparaison avec d'autres médicaments, la metformine est mal incorporée dans les cheveux, considérant que la dose quotidienne varie de 1 à 3 g. Bien que limitée dans le nombre de sujets, l'étude a permis de postuler une possible corrélation entre la dose quotidienne et la concentration dans les cheveux foncés, alors que pour les cheveux clairs aucune corrélation n'a été trouvée ***[Article 3].***

Article 3: Development of a new GC-MS/MS method for the determination of metformin in human hair

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SHORT COMMUNICATION

WILEY

Development of a new GC–MS/MS method for the determination of metformin in human hair

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Abstract

Diabetes mellitus is one of the most important public health challenges. Metformin (1,1-dimethylbiguanide) represents the "gold standard" for the treatment of diabetes mellitus type 2. Despite its important role in reducing mortality and morbidity in the diabetic population, metformin is associated with an increased risk of stroke. To document exposure to a drug, hair is considered to be the specimen of choice to complement blood and urine, since it provides historical detail of a subject's chronic exposure to drug(s). Measuring hair concentration of metformin can be important for forensic toxicologists investigating criminal poisoning or Munchausen's syndrome by proxy. In clinical toxicology, drug monitoring using hair to document metformin observance has not yet been described. To document the interest of hair analysis for metformin, the authors have developed and validated a method using a gas-chromatography tandem mass spectrometry system and applied it to authentic hair obtained from 9 diabetic patients under daily treatment. The validation procedure demonstrated a LOD an LOQ of 1 and 100 pg/mg, respectively and acceptable linearity, repeatability and reproducibility. The hair of the 9 patients tested positive in the low ng/mg range with concentrations ranging from 0.3 to 3.8 ng/mg. It seems obvious, in comparison with other drugs, that metformin is badly incorporated into hair, as the daily dosage varied from 1 to 3 g. Although limited in the number of subjects, the study allowed to postulate a possible correlation between daily dose and concentration in dark hair, while for light hair no correlation was found.

KEYWORDS

diabetes, GC–MS/MS, hair, hypoglycemic drug, Metformin

1 | INTRODUCTION

Metformin (1,1-dimethylbiguanide), an oral hypoglycemic drug, is the first line therapy for the treatment of diabetes mellitus type 2 (T2DM). Its pharmacological action consists in inhibiting the hepatic gluconeogenesis and increasing glucose peripheral uptake. This drug is available in 500, 850 and 1,000 mg tablets. Daily oral doses of metformin, as the hydrochloride salt, are normally within a range of 500–2,500 mg.[1] Factitious hypoglycemia is assessed as a manifestation of Munchausen's syndrome. Patients with this syndrome tend to deliberately cause a factitious illness in other people, usually their children, putting them at risk of death or irreversible damage.[2] In 2018 the American Association of Poison Control Centers' has reported 23,138 case of exposure to hypoglycemic agents, 2058 of which are children less than 5 years old. Among these, biguanides represent the most used with 906 case.[3]

Metformin is considered a safe drug, but several contraindications exist, including renal failure, liver disease, alcohol abuse or congestive heart failure. There are robust data which support that adequate use of metformin decrease morbidity and mortality in patient with T2DM without causing experiences of hypoglycemia. However, metformin intoxications can be severe, difficult to treat and are associated with

30% mortality.[4] Various analytical methods to test for metformin in biological samples such as blood and urine have been published. Metformin can be analyzed by liquid-chromatography coupled to UV detection,[5] mass spectrometry[6] or tandem mass spectrometry.[7] However, metformin testing by liquid chromatography presents some analytical difficulties, including high possibility of matrix effects. In addition, liquid–liquid extractions with buffer and various organic solvents are difficult to achieve given its high polarity, as metformin has a tendency to remain in the aqueous fraction. Finally, when using reverse phase chromatography as separation method, metformin is not well retained on the column, which results in a very short retention time and in the presence of numerous co-eluting substances.[8] To overcome the problems and therefore to prevent metformin elution in the solvent front and to obtain a good chromatographic separation, many publications have been published describing the use of chromatographic methods such as reversed phase ion pairing[9] or cation exchange chromatography.[10] These methods, however, also present some disadvantages including lack of sensitivity, use of a long and complex sample preparation or use of large volume sample. Many papers have described the use of gas chromatography for the analysis of metformin, coupled to a nitrogen detector[11] or to a mass spectrometer.[6]

Given the low concentrations that have to be measured in hair, sensitivity and specificity of mass spectrometry are required. To achieve a suitable chromatographic separation the gas chromatography seems to be the best choice.

Unfortunately, not much data is available in the literature for hair analysis of antidiabetics[12–14] and almost none for metformin. Only Binz et al.[12] have identified metformin in the hair with concentrations ranging from 11 to 101 pg/mg. They developed a reverse phase liquid chromatography screening method for several hypoglycemic drugs, including metformin, but the latter was eluted within the solvent front.

Hair should be the specimen of choice to complement urine or blood analysis as this specimen can increase the window of detection to weeks or months, pending its length. Drugs incorporated into hair remain relatively stable for many months or even years, thus hair represents a long proof record of drug intake, since it can reflect consumption over a time window wider than that of urine and blood (hours to days).[15] Thanks to these advantages, hair analysis has become routinely used in forensic investigations (drug-related deaths and criminal poisonings).[16]

The temporal stability of the molecules in the hair leads this matrix to be used not only in the forensic context but also in many other, especially in the clinical one. There is a great discordance in the literature regarding the application of hair testing for therapeutic drug monitoring (TDM) because of the discrepancies in the findings concerning the relationship between dose and concentrations in hair. Matsuno et al.[17] measured haloperidol concentration in hair of 59 psychiatric patients who had been taking the drug at fixed daily doses for more than 4 months. It was shown that the substance was excreted into hair in proportion to the doses given, and that the distribution of the drug along the hair length represented the month by-month dosage history. It was concluded that, for haloperidol and its metabolite, drug monitoring is possible in hair. While some authors find correlation, for other this relationship is lacking. Henderson et al.[18] measured cocaine-d5 administered to 25 volunteers under clinical conditions and they found poor correlation between drug incorporated and dose received by the subjects. Pragst et al.[19] found no correlation between the daily dosage of some tricyclic antidepressant (amitriptyline, clomipramine, doxepin, imipramine and maprotiline) and hair concentration of treated patients. The explanation could be that there is an inter-subject variability to which are added many variables including hair color, hair-growth rate and cosmetic treatments.

In the present study,we have developed a GC–MS/MS method for the detection and quantitation of metformin in human hair and applied it by testing hair specimens obtained from diabetic patients, in order to propose data for the interpretation of the measured concentrations.

2 | MATERIAL AND METHODS

2.1 | Chemical and reagents

Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) at a concentration of 1 mg/mL in methanol was purchased from LGC (Middlesex, UK). Metformin-d6 hydrochloride (1 mg) was supplied by Toronto Research Chemicals (Toronto, Canada). N-methyl-bis-(trifluoroacetamide) (MBTFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol for HPLC Isocatric Grade (MeOH) was obtained from V.W.R. Chemicals ProLabo (Fontenay-sous-Bois, France).

Metformin-d6 was used as internal standard (IS) at a concentration of 1 mg/L in methanol. For calibration standards, adequate dilutions were made in methanol from the initial solution of metformin at 1 mg/mL.

2.2 | Hair specimens

To get reference values and to obtain information about the correlation between the dose and the concentration found, hair specimens of 9 patients (men and women aged 80 to 85) on daily treatment with metformin since more than 10 years were analyzed. Given the short hair length of most patients, only the proximal segment (2 cm) was tested. For those whose length exceeded 6 cm, segmentation was achieved. None of the hair specimens were cosmetically treated. One of the authors collected all the specimens in an envelope and they were stored at ambient temperature at the laboratory before analysis. Blank hair for method validation was obtained from laboratory staff volunteers. An oral consent was obtained from each subject after the study has been explained. In France, hair collection is not considered to be an invasive maneuver.

2.3 | Metformin hair analysis

Calibrators and QC samples for method validation were prepared in blank hair obtained from the laboratory staff. Real hair samples (subjects under treatment) and blank hair, followed the same preparation. Hair specimens were decontaminated twice with 5 mL of dichloromethane for 2 minutes. After drying, the hair was cut into small pieces of less than 1 mm and 20 mg were incubated in the presence of 20 ng of metformin-d6 (20 µL of a 1 mg/L solution in methanol) in 1 mL of methanol and placed in a ultrasound bath for 2 h at room temperature. After centrifugation at 3000 rpm for 15 minutes, the supernatant was collected and evaporated to dryness at 50 °C under a nitrogen flow. The residues were derivatized with 40 µL of MBTFA at 80 °C for 60 min. 2 µL of the derivatized sample were injected into the GC–MS/MS system for analysis.

2.4 | Instrument conditions

GC separation was performed on an Agilent GC 8890 using an Agilent 30 m × 0.25 mm, 0.25 µm HP-5MS UI column for separation. A 2 µL splitless injection with a 1.2 mL/min flow of helium used as carrier gas was made. The gradient temperature was as follows: the initial oven temperature was set at 100 °C, and then the temperature was increased to 270 °C at a rate of 20 °C/min. The total run time was 7.5 min. A 7010B triple-quadrupole mass selective detector was used for analysis of the compounds. The tandem mass spectrometer was operated in electron ionization mode (EI) and multiple reaction monitoring mode (MRM) was used for the identification of metformin and metformin-d6. The following conditions were found to be optimal for the analysis of metformin and the IS: electron energy at 70 eV, front inlet temperature at 280 °C, ion source temperature at 230 °C and interface temperature at 280 ° C. The solvent delay was set at 4 min. Collision energy was adjusted to optimize the signal of the 2 most abundant products ions of the derivatized metformin: m/z 303 > 288.2, 303 > 125.1. For the internal standard the following transitions were used: m/z 309.1 > 291.1 and 309.1 > 128 (Table 1). The ions m/z 303 > 288.2 for metformin and 309 > 291.1 for IS were selected for quantification. MassHunter 10.0 software was used for quantification.

TABLE 1 Metformin and IS metformin-d6 specific transitions

	Precursor	Product ion	Electron energy (eV)	Collision energy (eV)
Metformin				
	303.0	288.2	70	15
	303.0	125.1	70	20
Metformin-d6				
	309.1	291.1	70	15
	309.1	128.0	70	25

2.5 | Validation procedure

The hair test method was validated for linearity, repeatability, reproducibility and detection and quantitation limits according to SFTA (Societé Française de Toxicologie analytique) guidelines.[20]

Three calibration curves which included six points (concentrations ranging from 0 to 50 ng/mg), obtained over a three-day period, were established for the study of linearity.

Quantification was achieved by plotting the peak area ratios of metformin to metformin-d6 versus concentration followed by linear regression analysis. Repeatability and reproducibility were determined for two QC levels, 0.1 ng/mg and 10 ng/mg. For repeatability, six replicates of each QC level were processed the same day. For 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 target and qualifier transitions. 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 a good linearity in the concentration range 0–50 ng/mg with a correlation coefficient ranging from 0.9873 to 0.9996 during the 3 tests. The LOD and the lower LOQ are 1 pg/mg and 100 pg/mg respectively. Repeatability was 15.5 and 7.2% at 0.1 and 10 ng/mg, respectively, and reproducibility was 17.3 and 9.5 at 0.1 and 10 ng/mg, respectively. Validation results are reported in Table 2. The extraction time was optimized by testing different incubation periods, varying from 1 hour to 3 hours, using an authentic specimen. A 2 hours incubation time was found suitable, as the signal for metformin did not increase after that period. Acetonitrile and methanol were tested and compared as extraction solvent. Best recovery and stability were observed with the use of methanol, in accordance with what was previously published.[21] It was not possible to establish an accurate extraction efficiency in authentic hair due to the lack of reference material (certified hair specimen containing a calibrated metformin amount)

TABLE 2 Validation parameters of metformin in hair

Linearity	0.1–50 ng/mg
Correlation coefficient	0.9873–0.9996
LOD	1 pg/mg
LOQ	100 pg/mg
QC 0,1 ng/mg	
CV repeatability (%)	15.5
CV reproducibility (%)	17.3
QC 10 ng/mg	
CV repeatability (%)	7.2
CV reproducibility (%)	9.5

available for validation. A typical chromatogram after extraction of the 0–2 cm segment of patient 4 is presented on Figure 1. As it can be observed, the chromatogram is clean and the peaks are well separated. The measured concentration was 0.3 ng/mg (the lowest measured concentration between the 9 subjects) and the intense response demonstrates the efficiency of the proposed method. No interference was found from chemicals, nor from endogenous compounds present in hair. Figure 2 is the chromatogram obtained after extraction of a blank hair sample. As it can be observed, the chromatogram is free from interference at the metformin retention time.

To gain suitable signal, prior to the GC analysis, metformin has to be converted to a volatile derivative. In a method developed by Uçaktürk in 2013[6] devoted to the determination of metformin in human plasma, the author tested various derivation reactions at different temperatures and different times and found that the acylation reaction obtained by adding MBTFA was the most suitable approach.

Hair analysis has proven to be very useful as it gives information on exposure to drugs over time. In cases of recurrent and severe hypoglycemic episodes in patients without treatment that could be associated with Munchausen's by proxy syndrome or in the context of criminal poisoning in general, it seems important that the most used hypoglycemic agent has to be screened in hair. In order to obtain reference values to be able to interpret concentrations found in metformin consumers, the developed method was successfully applied for the identificationand the quantification of metformin in hair of 9 diabetics patients on daily treatment. The daily dosage ranges from 1,000 to 3,000 mg. Table 3 presents the results, including dosage, hair color and metformin concentrations. Concentrations ranged from 0.3 to 3.8 ng/mg.

The toxicological significance of the measured concentrations was difficult to establish because there is only one paper in the literature presenting metformin concentrations in human hair [12]. The authors measured metformin concentrations ranging from 10.7 to 100.7 pg/mg in 9 patients treated with daily doses ranging from 780 to 2,652 mg/day. When comparing our concentrations with the results of Binz et al.[12] it must be pointed that their data are based on self-reported antidiabetics consumption by patients, and one cannot exclude incorrect adherence to the daily prescription. The hair preparation step is identical in both studies, as Binz et al. also used methanol incubation. No information was given by the authors regarding the presence or absence of cosmetic treatments (hair coloration or bleaching) which could affect metformin concentrations. One of the factors that could explain these different results might be the unknown incorporation rate of metformin in hair.

There are many factors that can affect the incorporation of a molecule into hair and this entails a great variability from subject to subject. Considering that metformin is a basic molecule, a property that has been shown to favor incorporation in hair, one can expect that its incorporation rate will be high.[15] As can be seen from the results presented in Table 3, despite the daily dosage varies from 1 to 3 g for patients under therapy for at least 10 years, measured concentrations were only in the low ng/mg range. However, the incorporation of a drug into hair not only depends of its pKa and melanin affinity but also on other factors such as itslipid solubility.

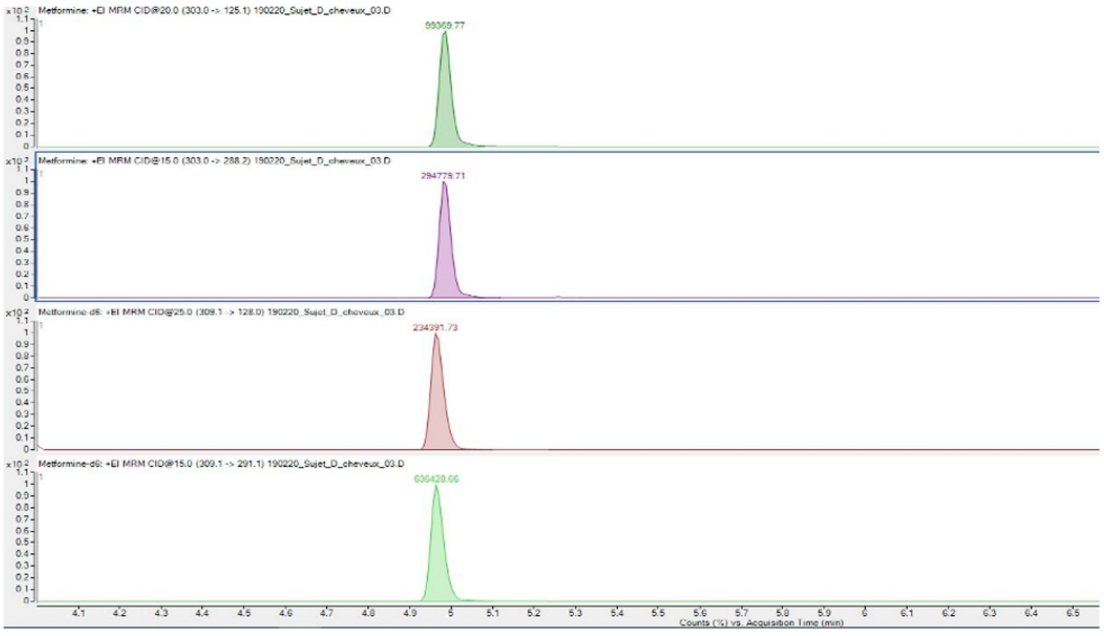

FIGURE 1 Chromatogram of subject's hair on daily treatment, 0–2 cm (from top to the bottom: 2 transitions of metformin and 2 transitions for IS metformin-d6). Concentration was 0.3 ng/mg [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Chromatogram of blank hair sample (from top to the bottom: 2 transitions of metformin and 1 transitions for IS metformin-d6) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Results of hair analysis

Subject	Dose (mg/daily)	Color	Metformin concentration (ng/mg)
1	2000	Blond	0.4
2	1,700	Blond	0.7
3	2,550	White	0.9
4	2,500	Blond	0.3
5	2000	Brown	1.9
6	1,000	Brown	1.1
7 (0–2 cm)	3,000	Brown	3.8
7 (2–4 cm)	3,000	Brown	3.6
7 (4–6 cm)	3,000	Brown	3.2
8	2000	White	0.6
9	1,500	White	1.4

The latter is a critical factor in determining the rate of transport from blood stream across the cell membrane into the growing root bulb.[15] Therefore, one could imagine that, given the high hydrophilicity of metformin, this molecule will be poorly incorporated into the keratinous matrix. In fact, in several studies, it was shown that many hydrophilic metabolites such as benzoylecgonine, morphine and amphetamine are less incorporated intohair than their lipophilic precursors (cocaine, 6-monoacetylmorphine or methamphetamine). In the same way, the tricyclic antidepressants amitriptyline, clomipramine, doxepin and imipramine accumulate more in hair than their corresponding metabolite.[22]

Although the number of subjects is limited in this study, one can consider that metformin stability in hair can be influenced by cosmetic treatments or normal hygiene practice. In subject 7 (the only one where segmentation was possible), it can be observed that the concentration of metformin gradually decreases from root to tip. The

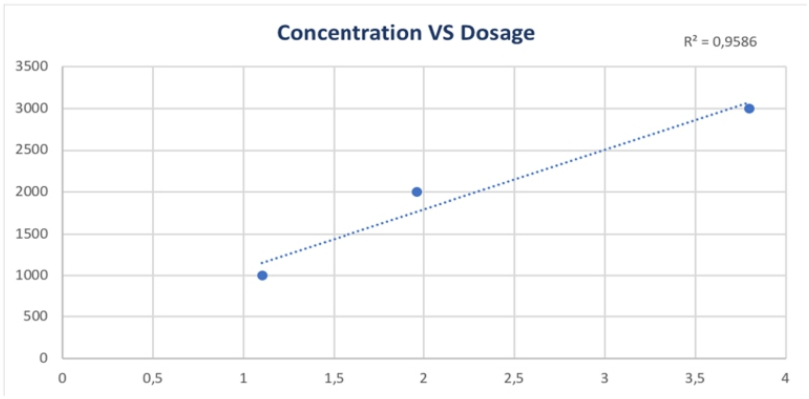

FIGURE 3 Concentration of metformin in dark hair versus daily dosage(n = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

concentrations found in the 3 segments are coherent because when a drug is ingested at the same amount each day, it is expected to notice a gradual decrease along the shaft as a result of loss during standard hygiene practices (shampoo washing). In hair testing science, it is very difficult to interpret analytical results when there is a lack of data in the literature, including the minimal detectable dose, the dose-concentration relationship and other parameters which can influence incorporation and stability. With respect to metformin, kinetics of incorporation into the keratin matrix and correlation between dosage and hair concentrations are not yet established. In this study, the authors tried to determine if there is a correlation between the dose and the concentrations measured in hair. As can be seen from the results presented in Table 3, a 1,000 mg/day dose produces a concentration of 1.1 ng/mg while a 3,000 mg/day dose an average concentration of 3.4 ng/mg. According to these findings, it can be anticipated that there is probably a correlation, at least a weak correlation between the metformin dose and the hair concentration, but only for dark hair (r^2 = 0.95) (Figure 3). These preliminary findings must be supported by additional tests to confirm this statement. Indeed, according to the results, it seems that there is a difference in metformin incorporation in dark hair when compared to light colored hair. Dark hair presents 5 times higher concentrations at the same dosage (Table 3). Several studies have investigated the different incorporation of drugs in relation to hair color. The findings supported the greater binding affinity of basic drugs to melanin which is the component of hair responsible for pigmentation. Dark hair contains more melanin than light colored hair, thus basic molecules have more affinity for them and are incorporated at a higher rate. [15] For example, Reid et al.[23] evaluated the degree of incorporation of benzoylecgonine in different hair colors after incubation in presence of the molecule and found that the levels were higher in black hair, followed by brown hair, and finally by blond hair. In another publication, Joseph et al.[24] observed a 10-time higher incorporation of cocaine-d3 into black hair than in blond hair. The observed phenomenon is explained as basic drugs are positively charged at physiological pH and interact through melanin polymer by electrostatic force between their cationic groups and the anionic carboxylic groups on the surface of the melanin.[15] As metformin is a basic molecule, it is theoretically better incorporated in dark hair, which corresponds to what was observed.

Relationships between hair concentrations and daily dose have been published for several drugs with highly variable results. For example, some authors did not find such a relationship. Gheddar et al.[25] in 2019 didn't find any correlation between the daily dose of hydrochlorothiazide and concentrations found in hair subjects under daily treatment. This was attributed to the acidic properties of hydrochlorothiazide. Puschel et al.[26] compared the concentrations of drugs of abuse present in the hair of drug abusers with self-reported consumption, but they were unable to establish any positive correlation. Although several studies have demonstrated that a linear relationship does not exist, controlled studies have supported a good correlation between the amount of drug taken and hair levels for some drugs. Polettini et al.[27] observed a good correlation between dosage and concentrations found in hair of subjects following a controlled regime of methamphetamine and amphetamine. Ropero-Miller et al.[28] demonstrated dose-related concentrations of cocaine and its metabolites in human hair after controlled cocaine administration. Many factors can affect this correlation, including the amount of drug estimated on the basis of a self-reported consumption, hair growth cycle, which is not homogenous, lipid solubility and pKa of the molecule, color of the hair, and cosmetic treatments.[15]

4 | CONCLUSION

An efficient and sensitive GC–MS/MS method for the detection of metformin in human hair was developed and its applicability was described for the first time. It was demonstrated to be applicable for the analysis of hair samples from diabetic patients. This study has given data on metformin concentrations that can be expected after therapeutic administration. Unfortunately, the number of subjects is not sufficient to conclude with certainty about the presence or absence of a correlation between dose and hair concentrations. In these limited number of cases, only for dark hair, a higher concentration of metformin corresponds to a higher drug consumption. These

findings may be the basis of future studies in understanding if there is a correlation and if it could be considered useful to use hair analysis in therapy drug monitoring. Furthermore, screening for the most widely used antidiabetic in the world could be very useful to complement blood and urine analysis for retrospective investigation of metformin consumption especially when associated with unclear hypoglycemia such as in the context of Munchausen by proxy syndrome.

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2.4. Applications à des cas d'expertises médico-judiciaires

Les méthodes développées pour l'identification et le dosage de la metformine dans les fluides biologiques et les cheveux ont été appliquées à des cas médico-légaux dont le décès était soupçonné être lié à un surdosage en metformine.

La méthode développée dans la matrice sanguine est également appliquée de manière routinière à des demandes hospitalières pour l'adaptation de la posologie et pour les demandes des services de réanimation en cas d'acidose lactique causée par un surdosage de metformine.

Une étude réalisée sur les cheveux de patients diabétiques sous traitement à la metformine a permis d'obtenir des valeurs de référence permettant d'interpréter les concentrations retrouvées dans les cas de surdosage mortel de metformine ***[Article 3].***
Ces cas ont donné lieu à une publication scientifique.

<u>Cas de décès lié à l'abus de metformine :</u>

Cas 1 ***[Article 3] :*** L'analyse des cheveux est très utile pour les enquêtes toxicologiques car, en offrant une fenêtre de détection plus large, elle donne la possibilité de réaliser une étude rétrospective sur la consommation historique d'une substance. Malheureusement, il n'y a pas de donnée disponible sur les concentrations capillaires dans les décès liés à la metformine. Dans cette étude, les auteurs présentent 2 cas d'intoxication fatale à la metformine dans lesquels, pour la première fois, l'analyse des cheveux a été réalisée en utilisant une méthode spécifique GC- MS/MS. La metformine a été testée positive dans le sang fémoral (112,3 mg/L et 64,7 mg/L respectivement) et le sang cardiaque (226,9 et 203,2 mg/L) des deux sujets. Pour le cas 1, d'autres échantillons ont également été testés positifs, notamment l'humeur vitrée (31,1 mg/L) et le contenu gastrique (773,5 mg/L). Pour le cas 2, la concentration de metformine a été mesurée à 844,9 mg/L dans l'urine. Les concentrations de metformine dans les cheveux étaient de 28,3-44,8 et 22,5 ng/mg pour les deux cas, respectivement. Les concentrations retrouvées dans les 2 cas de décès sont nettement supérieures à celles obtenues dans une étude précédente avec des sujets sous traitement (0,3 à 3,8 ng/mg) ou à celles retrouvées dans 3 cas post-mortem où le décès par metformine a été exclu (0,6 à 1,4 ng/mg). Une transpiration excessive pendant la phase agonique due à une hypoglycémie fatale pourrait expliquer ces concentrations élevées, car la sueur peut avoir contaminé les cheveux.

Cas 2 : Une femme de 65 ans, souffrant de diabète de type 2 et d'obésité, est retrouvée décédée à son domicile, trois jours seulement après une chirurgie bariatrique (by-pass gastrique Roux-en-Y, RYGB). Son traitement, comprenant de la metformine et de la dapagliflozine, avait été arrêté avant l'opération et n'avait pas été repris en postopératoire. Une autopsie médico-légale a été pratiquée. Du sang cardiaque et fémoral, de l'urine, du contenu gastrique et des cheveux ont été collectés pour des investigations toxicologiques.

Aucun signe de perforation digestive ou de péritonite n'a été identifié lors de l'autopsie, excluant une complication chirurgicale précoce comme cause du décès. Ces résultats ont été confirmés par l'analyse histologique qui a montré une réaction inflammatoire intestinale physiologique liée à la chirurgie récente. L'analyse toxicologique a révélé la présence d'acétone dans le sang (0,25 g/L) et dans l'urine (0,58 g/L), confirmant le diabète. La metformine a été testée positive dans le sang (148 mg/L), l'urine (49 mg/L), le contenu intestinal (> 100 mg/L) et les cheveux (1,9 ng/mg). La concentration sanguine était considérée comme potentiellement létale. La faible concentration dans l'urine indiquait une mort rapide après l'exposition. La concentration dans les cheveux se situait dans la fourchette thérapeutique.

Le médecin légiste a conclu que le décès était dû à une acidose lactique causée par un surdosage de metformine. En l'absence de preuve de suicide par ingestion de metformine, l'hypothèse soulevée par les auteurs était que la chirurgie bariatrique aurait pu provoquer des changements dans l'absorption de la metformine, conduisant à un surdosage rapide et au décès. Dans la littérature, il a été démontré chez les patients ayant subi un bypass gastrique qu'il existe une augmentation significative de la biodisponibilité de la metformine après administration orale. Le RYGB augmenterait le temps de transit intestinal et donc la durée d'exposition de la muqueuse de l'intestin grêle. Les modifications induites par la procédure chirurgicale ne sont probablement pas suffisantes pour expliquer les concentrations massives de metformine trouvées dans les échantillons *postmortem*, mais le contexte de la découverte du cadavre n'était pas évocateur d'un suicide. Comme il s'agit du premier cas de surdosage en metformine après une chirurgie bariatrique, d'autres cas seront nécessaires pour confirmer nos premières observations. On peut anticiper qu'une dose thérapeutique peut devenir toxique lorsqu'elle est administrée à un sujet ayant récemment modifié son équipement digestif. Ce cas souligne les doutes que peuvent rencontrer les pathologistes et les toxicologues en médecine légale et que les preuves doivent constamment être reconsidérées.

Article 4: Specific interpretation of hair concentrations in 2 fatal metformin intoxication cases

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Case Report

Specific interpretation of hair concentrations in 2 fatal metformin intoxication cases

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ABSTRACT

Hair analysis is very useful for toxicological investigations since, by providing a wider detection window, it gives the possibility to perform a retrospective study on the historical consumption of a substance. Unfortunately, there are no data available for hair concentrations in metformin-related deaths. In this study, the authors present 2 cases of fatal metformin intoxication in which, for the first time, hair analysis was performed using a specific GC–MS/MS method. Metformin was tested positive in femoral blood (112.3 mg/L and 64.7 mg/L respectively) and cardiac blood (226.9 and 203.2 mg/L) of the two subjects. For case 1, other samples were also tested positive, including vitreous humor (31.1 mg/L) and gastric contents (773.5 mg/L). In case 2, metformin was measured at 844.9 mg/L in urine. Metformin hair concentrations were 28.3–44.8 and 22.5 ng/mg for both cases, respectively. The concentrations found in the 2 fatal cases are clearly higher than those obtained in a previous study with subjects under treatment (0.3–3.8 ng/mg) or those found in 3 post-mortem cases where metformin death was excluded (0.6–1.4 ng/mg). Excessive sweating during the agonal phase due to fatal hypoglycemia could explain these elevated concentrations as sweat can have contaminated the hair.

1. Introduction

Metformin (Glucophage™) is the most worldwide used hypoglycemic agent for the treatment of type 2 diabetes mellitus (T2DM). Half a century ago, various biguanides were used for the treatment of T2DM (metformin, phenformin, buformin). In the 70 s they were all removed from the market due to a high association with lactic acidosis. However, the role of metformin has been reassessed in recent years and now represents the first-line therapy for type 2 diabetics [1]. Metformin is available as oral tablets in the immediate release form and in the extended release form at dosages of 500, 850 and 1000 mg to be taken two or three times a day [2].

Metformin is a small molecule (molecular weight of 129.2 Da) with a bioavailability between 50 and 60%. It is eliminated unchanged by the kidneys. The peaks plasma concentrations at therapeutic doses are between 1.5 and 3 mg/L [3]. The hypoglycemic action of metformin consists in inhibiting the production of glucose in the liver and in stimulating glucose uptake in peripheral tissues.

Although metformin is considered as one of the safest antidiabetics, fatal intoxications, whether accidental or intentional, are not rare events. Problems related to metformin are due to its hypoglycemic action and to the excessive production of lactic acid. In fact, metformin, by inhibiting liver gluconeogenesis, contributes to the increase of blood lactate level, which, under certain conditions and for certain patients, can be fatal. This side effect is known as the metformin-associated lactic acidosis (MALA). In subjects with normal renal function, metformin is eliminated as parent drug by glomerular filtration and tubular secretion. In case of kidney dysfunction, metformin will accumulate and produce increased lactate levels. Marked acidosis can cause significant vasodilation and depression of myocardial contractility. These mechanisms will lead to hypotension and hypo-perfusion, which in turn will cause acute renal failure followed by multisystem organ failure [4,5].

With respect to its hypoglycemic effect, unlike other antidiabetic agents such as sulfonylureas, which act directly by stimulating the release of insulin, metformin has a more complex effect, which allows maintaining a balance in blood glucose levels without causing significant decrease in concentration. Nevertheless, fatal hypoglycemia cases have already been reported, especially when metformin is used in combination with other antidiabetics [6].

Until now, in metformin-related death cases published in the medical

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literature, hair analysis has never been reported. Hair analysis has gained increasing importance in recent years both in the forensic and clinical practice, as it allows to establish the pattern of exposure to a drug and to evaluate, in some controlled circumstances the frequency and dosage of drug use during the previous months. The drug detection window in hair is extended to weeks, months or even years based on the length of the hair, in comparison to the standard hours/days for both blood and urine. For some drugs, if a correlation between the administered doses and the concentrations found in hair is established, hair test results can be useful in therapeutic drug monitoring. In a fatal case, segmental hair tests allow to verify treatment observance, which can be critical with hypoglycemic drugs [7].

The abuse of oral hypoglycemic agents for other purposes than therapeutic ones, has emerged in the more recent times [8–10]. In order to better explain unclear hypoglycemic events such as in the context of Münchausen or Münchausen by proxy syndromes or in situations of incapacitation, hair analysis seems to be a very useful to complement blood and urine since it gives retrospective data on drug exposure.

In this study, metformin was tested for the first time in the hair of two subjects deceased from fatal metformin overdose, using a new GC–MS/MS specific method.

2. Case report

2.1. Case 1

A 35-year-old woman with a history of diabetes, hypertension and depression was found dead by her partner in the bathroom of her home. It has been stated that the woman had experienced symptoms of extreme tiredness since the previous day. The woman was under treatment with metformin (1000 mg), extended-release insulin detemir (100 units/mL), paracetamol (500 mg), atenolol (50 mg), ramipril (10 mg) and mirtazapine (15 mg). External examination and medicolegal autopsy operations revealed an asphyxial syndrome associated to cyanosis of the extremities and multi-visceral congestion. Histological examination revealed pulmonary edema and hepatic steatosis. Since drug poisoning was suspected, samples of femoral and cardiac blood, vitreous humor, gastric content, urine and hair were taken for toxicological tests. The samples were stored at +4°C until analysis. Head hair specimen (23 cm, brown) was stored in an envelope at room temperature.

2.2. Case 2

A 57-year-old man was found dead in his bed. Based on the drugs found close to the corpse, it would appear that the subject was diabetic and depressed. In fact, the subject seemed to have been under treatment with drugs such as insulin lantus, metformin (1000 mg), zopiclone (7.5 mg), Prozac (20 mg) and pregabalin (200 mg). The body examination and the post-mortem body scanner revealed a marked asphyxiation syndrome with nail cyanosis. In order to perform toxicological tests, femoral and cardiac blood, urine and hair samples were collected. Samples of biological fluids were stored at + 4 °C until analysis. Head hair specimen (3 cm, dark brown) was stored in an envelope at room temperature.

3. Material and methods

3.1. Chemical and reagents

Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) at a concentration of 1 mg/mL in methanol was purchased from LGC (Middlesex, UK). Metformin-d6 hydrochloride (1 mg) was supplied by Toronto Research Chemicals (Toronto, Canada). N-methyl-bis- (trifluoroacetamide) (MBTFA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile for HPLC Isocratric Grade were obtained from V.W.R. Chemicals ProLabo (Fontenay-sous-Bois, France).

Metformin-d6 was used as internal standard (IS) at a concentration of 1 mg/L in methanol. For calibration standards, adequate dilutions were made in methanol from the initial solution of metformin at 1 mg/mL.

3.2. Metformin analysis

3.2.1. Blood analysis

For the extraction of metformin from blood samples, a method developed by Uçaktürk in 2013 was used [11]. A sample volume of 0.2 mL of human whole blood was subjected to protein precipitation for the extraction of metformin. The specimen was spiked with 20 µL of 100 mg/L deuterated internal standard (metformin-d6) and 3 mL of acetonitrile. The specimen was vortexed for 1 min, then centrifuged at 3500 rpm for 5 min. The supernatant was transferred to another tube and evaporated to dryness under a stream of nitrogen at 50 °C. Following dryness, the specimen was derivatized with 40 µL of MBTFA at 80 °C for 60 min and then transferred to injection vials and 1 µL was injected in the GC–MS/MS system.

A 7-point calibration curve was prepared using the following concentrations: 0, 1, 2, 5, 10, 20, 50, 250 mg/L of metformin in blood. Calibration, achieved in blood, was used for the other liquid specimens.

3.2.2. Hair analysis

Both hair specimens were brown in color. Hair of the first case was oriented root-tip, with a length of 23 cm. The first 6 cm were analyzed after being divided into 3 segments. In acute poisoning deaths, we do not find it interesting to search for a molecule beyond the first 6 cm. Hair of the second one was not oriented, with a length of 3 cm. In both cases, hair sampling was carried out following the guidelines of the SOHT (Society of Hair Testing) [12]. For metformin hair analysis, a method previously developed and validated by the authors was used [13]. Hair specimens were decontaminated twice with 5 mL of dichloromethane for 2 min. Once dry, they were cut into small pieces and 20 mg were incubated in the presence of 20 ng of metformin-d6 (20 µL of a 1 mg/L solution in methanol) in 1 mL of methanol and placed in an ultrasound bath for 2 h at room temperature. After centrifugation at 3000 rpm for 15 min, the supernatant was collected and evaporated to dryness at 50 °C under a nitrogen flow. The residues were derivatized with 40 µL of MBTFA at 80 °C for 60 min and 2 µL was injected into the GC–MS/MS system for analysis.

A 6-point calibration curve was prepared using the following concentrations: 0, 0.1, 1, 2, 5, 10, 50 ng/mg of metformin in hair.

3.2.3. Instrument (GC–MS/MS) conditions

GC separation was performed on an Agilent GC 8890 using an Agilent 30 m × 0.25 mm, 0.25 µm HP-5MS UI column for separation. A 1 µL split injection was made with a split ratio of 10:1 for blood analysis method and a 2 µL splitless injection was made for hair analysis. The flow rate of the carrier gas (helium) was set at 1.2 mL/min. The gradient temperature was as follows: the initial oven temperature was set at 100 °C, and then the temperature was increased to 270 °C at a rate of 20 °C/min. The total run was 7.5 min. A 7010B triple-quadrupole mass selective detector was used for analysis of the compounds. The tandem mass spectrometer was operated in electron ionization mode (EI) and multiple reaction monitoring mode (MRM) was used for the identification of metformin and metformin-d6. The following conditions were found to be optimal for the analysis of metformin and the IS: electron energy at 70 eV, inlet temperature at 280 °C, ion source at 230 °C and interface temperature at 280 °C. The solvent delay was set at 4 min. Collision energy was adjusted to optimize the signal of the 2 most abundant products ions of the derivatized metformin: m/z 303 > 288.2, 303 > 125.1. For the internal standard the following transitions were used: m/z 309.1 > 291.1 and 309 > 128. The ions m/z 303 > 288.2 for metformin and 309 > 291.1 for IS were selected for quantification. MassHunter 10.0 was used for quantification [13].

4. Results and discussion

Metformin was found in femoral blood, cardiac blood, vitreous humour and gastric contents of the subject 1 at the following concentrations: 112.3 mg/L, 226.9 mg/L, 31.1 mg/L, 773.5 mg/L. For subject 2 analyses revealed a femoral blood concentration of 64.7 mg/L, a cardiac blood concentration of 203.2 mg/L and a urine concentration of 844.9 mg/L. Metformin concentration in hair of the case 1 were: 28.3 ng/mg (root to 2 cm), 39.4 ng/mg (2–4 cm) and 44.8 ng/mg (4–6 cm), while for the case 2 we found a concentration of 22.5 ng/mg in the whole strand. (Table 1) Figs. 1 and 2 show the chromatograms obtained after extraction of subject's 1 head hair (root to 2 cm) and subject's 2 hair.

Further analyses, achieved during a comprehensive toxicological screening, revealed in femoral blood the presence of ethanol at a concentration of 2.4 g/L in subject 1 (concentration mainly linked to post-mortem bio-transformation) and zopiclone (448 ng/mL), clobazam (324 ng/mL), amitriptyline (1.51 mg/L), pregabalin (81.4 mg/L) and gliclazide (1.2 mg/L) in subject 2.

In both cases, an experienced pathologist attributed the cause of death to acute metformin intoxication. These findings are in agreement with those reported in the medical literature. According to a publication presented by Walz et al. [14] in 2019, the average concentration of metformin in fatal intoxications by single metformin is 48.5 mg/L (range 13–210 mg/L) and in multiple intoxications (death caused by metformin and other drugs) is 21 mg/L (4.40–95.0 mg/L). In 2018, Hess et al. [15] have indicated, in a study on 95 diabetic patients under chronic metformin treatment, that therapeutic blood concentrations have an average of 1.846 mg/L. The same authors analysed a case of metformin-related death, where they found a concentration of 119 mg/L in femoral blood and 166 mg/L in cardiac blood [15].

As it was previously noticed by others, higher concentrations of metformin were found in cardiac blood when compared to femoral blood, suggesting a possible post-mortem redistribution. Although the analysis of various biological samples has been extensively treated in the literature, metformin has never been tested in hair collected after death.

Hair test results are now recognized to be very useful in forensic toxicology as it allows to obtain information on drug consumption history. Furthermore, when performing hair segmentation, and using an average growth rate of 1 cm/month, it is possible to establish whether exposure occurred on single or multiple occasions.

Surreptitious drug administration in the context of drug-facilitated crimes has been well documented since the last 20 years, particularly with the introduction of bench-top LC-MS/MS tools. Especially, in the recent years, cases of hypoglycaemia induced deliberately in healthy subjects using antidiabetic drugs have been described. For example, Villain et al. [10] have demonstrated repeated exposure to glibenclamide in a non-diabetic subject. Hair analysis, in this case, was the only retrospective evidence of drug exposure. Given that metformin is the most prescribed antidiabetic in the world and considering its widespread use, it seems necessary to test it in cases of death or intoxication in which exposure to this drug is suspected. Hence, given the importance of hair analysis for retrospective information, in addition to test metformin in the standard samples, one should promote such investigations.

Table 1
Results of femoral blood, cardiac blood, vitreous humor, gastric contents, urine and hair analysis in the 2 reported cases.

	Femoral blood (mg/L)	Cardiac blood (mg/L)	Vitreous Humor (mg/L)	Gastric contents (mg/L)	Urine (mg/L)	Head hair (ng/mg)
Case 1	112.3	226.9	31.1	773.5	/	28.3 (root to 2 cm) 39.4 (2–4 cm) 44.8 (4–6 cm)
Case 2	64.7	203.2	/	/	884.9	22.5

Since there is no reference in the literature on metformin in hair in cases of subjects deceased from acute metformin overdose, the toxicological significance of the findings remains to be discussed. In a previous study [13] involving 9 diabetic patients under treatment (1000–3000 mg per day), hair concentrations ranging from 0.3 to 3.8 ng/mg were measured. Additionally, hair specimens from two cases where metformin was found at therapeutic concentrations in post-mortem blood and who died from a different cause, were analysed and concentrations varied from 0.6 to 1.4 ng/mg. The method used has been fully validated in this previous work [13]. LOD and LOQ were respectively 1 pg/mg and 0.1 ng/mg. Having found concentrations not below 0.3 ng/mg at therapeutic doses, we found no interest in going below 0.1 ng/mg as the lowest concentration of our calibration curve. Nothing has been published on the dosage of metformin in post-mortem hair. Only Binz et al. [16] measured metformin in the hair of subjects under treatment. The authors found concentrations ranging from 10.7 to 100.7 pg/mg in 9 patients treated with daily doses ranging from 780 to 2652 mg/day. As already well discussed in a previous work [13], if we compare our results with those obtained by Binz et al., it must be specified that we rely on self-reported consumption and that an incorrect adherence to therapy cannot be excluded. Furthermore, we have no information about the presence or absence of cosmetic treatments and the incorporation rate of metformin in the keratin matrix is unknown.

Although it cannot be generalized from 2 cases, there is evidence that the hair metformin concentrations are largely higher in case of acute fatal intoxication when compared to therapeutic use. This phenomenon can be explained through 2 hypotheses. First, one can suggest that the two subjects did not follow correctly their treatment but the difference between the 2 sets of concentrations is too high to account for this hypothesis. It must be considered that excessive sweating during the agonal phase due to fatal hypoglycemia can have contaminated the hair. The increase of sweating represents one of the neurogenic symptoms characteristic of hypoglycemia caused by an activation of the autonomic nervous system [17].

Drug incorporation into hair can take place not only through the bloodstream but also through sweat or other sebaceous secretions and sweat contamination is known to be difficult to eliminate by hair decontamination procedures. Molecules and metabolites present in sweat can be incorporated into hair and then become resistant not only to hygiene practices such as the use of shampoos but also to laboratory decontamination procedures [7]. Cone at al. [18] reported that codeine was found in hair after only 24 h post administration. The time between the administration of the substance and its appearance in the growing hair was too short to be caused by blood incorporation and suggested sweat implication. Henderson et al. [19] found cocaine in multiple hair segments after a single dose, supporting that sweat or other secretions are pathways for drug deposition in hair.

Sweat excretion depends on many factors, including molecular mass, pKa and hydrophilicity of the compound [20,21]. Since metformin is a highly hydrophilic, basic and small molecule, it is probably well excreted into sweat and this could explain hair contamination following situations of excessive sweating.

When analysing hair results of the first case reported, we can see that metformin concentrations in the hair increase going from root to tip (28.3–44.8 ng/mg). Since a possible contamination from sweat is assumed, a higher concentration is expected in the segment closest to the skin.

However, an important factor that must be considered is the porosity of the shaft. Today, it is accepted that the amount of a drug deposited from external contamination into the hair is depended on the porosity of

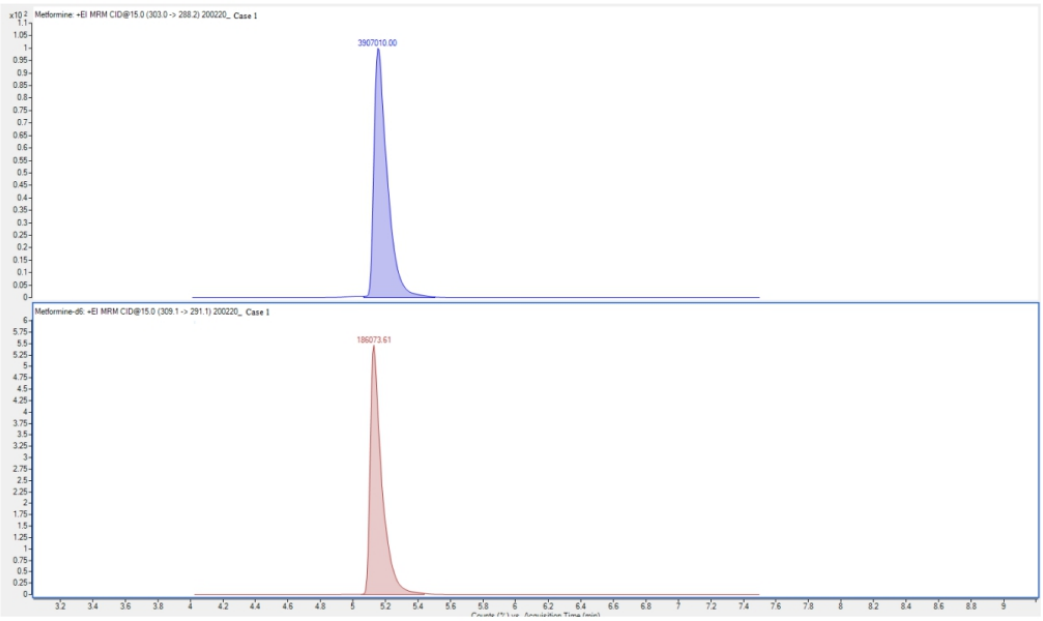

Fig. 1. Chromatogram of Subject's 1 hair, 0–2 cm (from top to the bottom: 1 transition of metformin and 1 transition for IS metformin-d6). Concentration was 28.3 ng/mg.

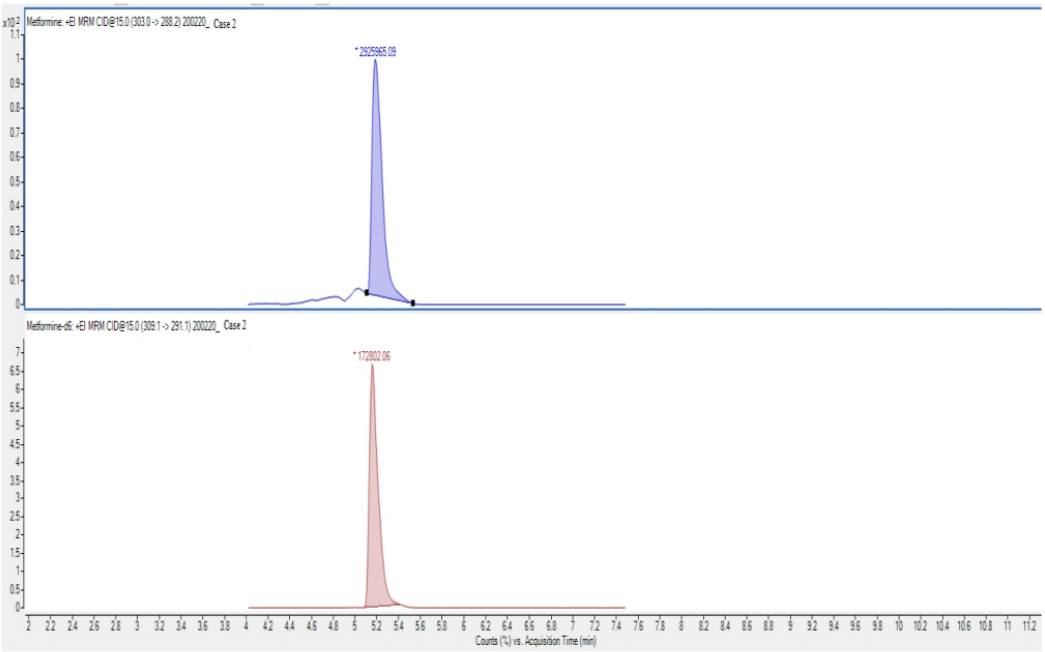

Fig. 2. Chromatogram of Subject's 2 hair (from top to the bottom: 1 transition of metformin and 1 transition for IS metformin-d6). Concentration was 22.5 ng/mg.

the hair shaft. Very porous specimen absorbs high amounts of substances. [22]. Moving away from the root to the tip, we find segments that have been more exposed over time to cosmetic treatments (routine shampooing, conditioning, thermal and chemical treatments). These cosmetic procedures can alter hair texture and, especially, disrupt the cuticular sheath resulting in increased porosity of the hair [23].

Analyzing the washing solutions obtained during the decontamination process, could help in explain the presence of high concentrations of metformin in overdose cases.

Unfortunately, the analysis of the washing solutions has proved to be of interest only later. Metformin quantification in hair was done only after washing and extracting the molecule from the hair, and the surprising result we found, allowed the authors to elaborate the hypothesis of sweat contamination, so the interest of the washing solutions was revealed only later. It would also be interesting for future studies, in case of metformin deaths, to analyze the washing solutions to be able or not to prove external contamination by sweat.

Sweat contamination of the hair have already been described for other molecules such as methadone. Methadone tends to cause excessive sweating in patients under treatment and in fact in many cases of exposure to methadone by children whom the drug was found in the hair, a possible contamination through the sweat of the parents has been suggested in some studies [24–26]. As there is no other element of comparison, it is scientifically acceptable, at this stage, that excessive sweating during the agonal phase of hypoglycaemia is responsible of high metformin concentrations found in the hair after acute fatal overdose.

5. Conclusion

Oral antidiabetic agents, despite the advantages in reducing morbidity and mortality of diabetic patients, represent a health problem due to their misuse, particularly in some situations of drug-facilitated crimes. In such cases, hair analysis can be very useful and sometimes can be the only evidence of exposure to a particular drug.

In this study the authors presented 2 cases of fatal metformin intoxication in which hair analysis was also performed. Metformin has never been tested in hair of subjects deceased after acute metformin overdose. Based on data collected, the concentrations found in fatal cases are clearly higher than concentrations found in subjects under treatment. Probably, excessive sweating caused by hypoglycemia is responsible for these findings.

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Troisième partie – Les autres Antidiabétiques Oraux

3.1. Présentation

3.1.1 Les sulfamides hypoglycémiants

Les sulfamides hypoglycémiants (également appelés sulfonylurées) sont une classe de médicaments antidiabétiques utilisés pour le traitement du diabète de type II.

Les sulfonylurées ont été découvertes en 1942, lorsque l'infectiologue Marcel Janbon et son équipe ont observé que certains antibiotiques sulfamidés (2254 RP (p-aminobenzènesulfamidoisopropylthiodiazole) provoquaient de l'hypoglycémie chez les animaux de laboratoire. Loubatiers et son équipe ont ensuite démontré que la capacité du 2254 RP à faire baisser la glycémie était due à la stimulation de la sécrétion d'insuline.

Ces études pionnières ont conduit au développement de plusieurs sulfonylurées pour le traitement du diabète non insulino-dépendant.

A partir de cette observation, le carbutamide (1-butyl-3-sulfonylurea) a été synthétisé.

Le carbutamide a été la première sulfonylurée utilisée pour traiter le diabète, mais il a ensuite été retiré du marché en raison de ses effets indésirables sur la moelle osseuse.

En 1956, le tolbutamide a été introduit et ce fut le premier de la classe des sulfanilurées de première génération.

À partir des années 1960, de nombreuses autres sulfamides ont été synthétisées et divisées en deux groupes. Les agents de première génération (tolbutamide, chlorpropamide, tolazamide et acétohexamide), qui ne sont plus utilisés, et les agents de deuxième génération (glibenclamide, gliclazide, glipizide, glimepiride et gliquidone).

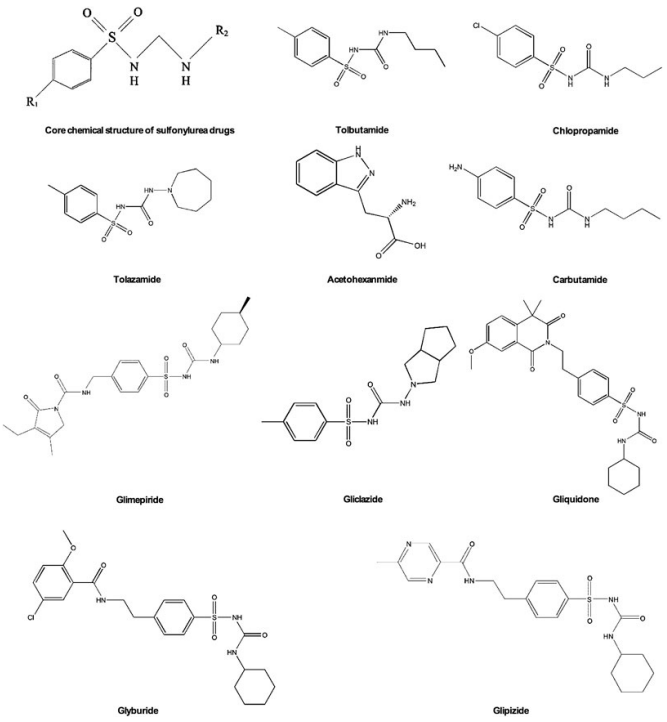

Figure 3. Structures chimiques des sulfamides hypoglycémiants [38]

La principale différence entre les sulfamides de première et de deuxième génération réside dans leurs propriétés pharmacocinétiques (absorption, métabolisme et dosage).

Les sulfanilurées font partie des médicaments antidiabétiques ayant un effet insulino-sécrétagogues et leur action principale est donc d'augmenter les concentrations plasmatiques d'insuline. C'est pourquoi des résidus de cellules bêta pancréatiques sont nécessaires pour que ces médicaments soient efficaces.

Leur action résulte de la liaison aux récepteurs spécifiques de l'insuline (SUR 1) présents dans les cellules bêta du pancréas. Ce lien bloque l'entrée de potassium (K^+) par le canal dépendant de l'ATP , le flux de potassium devient nul, la membrane cellulaire se dépolarise, supprimant ainsi l'écran électrique qui empêche la diffusion du calcium dans le cytosol.

L'augmentation du flux de calcium dans les cellules bêta provoque la contraction des filaments d'actomyosine responsable de l'exocytose de l'insuline, qui est donc rapidement sécrétée en grande quantité [18,19].

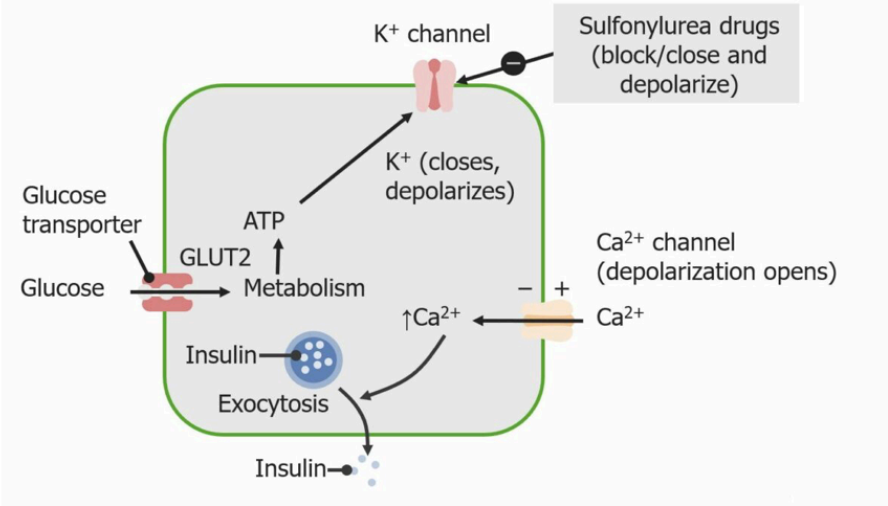

Figure 4. Mécanisme d'action des sulfamides hypoglycémiants [39]

Il est important de souligner que la libération d'insuline induite par les sulfonylurées est indépendante du taux de glucose, ce qui peut augmenter le risque d'hypoglycémie.

L'effet biologique des sulfonylurées se prolonge souvent bien au-delà de leur demi-vie plasmatique, en raison de l'interaction avec les récepteurs et de la formation de métabolites actifs, persistant 24 heures ou plus.

Par conséquent, en cas de surdosage, l'hypoglycémie peut persister pendant plusieurs heures et nécessiter une hospitalisation prolongée.

3.1.2 Les glinides

Les glinides sont une classe d'antidiabétiques insulinotropes, introduits en 1995 et dont l'utilisation clinique n'a été approuvée pour les patients atteints de diabète de type II que depuis les années 2000.
Le répaglinide a été le premier médicament de cette famille à être approuvé pour un usage clinique et est le seul à être commercialisé en France.

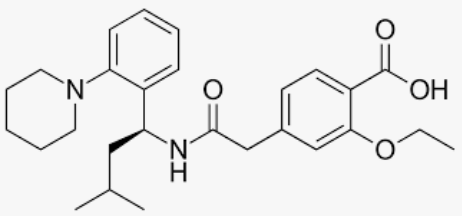

Figure 5. Structure chimique du répaglinide

Les glinides appartiennent à la famille des insulino-sécrétagogues et leur mécanisme d'action est le même que celui des sulfamides hypoglycémiants, qui stimulent la libération d'insuline en inhibant les canaux potassiques sensibles à l'ATP dans les membranes des cellules bêta-pancréatiques en se liant à un récepteur différent de celui des sulfamides (SUR1/KIR 6.2).

Bien que le mécanisme d'action soit identique à celui des sulfamides, ces médicaments ont des propriétés pharmacocinétiques et pharmacodynamiques différentes.
Ils sont rapidement absorbés et stimulent la libération d'insuline en quelques minutes. C'est pourquoi l'insuline sera facilement disponible pendant et juste après le repas après une administration préprandiale.

Le principal effet secondaire est l'hypoglycémie. Bien que le risque d'hypoglycémie soit moindre que pour les personnes traitées par des sulfamides, il s'agit toujours d'un effet secondaire potentiel grave qui peut mettre la vie en danger [20,21].

3.1.3 Les inhibiteurs de la dipeptidyl-peptidase 4 (DPP-4) (gliptines)

Les inhibiteurs de la dipeptidyl-peptidase 4 sont des agents antidiabétiques utilisés pour le traitement du diabète de type II.

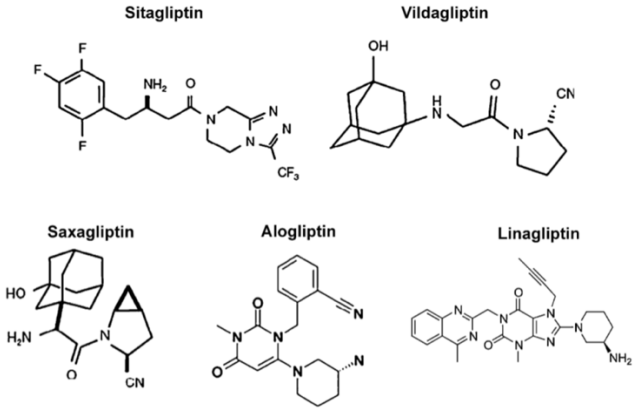

Figure 6. Structures chimiques des gliptines [40]

La dipeptidyl-peptidase IV (DPP-4) est une enzyme ubiquitaire qui peut être détectée dans l'endothélium de différents organes. Les incrétines, à savoir le glucagon-like peptide-1 (GLP-1) et le glucose-dependent insulinotropic peptide (GIP), sont les seuls substrats de la DPP-4. Ce sont des hormones peptidiques gastro-intestinales qui stimulent la sécrétion d'insuline suite à une augmentation postprandiale de la glycémie. La DPP-4 coupe et inactive le GLP- 1 en quelques minutes.

En raison de la rapidité du clivage et de l'inactivation, une thérapie avec le GLP-1 natif administré par voie parentérale n'a pas été considérée envisageable pour le traitement continu du diabète de type II.

Ces observations ont conduit à l'hypothèse que l'inhibition de la DPP-4 entraînerait une augmentation des taux de GLP-1 actif circulant, et pour cette raison les inhibiteurs de la DPP-4 ont été envisagés comme agents thérapeutiques pour le traitement du diabète de type II.

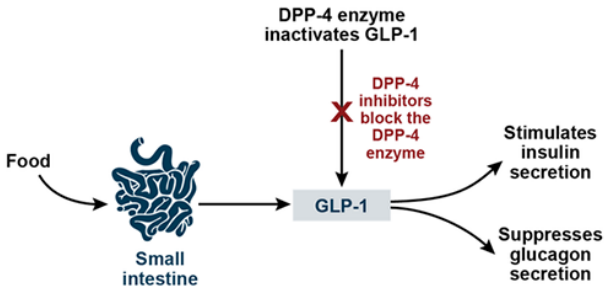

Figure 7. Mécanisme d'action des gliptines [41]

Le premier agent de cette classe est la sitagliptine qui a été approuvée en 2006 par la Food and Drug Administration (FDA) pour une utilisation en monothérapie et en association avec la metformine ou une thiazolidinedione. Par la suite, la sitagliptine a été approuvée pour une utilisation avec une sulfonylurée , et en plus de la metformine.

Le risque d'hypoglycémies dangereuses en cas de surdosages des gliptines semble être très limité. En effet, le pouvoir inhibiteur des gliptines atteint probablement un plateau malgré des concentrations plasmatiques très élevées. La puissante inhibition de la DDP-4 par les gliptines entraîne une augmentation substantielle du niveau des incrétines. Cependant, l'activité des incrétines est dépendante du glucose et par conséquent leur production diminue lorsque la glycémie baisse. Ainsi, le contrôle des incrétines sur la glycémie est calibré et adapté aux besoins, ce qui évite les situations d'hypersécrétion d'insuline et les hypoglycémies dangereuses qui en dérivent même après un surdosage [22,23].
Néanmoins, le risque peut être augmenté si d'autres substances hypoglycémiantes sont administrées en même temps.

3.1.4 Les inhibiteurs du cotransporteurs SGLT-2 (gliflozines)

Les inhibiteurs du cotransporteur SGLT-2 exploitent le rôle du rein dans le maintien de l'homéostasie glycémique.
Avec son activité constante, le rein filtre chaque jour 180 g de glucose et, en situation physiologique, en réabsorbe la quasi-totalité via le tubule proximal.
Ce rôle a été découvert lors d'études sur des patients atteints d'une maladie génétique rare (glycosurie rénale familiale) causée par des mutations du transporteur de Na-glucose

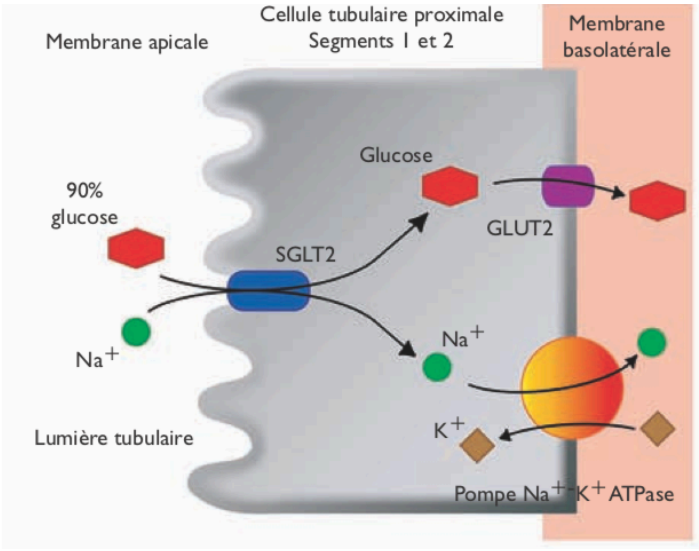

Figure 8. Mécanisme d'action des cotransportateurs SGLT-2 [42]

SGLT-2. Les molécules SGLT *(Sodium-dependant Glucose Transporter)* transportent le sodium et le glucose en exploitant le gradient transmembranaire créé par la pompe Na-K ATPase. Les SGLT concernés par les gliflozines sont les SGLT-2 que l'on trouve principalement dans la partie apicale des cellules tubulaires rénales proximales et qui permettent de réabsorber le glucose filtré par le glomérule.

Lorsque la glycémie dépasse 11 mol/L, la capacité de réabsorption des cotransporteurs est dépassée et la glycosurie apparaît.

L'effet anti-hyperglycémique de l'inhibition du SGLT-2 est connu depuis plus de 150 ans.

La phloridzine, un flavonoïde présent dans l'écorce de nombreux arbres fruitiers, a été isolée pour la première fois en 1835 par des chimistes français et étudiée sur des animaux de laboratoire pendant des siècles, car elle s'est avérée efficace pour réduire l'hyperglycémie et améliorer la sensibilité à l'insuline.

En raison de son manque de spécificité, de sa biodisponibilité orale limitée et de ses effets digestifs trop importants, elle a été abandonnée.

Depuis lors, des inhibiteurs plus sélectifs du SGLT-2, les gliflozines, ont été développés. Il s'agit de glycosides qui résistent aux enzymes gastro-intestinales et qui sont absorbés sans modification de leur structure.

Ils peuvent être utilisés en monothérapie ou en association avec d'autres antidiabétiques oraux ou de l'insuline. Contrairement à la plupart des antidiabétiques oraux, leur effet est totalement indépendant de l'insuline. En Europe, ces médicaments ont été approuvés en 2012 avec la dapagliflozine, premier représentant des gliflozines suivi par d'autres inhibiteurs de SGLT-2 avec canagliflozine, empagliflozine, ertugliflozine et sotagliflozine. En France, ils ne sont sur le marché que depuis 2020.

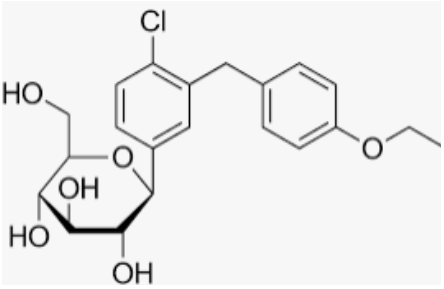

Figure 9. Structure chimique de la dapagliflozine

Leurs principaux effets secondaires sont les infections des voies génitales.

Le risque d'hypoglycémie est absent lors de l'utilisation de ces médicaments mais une exception est toujours faite lorsque d'autres antidiabétiques tels que les sulfonylurées et l'insuline sont utilisés en association [24].

De plus, en induisant une glycosurie de 70-100 g/j qui correspondrait à 280-400 kcal/j, ils entraînent une perte de poids. Par conséquent, leur utilisation détournée ne peut pas être exclue.

3.2 Développement d'une méthode d'identification et de quantification dans le sang (à l'exclusion des gliflozines)

En raison de l'utilisation de ces médicaments à des fins autres que thérapeutiques, afin de diagnostiquer les hypoglycémies dont l'origine n'est pas claire, mais aussi pour surveiller les concentrations plasmatiques pendant le traitement afin d'optimiser la posologie, une méthode de dépistage des antidiabétiques oraux semble nécessaire.
Dans le but d'accélérer l'analyse, il semble également nécessaire de disposer d'une méthode qui intègre un maximum d'agents antidiabétiques en une seule opération.
A ce propos, j'ai mis au point une méthode d'identification et de quantification de 13 antidiabétiques oraux dans le sang appartenant aux trois familles les plus prescrites pour le traitement du diabète (les sulfamides, le glinides et les inhibiteurs de la DDP-4).

Le principal problème rencontré lors de la mise au point de cette méthode de dépistage est de regrouper des molécules ayant des propriétés chimiques différentes.

Plusieurs approches ont été testées avant de choisir la méthode définitive.
Premier test : précipitation dans le méthanol et l'acétonitrile. Bien qu'il s'agisse d'une méthode rapide, elle ne permettait pas d'obtenir un taux satisfaisant de recouvrement et les chromatogrammes présentaient des interférences, ce qui gênait la lecture des concentrations faibles.

Deuxième test : extractions liquide-liquide pH basique (en utilisant un tampon borate saturé à pH 9,5) et à un pH acide (en utilisant un tampon acétate à pH 5,2). Avec un nombre important d'analytes aux propriétés chimiques différentes, le choix du pH du tampon d'extraction est essentiel. Un pH acide permet d'extraire les sulfamides et les glinides. En revanche, un pH trop acide empêche l'extraction des gliptines.

Pour cette raison, afin d'obtenir un taux de recouvrement acceptable pour toutes les molécules, la combinaison de deux extractions liquide-liquide à 2 pH différents (basique puis acide) a permis d'obtenir les meilleurs résultats.

Compte tenu de la présence de 3 familles structurellement différentes, 3 standards internes deutérés ont été utilisés (hydroxy-tolbutamide-d9, repaglinide-ethyl-d5, vildagliptin-d3), chacun appartenant à chaque famille d'antidiabétiques.

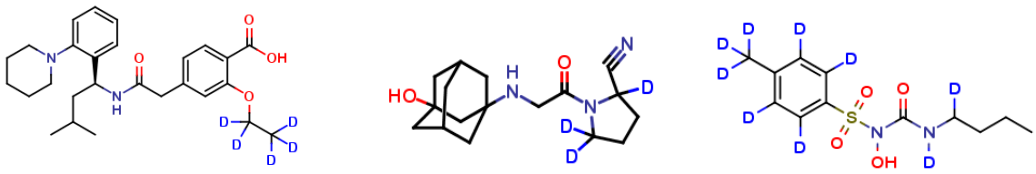

Figure 10. Répaglinide-éthyl-d5 (gauche), vildagliptine-d3 (milieu), hydroxy-tolbutamide-d9 (droite)

La méthode a été développée sur un système LC-MS/MS.

Les échantillons sont analysés en 15 minutes. La méthode a été optimisée et validée. Les paramètres de la méthode sont décrits dans l'article ci-dessous. Par ailleurs, la méthode a été appliquée avec succès au sang post mortem de 8 sujets diabétiques. Les échantillons de sang ont été testés positifs pour le gliclazide (246 – 3000 ng/mL), la sitagliptine (40 – 21400 ng/mL) et le répaglinide (15 ng/mL) ***[Article 5]***

Article 5: Développement et validation d'une méthode de criblage et de dosage de 13 antidiabétiques oraux dans le sang par LC-MS/MS : application à des cas post mortem

Nadia Arbouche, Jean-Sébastien Raul, Pascal Kintz

Toxicologie Analytique et Clinique. 2022; 12(9):1380-1386.

Toxicologie Analytique et Clinique

Développement et validation d'une méthode de criblage et de dosage de 13 antidiabétiques oraux dans le sang par LC-MS/MS: application à des cas post mortem

--Projet de manuscrit--

Numéro du manuscrit:	TOXAC-D-22-00009R1
Type d'article:	Article original / Original Article
Section/Catégorie:	Toxicologie analytique / Analytical Toxicology
Mots-clés:	antidiabétiques, LC-MS/MS, diabète, hypoglycémie
Auteur correspondant:	Nadia Arbouche FRANCE
Premier auteur:	Nadia Arbouche, Doctorante
Ordre des auteurs:	Nadia Arbouche, Doctorante
	Jean-Sébastien Raul, Professeur
	Pascal Kintz, Professeur
Résumé:	Le dépistage des antidiabétiques oraux, agents hypoglycémiants utilisés pour le traitement du diabète de type II, représente un défi dans la pratique médico-légale. En cas d'hypoglycémie d'origine douteuse, la recherche des antidiabétiques oraux permet de différencier les hypoglycémies factices (tentative de suicide, syndrome de Münchhausen par procuration) induites par les antidiabétiques des hypoglycémies vraies (ex. insulinome). Au niveau clinique, au cours d'un traitement, il peut être important de surveiller leurs concentrations plasmatiques afin d'optimiser la posologie. Dans cette étude, une méthode utilisant la chromatographie liquide couplée à la spectrométrie de masse en tandem a été développée et validée pour l'analyse de 13 antidiabétiques oraux dans le sang total humain après une double extraction liquide-liquide à deux pH différents. La procédure de validation a montré une linéarité acceptable pour tous les composés entre 1 et 5000 ng/mL pour le gliclazide, glibornuride et le natéglinide et entre 1 et 1000 ng/mL pour tous les autres antidiabétiques. Les limites de détection et de quantification étaient respectivement de 0,1 e 1 ng/mL pour toutes les molécules. La répétabilité et la fidélité intermédiaire étaient inférieures à 20 % pour les 13 molécules. La méthode a été appliquée avec succès au sang post mortem de 8 sujets diabétiques. Les échantillons de sang ont été testés positifs pour le gliclazide (246 – 3000 ng/mL), la sitagliptine (40 – 21400 ng/mL) et le répaglinide (15 ng/mL).

Développement et validation d'une méthode de criblage et de dosage de 13 antidiabétiques oraux dans le sang par LC-MS/MS : application à des cas post mortem

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Résumé

Le dépistage des antidiabétiques oraux, agents hypoglycémiants utilisés pour le traitement du diabète de type II, représente un défi dans la pratique médico-légale. En cas d'hypoglycémie d'origine douteuse, la recherche des antidiabétiques oraux permet de différencier les hypoglycémies factices (tentative de suicide, syndrome de Münchhausen par procuration) induites par les antidiabétiques des hypoglycémies vraies (ex. insulinome). Au niveau clinique, au cours d'un traitement, il peut être important de surveiller leurs concentrations plasmatiques afin d'optimiser la posologie.

Dans cette étude, une méthode utilisant la chromatographie liquide couplée à la spectrométrie de masse en tandem a été développée et validée pour l'analyse de 13 antidiabétiques oraux dans le sang total humain après une double extraction liquide-liquide à deux pH différents.

La procédure de validation a montré une linéarité acceptable pour tous les composés entre 1 et 5000 ng/mL pour le gliclazide, glibornuride et le natéglinide et entre 1 et 1000 ng/mL pour tous les autres antidiabétiques. Les limites de détection et de quantification étaient respectivement de 0,1 e 1 ng/mL pour toutes les molécules. La répétabilité et la fidélité intermédiaire étaient inférieures à 20 % pour les 13 molécules. La méthode a été appliquée avec succès au sang post mortem de 8 sujets diabétiques. Les échantillons de sang ont été testés positifs pour le gliclazide (246 – 3000 ng/mL), la sitagliptine (40 – 21400 ng/mL) et le répaglinide (15 ng/mL).

Mot-clés: antidiabétiques, LC-MS/MS, diabète, hypoglycémie

Title

Testing for 13 antidiabetic drugs using LC-MS/MS in whole blood. Application to postmortem specimens

Abstract

The detection of oral antidiabetics or hypoglycaemic agents used for the treatment of type II diabetes, represents a challenge in forensic practice. In the case of hypoglycaemia with unclear origin, the detection of oral antidiabetic drugs allows discrimination between hypoglycaemia of factitious origin (suicide attempt, Munchausen's syndrome by proxy) induced by antidiabetic drugs and true hypoglycaemia (e.g. insulinoma). In addition, during treatment with oral antidiabetic drugs, it is important to monitor their plasma concentration in diabetic patients in order to adjust their dosage.

In this study, a method using liquid chromatography-tandem mass spectrometry was developed and validated for the analysis of 13 oral antidiabetics in human whole blood after a double liquid-liquid extraction at two different pH values.

The validation procedure showed acceptable linearity for all compounds between 1 and 5000 ng/mL for gliclazide, glibornuride and nateglinide and between 1 and 1000 ng/mL for all other antidiabetics. The limits of detection and quantification were 0,1 and 1 ng/mL respectively. Repeatability and intermediate precision were below 20% for all 13 molecules. The method was successfully applied to postmortem blood of 8 diabetic subjects. Blood samples tested positive for gliclazide (240 - 3000 ng/mL), sitagliptin (40 - 21400 ng/mL) and repaglinide (15 ng/mL).

Keywords: antiabetic drugs, LC-MS/MS, diabetes, hypoglycaemia

Introduction

Les antidiabétiques oraux sont des médicaments utilisés pour le traitement du diabète de type II, également appelé diabète non insulinodépendant. Il s'agit d'une pathologie caractérisée par une hyperglycémie chronique due au développement d'une résistance périphérique à l'action de l'insuline [1]. Selon les derniers chiffres de 2020, 3,5 millions de personnes en France étaient sous traitement antidiabétique [2].

Bien qu'il n'existe pas de traitement curatif pour le diabète, un style de vie sain et l'administration de médicaments hypoglycémiants permettent de contrôler la glycémie chez les personnes atteintes de diabète [1]. Actuellement, plusieurs classes d'antidiabétiques oraux avec des structures chimiques et des mécanismes d'action différents sont inscrits à la Pharmacopée française. Ces classes pharmacologiques comprennent des agents qui 1. stimulent la sécrétion pancréatique d'insuline (sulfamides et glinides), 2. réduisent la résistance à l'insuline et la production hépatique de glucose (biguanides), 3. ralentissent l'absorption intestinale du glucose (inhibiteurs de l'alpha-glucosidase), 4. augmentent la sécrétion d'insuline en inhibant l'activité de l'enzyme dipeptidyl peptidase-4 (DDP-4) responsable de la dégradation des incrétines (vildagliptine, sitagliptine, saxagliptine, alogliptine, linagliptine) et 5. améliorent la sensibilité périphérique à l'insuline en activant le récepteur nucléaire PPARγ (thiazolidinediones).

Les principaux effets indésirables liés à l'utilisation de ces médicaments sont l'acidose lactique pour la metformine (biguanides en général), et l'hypoglycémie pour les sulfamides et les glinides [3].

Le traitement du diabète de type II a considérablement évolué aux cours des dernières années et les médicaments qui existent aujourd'hui permettent de contrôler cette pathologie en limitant les effets secondaires. Bien que les antidiabétiques de nouvelle génération ne devraient pas provoquer d'hypoglycémie, des études récentes ont montré que le taux d'incidence des hypoglycémies n'a pas changé [4].

Lorsque ces épisodes surviennent chez des sujets diabétiques, l'origine de l'hypoglycémie peut être déterminée facilement, alors que chez les sujets sans antécédents d'exposition aux antidiabétiques, identifier la cause reste un défi. Ces épisodes d'hypoglycémie peuvent être soit accidentels, en raison de dosages inadaptés ou du non-respect du traitement de la part du patient, soit volontaires dans le cadre d'une tentative de suicide, de meurtre ou dans le cadre de troubles psychiatriques tels que le syndrome de Münchhausen par procuration.

Les hypoglycémies intentionnelles restent des défis diagnostiques auxquels les endocrinologues sont confrontés dans leur pratique et qui conduisent à des investigations coûteuses et inutiles pour éliminer d'autres causes d'hypoglycémie.

Après l'injection d'insuline exogène, le taux d'insuline augmente mais pas celui du peptide C, ce qui permet une discrimination efficace. A contrario avec l'administration d'antidiabétiques insulino-sécrétagogues (tels que les sulfamides et les glinides), le tableau clinique peut ressembler à un insulinome avec des taux plasmatiques élevés de peptide C et d'insuline [5].

L'intoxication par antidiabétiques représente également un défi dans la pratique médico-judiciaire car l'interprétation est souvent douteuse puisqu'il n'est pas toujours facile de discriminer les décès liés à des causes accidentelles de ceux causés volontairement par l'utilisation de xénobiotiques hypoglycémiants.

Bien que l'utilisation détournée de ces substances à des fins autres que thérapeutiques ait été décrite, la recherche des antidiabétiques n'est pas systématique dans la plupart des laboratoires.

Il est donc évident qu'en cas d'intoxication par des antidiabétiques, un laboratoire de toxicologie doit être en mesure d'effectuer un dépistage et une confirmation. En outre, une méthode de dépistage sensible et fiable permettrait son utilisation dans le cadre de la surveillance thérapeutique des antidiabétiques qui est un élément important pour l'adaptation de la posologie, le contrôle et le diagnostic.

Différentes méthodes ont été décrites dans la littérature pour l'identification et la quantification des médicaments antidiabétiques dans le sang, dans les urines et dans les cheveux en utilisant la chromatographie liquide couplée à une détection UV [6] et la chromatographie liquide et gazeuse couplées à la spectrométrie de masse [7-8] ou à la spectrométrie de masse en tandem [9-15]. Il s'agit néanmoins de méthodes qui ciblent une molécule spécifique ou seulement une famille thérapeutique. Seuls trois articles décrivent des méthodes qui regroupent plusieurs familles [7,16-17]. Les screening de Korman et al. [16] et de Gonzalez et al. [17] ciblent les familles des sulfamides, glinides et thiazolidinediones. L'intérêt de ces méthodes ne concerne principalement que 2 familles (sulfamides et glinides) car les thiazolidinediones ont été retirées du marché en France depuis 2011 en raison d'une augmentation d'événements cardiovasculaires [18]. La méthode de Hess et al. [7], en revanche, inclut également la nouvelle famille des gliptines (également appelés inhibteurs de la DDP-4). Cependant, les auteurs ont considéré que

cette méthode n'était pas adaptée à la quantification des gliptines puisque de nombreux paramètres de validation étaient hors limites.

Dans cet article, les auteurs présentent une méthode pour le dépistage de 13 antidiabétiques oraux appartenant à 3 familles différentes dans le sang total humain. Cette méthode a été appliquée à des échantillons authentiques de sujets diabétiques décédés alors qu'ils étaient sous traitement.

Matériels et méthodes

Agents chimiques et réactifs

Les poudres certifiées de gliclazide, glibenclamide, glimépiride et gliquidone ont été achetées auprès de LGC (Molsheim, France). Le glipizide, le glibornuride, la vildagliptine, la sitagliptine, la saxagliptine, l'alogliptine, la linagliptine, le répaglinide, le natéglinide, le répaglinide-éthyl-d5, l'hydroxy tolbutamide-d9 et la vildagliptine-d3 ont été fournis par Toronto Research Chemicals (Toronto, Canada).

Tous les standards ont été conditionnés dans des volumes appropriés de méthanol pour obtenir des solutions de départ de 1 mg/mL. Le méthanol, l'acétonitrile, le dichlorométhane et l'éther diéthylique de qualité HPLC ont été obtenus auprès de VWR Chemicals ProLabo (Fontenay-sous-bois, France).

Le formiate d'ammonium a été fourni par VWR Chemicals (Leuven, Belgique). L'acide formique de qualité LC-MS a été obtenu auprès de Carlo Erba (Val-de-Reuil, France). L'hydroxyde de sodium (NaOH) a été acheté auprès de Merck (Darmstadt, Allemagne).

Le tétraboratedecahydrate de disodium et l'acétate d'ammonium ont été obtenu auprès de Sigma Aldrich (Saint-Quentin-Fallavier, France).

Préparation des échantillons

Les calibrateurs et les contrôles qualité (CQ) pour la validation de la méthode ont été préparés dans du sang total blanc obtenu auprès de l'Etablissement Français du Sang (EFS). Le sang

blanc a été choisi après avoir vérifié l'absence de réponse aux temps de rétention des différents antidiabétiques et des standards internes.

1 mL de sang a été extrait avec 1 mL de tampon borate saturé à pH 9,5 et 5 mL d'un mélange d' éther diéthylique/dichlorométhane (80 :20) en présence de trois standards internes deutérés (hydroxy tolbutamide -d9, répaglinide-ethyl-d5 et vildagliptin-d3) à une concentration de 1 mg/L. Après agitation pendant 15 minutes et centrifugation (15 minutes, 3000 rpm), la phase organique a été recueillie dans un tube à essai en verre et conservée.

500 µL de tampon acétate 1 M pH 4 ont été additionnés dans le sang restant puis, 5 ml d'un mélange éther/dichlorométhane (80 :20) ont été ajoutés et l'échantillon a été agité pendant 15 minutes et centrifugé (15 minutes, 3000 rpm). La phase organique a été recueillie, ajoutée au même tube à essai contenant la première extraction et évaporée à sec sous un flux d'azote.

Le résidu sec a été reconstitué dans 30 µL de tampon formiate d'ammonium 5 mM ajusté à pH 3.

Instrumentation

La séparation LC a été réalisée à l'aide d'une colonne C18 HSS Acquity de Waters (150 x 2,1 x 1,8 µm). Le compartiment de la colonne était régulé à 50 °C. Une injection de 2 µL avec un débit de 0,4 mL/min de tampon formate ajusté à pH 3 (A) et d'acide formique à 0,1 % dans l'acétonitrile (B) a été utilisée. L'élution en gradient était la suivante : la composition initiale (13 % B) a été augmentée de 13 à 50 % en 10 minutes, de 50 % à 95% en 2,25 minutes et est revenue aux conditions initiales en 2,25 minutes. Le temps de run total est de 15 minutes. Les temps d'élution des antidiabétiques et des étalons internes sont décrits dans le tableau 1.

Un spectromètre de masse triple quadrupole XEVO TM TQS-µ (Waters Corporation, Milford, MA, USA) équipé d'une source d'ionisation électrospray (ESI) Z-sprayTM utilisée en mode d'ionisation positive et négative (ES+/-) a été utilisé pour l'analyse des composés.

Les conditions suivantes se sont avérées optimales pour l'analyse des antidiabétiques et des étalons internes : tension capillaire de 2,5 kV ; température du bloc source de 150 °C ; azote gazeux de désolvatation chauffé à 600 °C et délivré à un débit de 1 000 L/h. Afin d'établir une condition appropriée de surveillance des réactions multiples, la tension du cône a été ajustée pour maximiser l'intensité de l'ion moléculaire protoné et une dissociation induite par collision

a été réalisée. La tension du cône et l'énergie de collision ont été ajustées pour optimiser le signal des ions plus abondants produits des antidiabétiques et des standards internes.

La tension du cône, l'énergie de collision et les transitions m/z sont décrites dans le tableau 1.

Le logiciel MassLynx V 4.2 a été utilisé pour la quantification.

Procédures de validation

La méthode d'analyse a été validée pour la linéarité, la répétabilité, la fidélité intermédiaire et les limites de détection et de quantification conformément aux directives de la SFTA (Société Française de Toxicologie Analytique) [19]. Trois courbes d'étalonnage comprenant huit points (1, 5, 10, 50, 100, 500, 1 000, 5 000 ng/mL), obtenus sur une période de trois jours, ont été réalisées pour l'étude de la linéarité.

La quantification a été réalisée en traçant les rapports d'aires sous les pics entre les sulfamides et l'hydroxy tolbutamide-d9, les gliptines et la vildagliptine-d3 et des glinides et le répaglinide-éthyl-d5 en fonction de la concentration, puis en effectuant une analyse de régression linéaire.

La répétabilité et la fidélité intermédiaire ont été évalués à trois niveaux de contrôles de qualité (10, 100 et 1 000 ng/mL). Pour la répétabilité, six aliquots de chaque niveau de CQ ont été traitées le même jour. Pour la fidélité intermédiaire, chaque niveau de CQ a été traité trois fois sur quatre jours différents sur une période de deux semaines.

La limite de détection (LD) est la plus faible concentration du composé qui peut être détectée avec un rapport signal/bruit supérieur à 3:1 pour la transition spécifique. La limite de quantification (LQ) a été définie comme le premier point de la courbe d'étalonnage.

En ce qui concerne l'effet de matrice, celui-ci a été déterminé en analysant 5 échantillons différents de sang blanc surchargés avec les analytes à une concentration de 100 ng/mL après extraction et évaporation. Ces échantillons ont ensuite été comparés à 5 solutions méthanoliques contenant la même concentration d'antidiabétiques. L'effet matrice a été définis comme le pourcentage des aires de pic des cinq extraits surchargés par rapport aux aires des pics des cinq solutions de méthanol aux mêmes concentrations.

Pour établir le taux de recouvrement, 5 échantillons de sang surchargés avec 100 ng/mL d'antidiabétiques oraux ont été extraits et comparés avec des solutions standard d'antidiabétiques dans du méthanol à la même concentration.

La contamination inter-échantillons a également été déterminée en analysant pendant 5 jours, 3 échantillons de sang surchargés d'une concentration de 500 ng/mL suivis de 3 échantillons de sang blanc sans standard. Le pourcentage de contamination inter-échantillon a été calculé en faisant le rapport entre la différence entre la moyenne des valeurs des blancs passés juste après la séquence des valeurs élevées et la moyenne des valeurs des blancs passés en dernier et la différence entre la moyenne des valeurs élevées et la moyenne des derniers blancs.

Résultats et discussion

Les résultats de la validation de la méthode sont résumés dans le tableau 2. La méthode était linéaire de la limite de quantification aux concentrations thérapeutiques élevées (et aux concentrations de surdosage) avec un coefficient de corrélation allant de 0,96 à 0,99 pour les 3 essais. Les gammes de linéarité des antidiabétiques qui peuvent être quantifiés par cette méthode sont présentées dans le tableau 2. La validation de méthode a été effectuée avec des répétabilités et des fidélités intermédiaires acceptables (CV < 20 %). La répétabilité a été déterminée à trois concentrations et variait de 2,0 à 18,8% ; la fidélité intermédiaire variait de 4,8 % à 19,8 %. Les taux de recouvrement sont satisfaisants pour tous les composés. L'effet de matrice a été établi pour toutes les analytes et a été évalué entre 71 et 128 %. L'évaluation de la contamination inter-échantillons a démontré une absence de contamination fournissant des valeurs inférieures à 0,1% pour tous les analytes.

Les limites de détection étaient inférieures à 1 ng/mL pour tous les composés.

Si les échantillons réels présentent des concentrations supérieures au point le plus élevé de la gamme d'étalonnage, les échantillons doivent être dilués.

Plusieurs approches ont été testées avant d'établir la méthode définitive. Dans un premier temps, une simple précipitation dans le méthanol et l'acétonitrile a été évaluée, puisque utilisée par plusieurs auteurs [20-21]. Bien qu'il s'agisse d'une méthode rapide, elle ne permettait pas d'obtenir un taux satisfaisant de recouvrement et les chromatogrammes présentaient des

interférences, ce qui gênait la lecture des concentrations faibles. Dans un deuxième temps, deux extractions liquide-liquide ont été testées à la fois à un pH basique (en utilisant un tampon borate saturé à pH 9,5) et à un pH acide (en utilisant un tampon acétate à pH 5,2) et en essayant différents solvants organiques. Avec un nombre important d'analytes aux propriétés chimiques différentes, le choix du pH du tampon d'extraction est essentiel. Un pH acide permet d'extraire les sulfamides et les glinides. En revanche, un pH trop acide empêche l'extraction des gliptines.

Pour cette raison, afin d'obtenir un taux de recouvrement acceptable pour toutes les molécules, la combinaison de deux extractions liquide-liquide à 2 pH différents (basique puis acide) a permis d'obtenir les meilleurs résultats.

Différents solvants organiques ont été comparés (Tert-butyl-méthyl éther, n-héxane/acétate d'éthyle, dichlorométhane/diéthyl éther). Le mélange dichlorométhane/éther a été choisi car il permet d'obtenir le meilleur taux de recouvrement.

L'étalon interne idéal doit être une substance dont la structure est similaire et qui possède donc des propriétés chimiques et physiques identiques. Compte tenu de la présence de 3 familles structurellement différentes, 3 standards internes deutérés ont été utilisés, chacun appartenant à chaque famille d'antidiabétiques. Un chromatogramme typique obtenu après extraction de sang surchargé à 10 ng/mL avec chaque analyte est présenté Figure 1.

Cette méthode a été appliquée à l'analyse d'échantillons authentiques de sang humain post mortem obtenus lors de l'autopsie de 8 sujets diabétiques sous traitement. Dans chacun de ces cas, le décès n'a pas été attribué à un surdosage d'antidiabétique, mais à une autre cause, qu'elle soit traumatique ou d'origine toxique. Des chromatogrammes typiques sont présentés Figure 2. La posologie quotidienne des divers médicaments ne nous a pas été fournie. Le tableau 3 résume les concentrations mesurées pour chaque molécule. Ces concentrations ont varié entre 240 ng/mL et 3000 ng/mL pour le gliclazide, 40 et 21400 ng/mL pour la sitagliptine et une seule concentration de 15 ng/mL pour le répaglinide a été mesurée.

Il est possible d'interpréter ces résultats en regard de la littérature scientifique. Binz et al., en 2012 [20], ont présenté une méthode de détection et de quantification de 4 sulfamides dans le sérum par une technique de précipitation utilisant la chromatographie liquide couplée à la

spectrométrie de masse en tandem. Cette méthode a été appliqué à des échantillons réels. Le gliclazide a été identifié et dosé dans un seul échantillon à une concentration de 480 ng/ml. Zhong et al. [22] ont développé une méthode pour identifier le gliclazide dans le sang humain appliquée à une étude de bioéquivalence, en utilisant également la précipitation comme méthode d'extraction et la LC-MS/MS comme instrument de détection. Après l'administration de 40 mg de gliclazide à 20 sujets, des concentrations allant de 4386,5 à 6146,9 ng/mL ont été mesurées.

Les concentrations de gliclazide présentées dans le Tableau 3 se trouvent dans la fourchette thérapeutique (240 ng/mL - 3000 ng/mL). En effet, il a été publié qu'en moyenne une concentration de 580 +/- 120 ng/mL est mesurée suite à la prise de 30 mg par jour. Dans le cas des formes à libération prolongée à 60 mg, une concentration moyenne de 2030 +/- 1190 ng/mL est observée [23].

Peu de cas de surdosage de gliclazide ont été rapportés dans la littérature, et par conséquent sa cinétique en cas de surdosage est peu décrite. Les cas de la littérature concernaient des tentatives de suicide suite à l'administration de grandes quantités de gliclazide (dosage supérieure à 2 g) aux effets principalement neuroglycopéniques. Le danger est principalement lié aux formules à libération prolongée, qui peuvent provoquer des hypoglycémies pouvant durer plusieurs jours et nécessiter de longues périodes d'hospitalisation. [24-25].

Pour le sujet numéro 2 (Tableau 3), il a été possible de faire une comparaison entre la concentration mesurée dans le sang périphérique et le sang cardiaque. Dans ce cas, une concentration 7 fois plus élevée dans le sang cardiaque (1700 ng/mL) a été observée par rapport au sang fémoral (240 ng/mL).

La littérature ne contient pas de données de redistribution post mortem. Cependant, l'article de Kintz et al. rapportent un cas de décès dans lequel une faible différence a été constatée entre la concentration de gliclazide mesurée dans le sang cardiaque (2200 ng/mL) et celle mesurée dans le sang périphérique (1900 ng/mL). La redistribution post-mortem d'un médicament est influencée par des facteurs physiques tels que la concentration du médicament au niveau gastro-intestinal, le temps écoulé entre le décès et le prélèvement et d'autres facteurs comme la lipophilie, la liaison aux protéines plasmatiques, le pKa et le volume de distribution de la molécule. Le gliclazide est une molécule acide ayant une valeur de volume de distribution très faible (0,27 L/kg). Cependant, étant donné que la diffusion gastro-intestinale peut affecter tous les médicaments, il est possible que le long délai entre le décès et le recueil de l'échantillon et

une éventuelle distribution ante mortem incomplète au moment du décès aient conduit à la diffusion dans les chambres cardiaques de quantités non absorbées dans l'estomac [26].

Dans cet article, la sitagliptine a été détectée dans le sang de 3 sujets décédés. Les concentrations mesurées chez deux sujets se trouvaient dans la fourchette thérapeutique (40 et 251 ng/mL). Pour le sujet numéro 5, une concentration de 21 400 ng/mL a été observée, ce qui est 100 fois plus élevé que les concentrations mesurées à des doses thérapeutiques.

Des méthodes analytiques pour le dosage des gliptines dans le sang, et en particulier de la sitagliptine, ont également été décrites [13,21,27-30]. Loh et al., en 2020 [27], ont développé et validé une méthode pour le dosage de la sitagliptine dans le plasma humain et l'ont appliqué à une étude de bioéquivalence. Des concentrations allant de 251 à 404 ng/mL ont été mesurées chez des sujets testés après l'administration de 100 mg de sitagliptine en utilisant un système LC-MS/MS. Burugula et al. [28] ont appliqué leur méthode à une étude pharmacocinétique pour la sitagliptine chez des sujets volontaires et ont trouvé des concentrations allant de 7 à 389 ng/mL après l'administration de 100 mg. Deux cas dans la littérature mentionnent des tentatives de suicide par sitagliptine [31-32]. Un article de Fukurawa et al. [31] présente le cas d'un sujet non diabétique chez qui une concentration de l'ordre de 1500 ng/mL a été retrouvée, ce qui correspond à 4,5 fois la valeur haute en thérapeutique. Dans ce cas, le sujet a survécu sans séquelle. En effet, le pouvoir inhibiteur des gliptines atteint probablement un plateau malgré des concentrations plasmatiques très élevées. La puissante inhibition de la DDP-4 par les gliptines entraîne une augmentation substantielle du niveau des incrétines. Cependant, l'activité des incrétines est dépendante du glucose et par conséquent leur production diminue lorsque la glycémie baisse. Ainsi, le contrôle des incrétines sur la glycémie est calibré et adapté aux besoins, ce qui évite les situations d'hypersécrétion d'insuline et les hypoglycémies dangereuses qui en dérivent même après un surdosage en sitagliptine [31]. Pour cette raison, un surdosage de gliptine ne devrait pas entraîner d'hypoglycémie fatale. Dans notre cas, le décès a été attribué à un surdosage de métoprolol, un ß-bloquant, présent dans le sang à 74 mg/L. Malgré les effets indésirables réduits pour la sitagliptine, le risque de mortalité associé à l'hypoglycémie est accru lors de l'utilisation de ß-bloquants tels que le métoprolol. En effet, le traitement par ß-bloquants augmente le risque d'hypoglycémie grave ou prolongée car ils affectent la remontée du niveau de glucose après une hypoglycémie chez les patients diabétiques. Suite à une hypoglycémie sévère, l'action hyperglycémiante de l'adrénaline, qui stimule la production de glucose et limite son utilisation par les organes non vitaux, permet de restaurer la glycémie. Puisque l'action de l'épinéphrine est principalement médiée par les récepteurs ß-adrénergiques, l'action antagoniste

du métoprolol sur ces récepteurs empêche la restauration du glucose après une hypoglycémie [33].

Au final, il convient d'ajouter que l'analyse de la metformine, substance très polaire, ne peut pas se faire par l'approche analytique développée et nécessite une méthode dédiée [15].

Conclusion

Le dépistage des antidiabétiques oraux peut être utile pour le diagnostic des hypoglycémies dont l'origine n'est pas déterminée de façon claire. Afin d'accélérer la réalisation de l'analyse et de faciliter le travail des enquêteurs, il semble nécessaire pour un laboratoire de toxicologie de disposer d'une méthode qui intègre le plus grand nombre d'agents antidiabétiques en une seule opération. Ce travail présente une méthode pour l'identification et la quantification, dans le sang humain, de 13 antidiabétiques oraux appartenant aux trois familles d'antidiabétiques les plus prescrites pour le traitement du diabète.

Les résultats montrent que la méthode décrite peut être utilisée en routine pour l'analyse médico-légale des échantillons de sang et pour le suivi thérapeutique des antidiabétiques oraux. La méthode a été appliquée avec succès aux échantillons de sang post mortem chez huit sujets diabétiques.

Conflit d'intérêts : aucun

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Tableau 1. Transitions spécifiques des antidiabétiques oraux et de leurs étalons internes

Famille	Analyte	RT (min)	Transition MRM	Cone (V)	EC (eV)	Polarité ESI
Sulfamides	Gliclazide	10,18	324,25 > 110,13	32	20	+
			324,25 > 91,07	30	36	
	Glibenclamide	11,26	494,14 > 168,86	48	30	+
			494,14 > 368,88	48	12	
	Glipizide	8,87	446,09 > 320,96	28	14	+
			446,09 > 102,89	28	36	
	Glibornuride	10,98	365,02 > 169,90	42	20	-
			365,02 > 105,88	42	44	
	Glimépiride	11,4	489,12 > 224,88	58	32	-
			489,12 > 363,99	58	22	
	Gliquidone	11,58	528,41 > 403,28	2	10	+
			528,41 > 103,10	2	54	
	Hydroxy tolbutamide-d9	5,02	296,3 > 83,20	24	14	+
			296,3 > 107,07	24	24	
Glinides	Répaglinide	11,42	453,42 > 230,27	32	28	+
			453,42 > 86,12	32	28	
	Natéglinide	11,35	318,33 > 125,14	28	16	+
			318,33 > 120,06	28	16	
	Répaglinide-éthyl-d5	11,5	458,33 > 230,21	46	30	+
			458,33 > 86,09	46	30	
Gliptines	Vildagliptine	1,48	304,29 > 154,16	30	14	+
			304,29 > 97,06	30	30	
	Sitagliptine	4,47	408,15 > 174,09	38	28	+
			408,15 > 193,08	38	28	
	Saxagliptine	2,66	316,3 > 180,23	22	20	+
			316,3 > 92,63	22	54	
	Alogliptine	2,98	340,29 > 116,11	36	32	+
			340,29 > 323,28	34	16	
	Linagliptine	5,3	473,38 > 420,35	30	22	+
			473,38 > 116,16	30	74	
	Vildagliptine-d3	1,52	307,26 > 157,2	48	16	+
			307,26 > 99,99	48	28	

Tableau 2. Paramètres de validation des antidiabétiques dans le sang.

Analyte	Gamme de linéarité (ng/mL)	LD (ng/mL)	LQ (ng/mL)	Taux de recouvrement à 100 ng/mL (%)	Concentration (ng/mL)	CV répétabilité (%)	CV fidélité intermédiaire (%)	Effet matrice (%)
Gliclazide	1-5000	0,1	1	102	10	10,8	14	128
					1000	9,4	13,2	
Glibenclamide	1-1000	0,1	1	95	10	8,3	13,4	73
					1000	5,4	10,8	
Glipizide	1-1000	0,1	1	101	10	11,8	19,1	92
					100	9,1	17,7	
Glibornuride	1-5000	0,1	1	36	10	6	13,1	103
					1000	3,9	4,8	
Glimépiride	1-1000	0,1	1	74	10	13	15,8	98
					100	7	10,6	
Gliquidone	1-1000	0,1	1	48	10	11,8	15,4	75
					100	7,6	10,6	
Répaglinide	1-1000	0,1	1	104	10	5,7	8,9	110
					100	3,2	6,0	
Natéglinide	1-5000	0,1	1	102	100	5,4	7,8	71
					1000	5,7	5,2	
Vildagliptine	1-1000	0,1	1	101	10	6,5	14,1	89
					100	4,30	10,0	
Sitagliptine	1-1000	0,1	1	98	10	4,6	16,1	120
					100	2	8,5	
Saxagliptine	1-1000	0,1	1	103	10	16,9	19,8	105
					100	12,4	15,1	
Alogliptine	1-1000	0,1	1	107	10	18,8	16,3	95
					100	11,0	12,6	
Linagliptine	1-1000	0,1	1	95	10	13,2	17,6	101
					100	3,5	11,4	

Tableau 3. Résultats de l'analyse des échantillons de sang post-mortem.

Analyte	Sujet	Origine sang	Concentration (ng/mL)
Gliclazide	1	Sang fémoral	245
Gliclazide	2	Sang fémoral	240
Gliclazide	2	Sang cardiaque	1700
Gliclazide	3	Sang fémoral	2200
Gliclazide	4	Sang fémoral	3000
Sitagliptine	5	Sang fémoral	21400
Sitagliptine	6	Sang fémoral	251
Sitagliptine	7	Sang fémoral	40
Répaglinide	8	Sang fémoral	15

Figure 1. Chromatogrammes des transitions quantitatives pour un échantillon de sang contenant 10 ng/mL de chaque analyte.

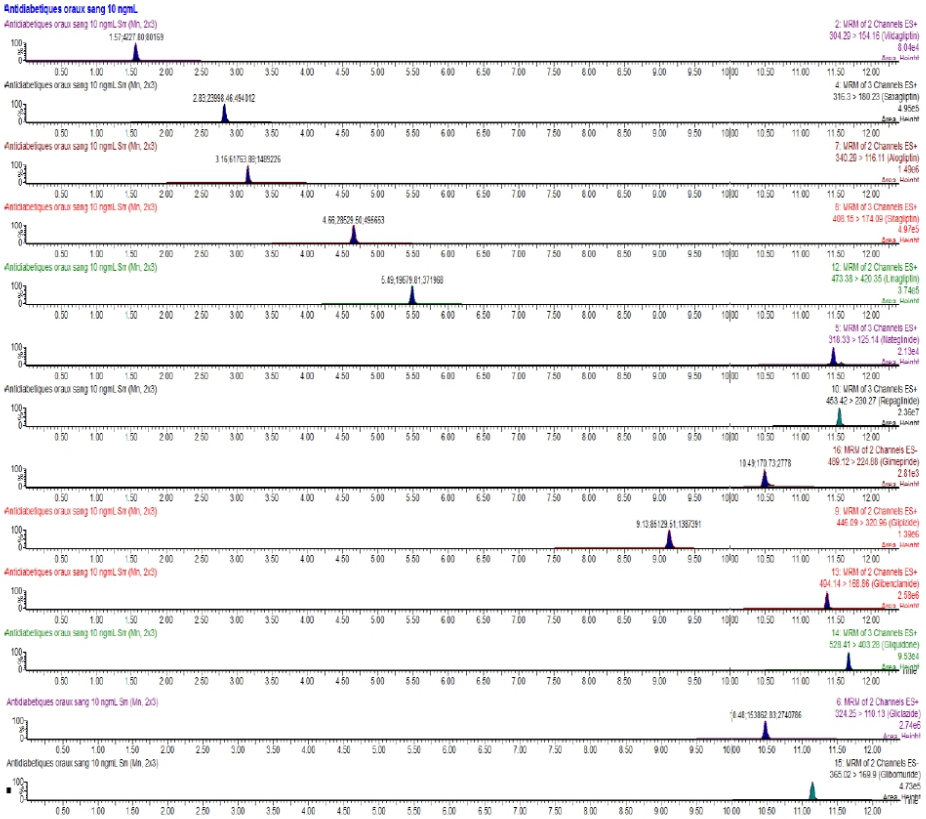
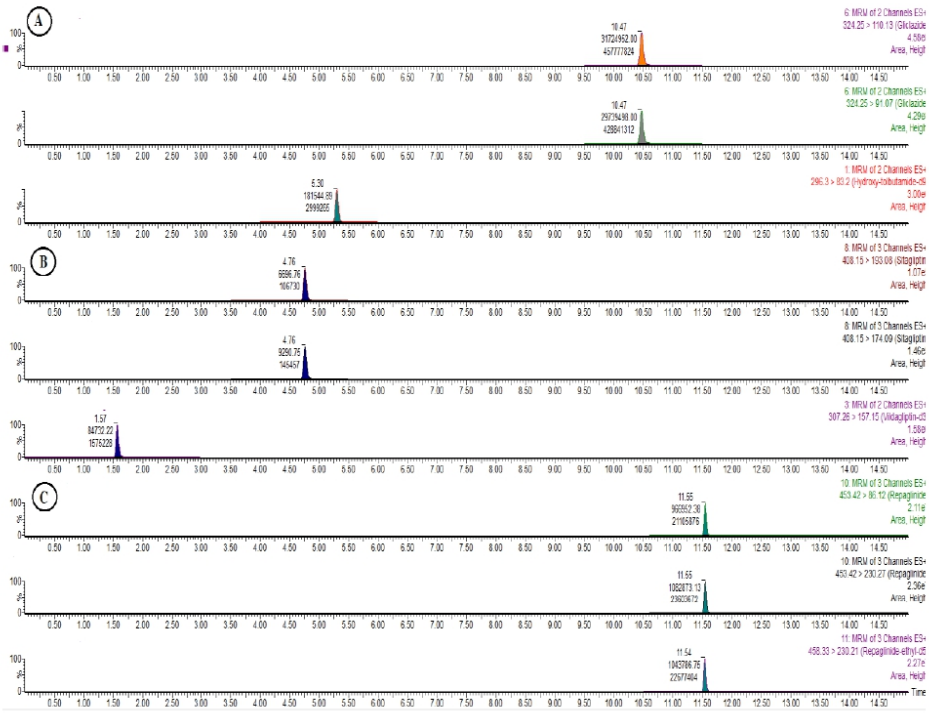


Figure 2. Chromatogrammes des transitions ioniques d'échantillons de sang *postmortem* contenant a) 2 transitions de gliclazide et 1 transition de l'IS (hydroxy tolbutamide-d9), la concentration était de 3000 ng/mL ; b) 2 transitions de sitagliptine et 1 transition de l'IS (vildagliptin-d3), la concentration était de 251 ng/mL ; c) 2 transitions de repaglinide et 1 transition de l'IS (repaglinide éthyl-d5), la concentration était de 15 ng/mL.



3.3 Développement d'une méthode d'identification et de quantification dans les cheveux (à l'exclusion des gliflozines)

La recherche des antidiabétiques oraux dans les cheveux peut être d'une grande importance dans les cas d'hypoglycémies inattendues, car elle permet de distinguer les hypoglycémies dues aux antidiabétiques de celles dues à d'autres causes (par exemple, l'insulinome). Il est donc important pour un laboratoire de toxicologie de rechercher les antidiabétiques dans les cheveux en raison de la large fenêtre de détection que permet cette matrice associée à sa longue stabilité dans le temps.

Au cours de cette thèse j'ai donc développé et validée une méthode pour l'analyse simultanée de 13 antidiabétiques oraux dans les cheveux par LC-MS/MS.
Les paramètres de la méthode sont décrits dans l'article ci-dessous.
La méthode a été appliquée avec succès aux cheveux de 18 patients diabétiques sous traitement antidiabétique oral. Les cheveux ont été testés positifs pour le gliclazide (3- 21,400 pg/mg), la sitagliptine (1.4 - 1.8 pg/mg), la vildagliptine (3.3 - 1,740 pg/mg) et le répaglinide (14.1 pg/mg) ***[Article 6]***.

Article 6: Development of a new LC-MS/MS method for the simultaneous identification and quantification of 13 antidiabetic drugs in human hair

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Development of a new LC-MS/MS method for the simultaneous identification and quantification of 13 antidiabetic drugs in human hair

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Abstract:	Oral antidiabetics are the drugs used to control blood sugar in diabetic subjects. The greatest risk of using these drugs is hypoglycaemia, which can be fatal if managed inappropriately. The diagnosis of hypoglycemia may be simple in diabetic subjects but can become a challenge in subjects with no history of exposure to these drugs. The major interest of testing for these compounds in hair is in the case of unexpected hypoglycaemias, as it enables discrimination between hypoglycaemias caused by antidiabetics and other reasons (e.g. insulinoma). Therefore it is important for a toxicology laboratory to screen for antidiabetics in hair due to the large window of detection this matrix allows associated to its long stability over time. In this study, a method has been developed and validated using liquid-chromatography coupled to tandem mass spectrometry for the analysis of 13 oral antidiabetics in hair. After addition of three different internal standards (hydroxy-tolbutamide-d9 for sulfonylureas, repaglinide-ethyl-d5 for glinides and vildagliptin-d3 for gliptins) and incubation in an ultrasonic bath in methanol, the hair was dissolved in NaOH and then subjected to liquid-liquid extraction. The validation procedure demonstrated an acceptable linearity for all compounds between 1 and 50,000 pg/mg. LOD and LOQ were between 0.5 – 5 pg/mg and 1- 10 pg/mg respectively. Repeatability and reproducibility were below 20 % at two concentrations for all the analytes. The method was successfully applied to the hair of 18 diabetic patients under treatment of oral antidiabetics. The hair tested positive for gliclazide (3- 21,400 pg/mg), sitagliptin (1.4 - 1.8 pg/mg), vildagliptin (3.3 – 1,740 pg/mg) and repaglinide (14.1 pg/mg).
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Highlights

- Antidiabetic intoxications can be fatal
- Hair analysis is important to analyze the historical consumption of antidiabetics in cas of factitious hypoglycaemias
- A method for identifying and quantifying antidiabetic agents in hair is needed
- In the method developed by the authors, it is possible to simultaneously identify and quantify 13 different antidiabetics

Abstract

Oral antidiabetics are the drugs used to control blood sugar in diabetic subjects. The greatest risk of using these drugs is hypoglycaemia, which can be fatal if managed inappropriately. The diagnosis of hypoglycemia may be simple in diabetic subjects but can become a challenge in subjects with no history of exposure to these drugs. The major interest of testing for these compounds in hair is in the case of unexpected hypoglycaemias, as it enables discrimination between hypoglycaemias caused by antidiabetics and other reasons (e.g. insulinoma). Therefore it is important for a toxicology laboratory to screen for antidiabetics in hair due to the large window of detection this matrix allows associated to its long stability over time.

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Development of a new LC-MS/MS method for the simultaneous identification and quantification of 13 antidiabetic drugs in human hair

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Abstract

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Keywords: diabetes, LC-MS/MS, hair, antidiabetic drugs

1. Introduction

Type II diabetes, also known as non-insulin dependent, is a disease characterized by chronic hyperglycaemia and represents the most common chronic diseases in the world. In 2020, it has concerned about 3.5 million people in France (5.3 % of the population). It affects 95 % of the total diabetic population and in 2019 it was the ninth leading cause of death in the world with 1.5 million deaths [1].

Even if there is no real cure for diabetes, a healthy diet and the use of a wide class of appropriate oral antidiabetic agents can help control blood glucose in these patients. This pharmacological class includes agents that stimulate pancreatic insulin secretion (sulfonylureas and glinides), reduce insulin resistance and hepatic glucose production (biguanides), slow intestinal glucose absorption (alpha glucosidase inhibitors), and drugs that increase insulin secretion by inhibiting the activity of the dipeptidyl peptidase-4 enzyme responsible for incretin degradation (gliptins) [2].

Most oral antidiabetic drugs can cause severe hypoglycaemia that can become fatal. With more than 3,565 cases of poisoning reported in the US in 2019, sulfonylureas are considered the most problematic hypoglycaemic drugs [3-4].

Severe hypoglycaemia is defined as a blood glucose fall below 0.4 g/L. When this drop is particularly marked, a metabolic alteration is generated, causing neuroglycopenic effects due to the lack of glucose in the brain, which can quickly lead to a hypoglycaemic coma and death [5]. Other insulinotropics such as glinides are associated with a lower frequency of undesirable hypoglycaemic episodes, whereas for drugs such as biguanides the main adverse effect is lactic acidosis, which can often become fatal in case of overdose [6].

The risk of developing hypoglycaemia may be increased by the presence of other medical conditions such as renal insufficiency or by the simultaneous intake of drugs that may potentiate the hypoglycaemic effect or by non-compliance with treatment [3].

Because of the dangerous nature of these drugs and the increase in the prevalence of diabetes in the recent years and therefore their availability, the risk of misuse in criminal contexts is also of major concerns.

Despite the increasing number of unclear hypoglycaemic episodes reported by poison centres, in clinical and forensic toxicology laboratories, these substances are seldom investigated.

Various analytical methods for the detection of oral antidiabetics in blood and urine have been described in the literature. Antidiabetics can be detected in blood and urine by liquid chromatography coupled with classical mass spectrometry [3] or tandem mass spectrometry [7-12], using UV [13] or gas chromatography coupled with mass spectrometry [14] or tandem mass spectrometry [15].

Blood and urine are the traditional specimens for documenting drug exposure. Cases of intoxication with antidiabetic drugs have been described over time in the context of drug-facilitated crimes and especially in the context of Munchausen syndrome by proxy [16]. The latter is generally a form of child

abuse in which an adult parent deliberately causes an illness in their child to attract the attention and sympathy of medical personnel. In this context, unnecessary treatments are usually administered to the child to treat the factitious illness. The inability to identify factitious hypoglycaemia due to a lack of analytical methods has led over time to drastic events such as unnecessary pancreatectomies. Antidiabetic drugs have been reported repeatedly as agents to cause factitious illness and in particular sulphonylureas such as gliclazide and glibenclamide [17-19].

Although various methods on blood and urine analysis have already been published, these matrices have a limited detection window. This is the reason why hair analysis is becoming a widely used tool for measuring long-term drug exposure and thus, complements blood and urine analyses. A forensic interest has been observed in the context of drug-facilitated crimes as it is possible to differentiate, by segmentation, a single exposure from a chronic intake [20].

In the literature, there is a lack of available data on the identification of antidiabetic agents in hair. The limited papers have demonstrated the utility of using this matrix. Given the weak correlation between blood levels and hair concentrations, segmented hair analysis is able, in theory, to reflect the consumption of a drug over a period of time and thus be useful for therapeutic drug monitoring or for forensic pathologists investigating the cause of death in a suspected poisoning.

In the paper by Kintz et al. [21] the segmented hair analysis of a case of a diabetic who died with a therapeutic concentration of gliclazide revealed a non-compliance of the treatment by the patient which resulted in fatal hypoglycaemia despite the therapeutic concentration in the blood. In another case described by Villain et al. [22] the detection of glibenclamide in the hair of a non-diabetic-deceased patient was the only evidence of administration of this drug, as the blood test tested negative given a late collection.

Because of their possible misuse, antidiabetics should be screened in cases of hypoglycaemia whose nature is unclear. It seems essential for a laboratory to dispose of analytical methods involving most of the commercially available hypoglycaemic drugs.

In this study, the authors present for the first time a method using LC-MS/MS technology to simultaneously identify and quantify 13 oral antidiabetic agents in hair. This method was applied to hair samples obtained from patients under treatment in order to provide data for the interpretation of the measured concentrations. Metformin, another antidiabetic agent, was not included in this list as it is structurally very different from these drugs and therefore, needs a specific approach for its extraction [15].

2. Material and Methods

2.1 Chemical and reagents

Certified powders of gliclazide, glibenclamide, glimepiride and gliquidone were purchased from LGC (Molsheim, France). Glipizide, glibornuride, vildagliptin, sitagliptin, saxagliptin, alogliptin, linagliptin, repaglinide, nateglinide, repaglinide-ethyl-d5, hydroxy tolbutamide-d9 and vildagliptin-d3 were supplied by Toronto Research Chemicals (Toronto, Canada).

All standards were conditioned in appropriate volumes of methanol to obtain starting solutions of 1 mg/mL. HPLC-grade methanol, acetonitrile, dichloromethane and diethyl ether were obtained from V.W.R. Chemicals ProLabo (Fontenay-sous-bois, France).

Ammonium formate was provided by VWR Chemicals (Leuven, Belgium). Formic acid for LC-MS was obtained from Carlo Erba (Val-de-Reuil, France). NaOH was purchased from Merck (Darmstadt, Germany).

2.2 Hair specimens

To get reference values about the correlation between doses and concentrations, hair specimens from 18 diabetic patients (8 men and 10 women aged 65 to 92 years) on daily treatment with oral antidiabetics since 1 to 20 years were analyzed. This was part of their medical evaluation and oral consent was obtained from all the participants after the purpose of the study was clearly exposed. In France, hair collection is not considered to be an invasive operation.

Given the short hair length of most patients, only the proximal segment (0 to 2-3 cm) was tested.

For those whose length exceeded 6 cm, segmentation was achieved. None of the hair specimens was cosmetically treated. One of the authors collected all the specimens in an envelope and they were stored at ambient temperature at the laboratory before analysis.

Blank hair for method validation was obtained from laboratory staff volunteers.

2.3 Antidiabetics hair analysis

Calibrators and QC samples for method validation were prepared in blank hair obtained from the laboratory staff. Blank hair was chosen after having verified the absence of response at the retention times of the various antidiabetics and internal standards.

Real hair samples (subjects under treatment) and blank hair followed the same preparation. Hair specimens were decontaminated twice with 5 mL of dichloromethane for 2 minutes.

After drying, the hair was cut into small pieces of less than 1 mm and 20 mg were incubated in 2 mL of methanol in the presence of a mix of deuterated standards (hydroxy tolbutamide-d9, repaglinide-ethyl-d5 and vildagliptin-d3) at a concentration of 1 ng/mg and placed in an ultrasound bath for 2 h at room

temperature. After centrifugation at 3000 rpm for 15 minutes, the supernatant was collected in a glass test tube and stored.

The remaining hair were spiked with 1 mL NaOH and incubated at 95°C for 15 minutes. Then, 5 mL of a mixture of ether/dichloromethane (80:20) was added. After agitation for 15 minutes and centrifugation (15 minutes, 3000 rpm), the organic phase was collected, added to the same test tube containing the methanol and evaporated to dryness under a nitrogen flow.

The residue was reconstituted in 30 µL of 5 mM ammonium formate buffer adjusted to pH 3.

2.4 Instrument conditions

LC separation was performed using a Waters Acquity HSS C18 column (150 x 2.1 x 1.8 µm). The column compartment was regulated at 50 °C. A 2-µL injection with a 0.4 mL/min flow of formate buffer adjusted to pH 3 (A) and 0.1 % formic acid in acetonitrile (B) was used. The gradient elution was as follows: the initial composition (13 % B) was increased from 13 to X % over 2.5 minutes, X % to X % over 6.5 minutes and returned to initial conditions over X minutes. The elution times for the antidiabetics and internal standards are described in Table 1.

An XEVO ™ TQS-µ triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) provided with a Z-spray™ electrospray ionization source (ESI) used in positive and negative ionization mode (ES+/-) was used for analysis of the compounds.

The following conditions were found to be optimal for the analysis of antidiabetics and internal standards: capillary voltage at 2.5 kV; source block temperature at 150 °C; desolvation gas nitrogen heated at 600 °C and delivered at a flow rate of 1,000 L/h. In order to establish an appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation was performed. Cone voltage and collision energy were adjusted to optimize the signal of the most abundant product ions of antidiabetics and internal standards. The cone voltage, the collision energy and *m/z* transitions are described in Table1.

MassLynx V 4.2 software was used for quantification.

2.5 Validation procedure

The hair test method was validated for linearity, repeatability, reproducibility and detection and quantitation limits according to the SFTA (Societé Française de Toxicologie Analytique) guidelines [23]. Three calibration curves that included ten points (1, 5, 10, 50, 100, 500, 1,000, 5,000, 10,000, 50,000 pg/mg), obtained over a three-day period, were established for the study of linearity.

Quantification was achieved by plotting the peak area ratios of sulfonylureas to hydroxy tolbutamide-d9, of gliptins to vildagliptin-d3 and of glinides to repaglinide-ethyl-d5 versus concentration followed by linear regression analysis.

Repeatability and reproducibility were determined for three quality controls (QC) levels at 10, 100 and 1,000 pg/mg. 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.

Results and Discussion

For the method validation, the results are summarized in Table 2. The method demonstrated good linearity in the concentration ranges defined in Table 2 with a correlation coefficient ranging from 0.975 to 0.999 during the 3 tests. The LODs and the lower LOQs were considered satisfactory considering that the method integrates 13 molecules with different chemical properties. The method showed good repeatability and reproducibility with CVs < 20% for all the analytes. Due to the lack of certified reference material (hair specimen containing calibrated antidiabetics amounts) an accurate extraction efficiency could not be established.

Prior to the selection of the method described in section 2.3, various tests were performed in order to choose the extraction that gives the best results for all the 13 different compounds. At first, an overnight incubation in an acid buffer (phosphate buffer pH 5.2) and extraction in a mix of organic solvents (dichloromethane/ diethyl-ether) was performed (Method 2). In a second test, a simple incubation in methanol in an ultrasound bath was performed (Method 3).

As the *pKa* values of these antidiabetics are different due to their different chemical structures, the association of incubation in methanol in an ultrasound bath followed by dissolution of hair in NaOH and liquid-liquid extraction was considered the most appropriate as it provided the best recoveries (Method 1). For each compound, the area was compared after extraction with methods 2 and 3 to the method 1. The results are expressed in terms of gain and loss of methods 2 and 3 compared to method 1. Methods 2 and 3 gave similar results. However, method 3 failed to detect vildagliptin even at a concentration of 100 pg/mg. Method 1 allows a significant gain in response especially for DDP-4 inhibitors which have very high pKa, while maintaining satisfactory values even for sulfonylureas and glinides which instead have very low pKas. Method 1 was therefore chosen as the most appropriate for the screening of 13 antidiabetics.

The price to pay for involving 13 compounds with different chemical structures into one screening method was a slight loss of recovery for glibenclamide, glibornuride and nateglinide.

Different organic solvents were compared (TBME, n-hexane/acetate ethyl, dichloromethane/diethyl-ether). The DCM/ether mix was chosen as it provided the best recoveries. Different incubation periods were tested ranging from 1 to 3 hours using authentic samples to optimize the extraction time. An incubation time of 2 hours was found suitable as the signal of most oral antidiabetics did not increase after that period. The use of methanol and acetonitrile as extraction solvents has been compared. Best recovery was observed with methanol, in agreement with what previously published, particularly because acetonitrile does not swell [15,24].

A typical chromatogram after extraction of spiked hair with 100 pg/mg of each analyte is presented in Figure 1. Typical chromatograms obtained after extraction of the proximal hair segment of 4 patients are presented in Figure 2. As can be observed, the chromatograms are clean and the peaks are well separated.

Hair analysis has become a widely used matrix to complete blood and urine tests. The main advantage over other these later specimens, is that hair allows retrospective data of drug consumption. Consequently, it is of relevance both from a clinical point of view and for forensic toxicologists in investigating possible poisoning. Evidence of inappropriate use for something different than curative purposes, such as in the context of Munchausen syndrome poisonings and Munchausen by proxy or for other criminal purposes, can be facilitated by hair analysis due to an increased window of detection (months or even years, depending on the length of the hair) in contrast to matrices such as blood and urine where the molecule remains for a limited time [20].

In order to obtain reference values to interpret the concentrations found in the hair of diabetics, the method was applied to quantify antidiabetic drugs in the hair of 18 patients under daily treatment. Daily dosages ranged from 60 to 80 mg for gliclazide, from 50 and 100 mg for vildagliptin and sitagliptin, and was 1 mg for repaglinide, respectively.

Table 4 summarizes the test results, including daily dosages, hair color and measured concentrations. These concentrations ranged between 3 and 21,400 pg/mg for gliclazide, between 1 and 2 pg/mg for sitagliptin, between 10 and 1,740 pg/mg for vildagliptin, and the only repaglinide result gave a concentration of 14 pg/mg.

The toxicological significance of these results is rather difficult to establish as there is a lack of data in the literature concerning antidiabetic agents in hair. There are only 4 articles reporting antidiabetic values in hair and they only involve sulphonylureas and metformin [15,21,22,25]. In 2012 Binz et al. [25] published a method for the screening of 5 oral antidiabetic drugs in hair (4 sulphonylureas and metformin). They report gliclazide values ranging from 32.2 to 100.7 pg/mg in patients with daily doses ranging from 80 to 240 mg. In 2020, Kintz et al. [21] reported the case of a diabetic who died with a therapeutic blood concentration of gliclazide. The concentrations measured in the hair following segmentation were 7 and 3 pg/mg. The hair analysis in this case was useful to the pathologist who concluded that the death occurred due to inappropriate use of gliclazide.

The advantage of hair analysis is also to discriminate between chronic use of a substance and a single administration (although it is not possible to prove this for all compounds). In the case of Villain et al. [22] the authors demonstrated that a single dose of 5 mg of glibenclamide is detectable in hair (5 pg/mg). In a subject under daily treatment with glibenclamide for one year, they measured 650 pg/mg. The detection of this sulfonylurea in hair allowed to demonstrate an exposure to glibenclamide in a criminal context of a non-diabetic subject who was exposed several times to this compound before his death.

When comparing the results obtained with this method in patients treated with gliclazide at different concentrations, the very large variations may be due to the unknown rate of incorporation of gliclazide into the hair, probably linked to hair colour and hair damage over time and to the fact that the doses are self-reported. Despite the limited number of cases, it can be observed that higher concentrations of gliclazide are found in subjects with black hair. In the study by Binz et al. [22] no information is given about the colour of the patients' hair and therefore no comparison is possible.

While the detection of some sulphonylureas in hair has already been described in the literature, there is no data on DDP-4 inhibitors in hair. Therefore, it is difficult to interpret the values obtained in our cases. As can be seen from Table 4, the concentrations of vildagliptin (the gliptin most represented in our cases) the concentrations measured range from 3 to 1,740 pg/mg. Concentrations around 1 ng/mg are only measured for doses of 100 mg and in patients who have been on treatment for at least 10 years. In the mechanism of incorporation of a compound in hair, several factors are involved: pKa, lipid solubility, size and affinity for melanin. The lipid solubility of a substance is a critical factor in determining the rate of transport of a substance from the bloodstream through the cell membrane of the growing bulb. In addition, the pH gradient from neutral plasma to acid melanocytes gives basic molecules an advantage in incorporation over acid molecules [20]. Given the slightly lipophilic character of glyptins ($0.6 < \text{LogP} < 1.9$) and the basicity of these molecules ($8.6 < \text{pKa} < 14.7$), they should incorporate fairly well. Hair colour does not seem to play an important role, as in the white hair of patient 8, a vildagliptin concentration of 1300 pg/mg was measured, while in the brown hair of patient 16 a concentration of only 10 pg/mg was measured. This lack of correlation is difficult to explain at this stage, given the limited reports on this topic.

Most studies have shown that basic molecules accumulate more in melanin-rich hair due to the strong ionic interaction between positively charged molecules and the polyanionic melanin polymer. Kronstrand et al. [26] analysed the hair of Parkinson's patients treated with selegiline to show a correlation between dose and concentration in the hair. Although a preference of selegiline metabolites for dark hair was revealed, the presence of selegiline metabolites in white hair showed that pigmentation is not the only factor to be taken into consideration, but that binding to keratin and other hair proteins is also a significant part of the accumulation of a substance.

Finally, the lack of correlation between dose and concentration measured in the hair is in agreement with the reports in the literature. This can be due to inter-individual variations. The concentrations of a substance in hair can vary from one individual to another due to washing, drying, cosmetic treatments,

daily exposure to sunlight and atmospheric agents, which have been shown to reduce the concentration of many molecules over time [20]. In most studies, it is reported that measuring the concentration of a substance in hair does not allow to establish the dose consumed.

In 2007 Kronstrand et al. [29] showed that the correlation dose-concentration increases if results are normalised by melanin content. There are discordant opinions about, the inter-individual variations mainly related to the patient's compliance to the treatment which is often not known and this may obscure a possible correlation between the dose administered and concentration of a drug measured in the hair.

Conclusion

The inappropriate use of antidiabetic drugs is well known. It is essential that a toxicology laboratory is able to identify and quantify most of the antidiabetic drugs on the market in order to explain unclear episodes of hypoglycemia.

In this study, the authors developed and validated a method for the identification of 13 oral antidiabetic agents including 3 different families. The method was successfully applied to hair from 18 patients and for the first time, data on concentrations of gliptins and glinides in hair are provided.

This method allows documentation of long-term exposure to oral antidiabetic drugs and is therefore useful in facilitating investigations in cases of unexplained hypoglycemia.

Since these are the first data, the interpretation of the measured concentrations is a difficult task and further developments are needed to better explain the results.

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Figure 1. Chromatograms of the quantitative ion transitions for spiked hair sample with 100 pg/mg of each analyte.

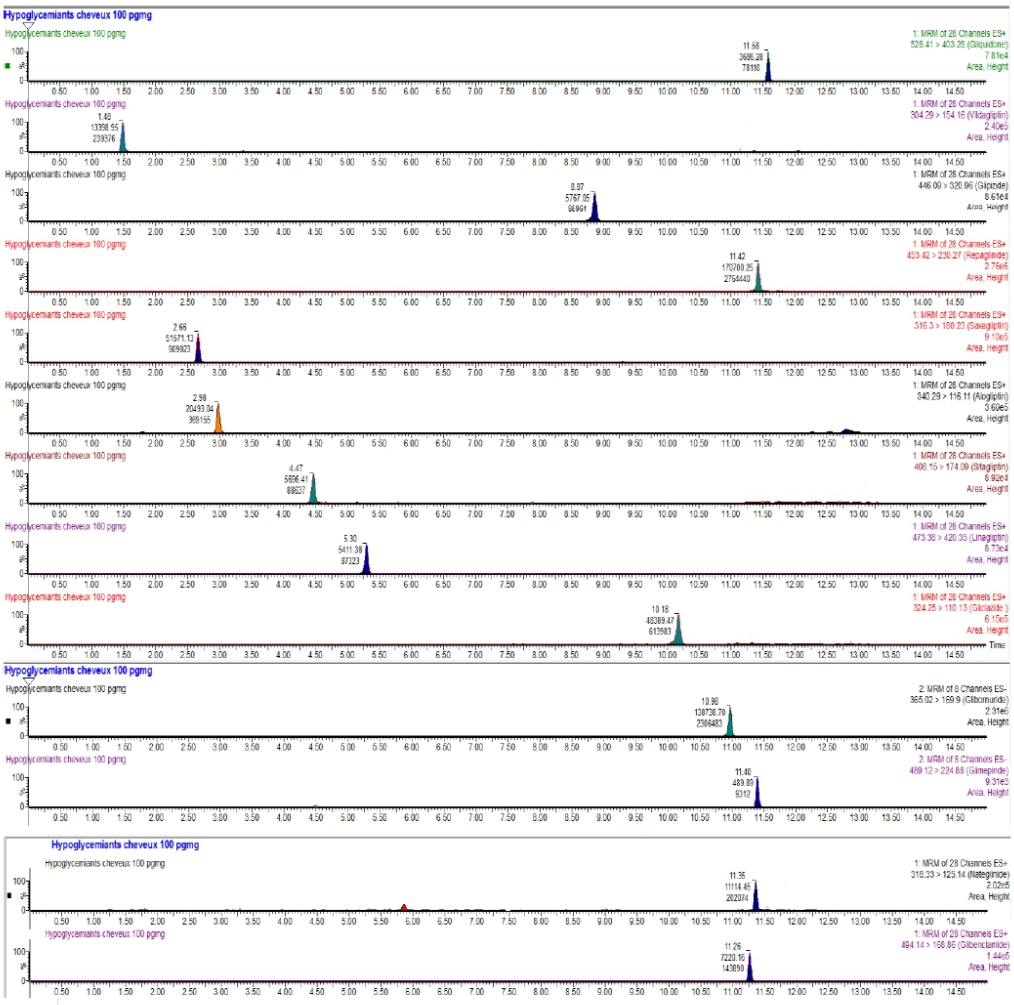

Figure 2. Chromatograms of the ion transitions of real hair samples containing a) 2 transitions of gliclazide and 1 transition of the IS (hydroxy tolbutamide-d9), the concentration was 132 pg/mg; b) 2 transitions of sitagliptin and 1 transition of the IS (vildagliptin-d3), the concentration was 2 pg/mg; c) 2 transitions of vildagliptin and 1 transition of the IS (vidagliptin-d3), the concentration was 3 pg/mg; d) 2 transition of

repaglinide and 1 transition of IS (repaglinide ethyl-d5), the concentration was 14 pg/mg.

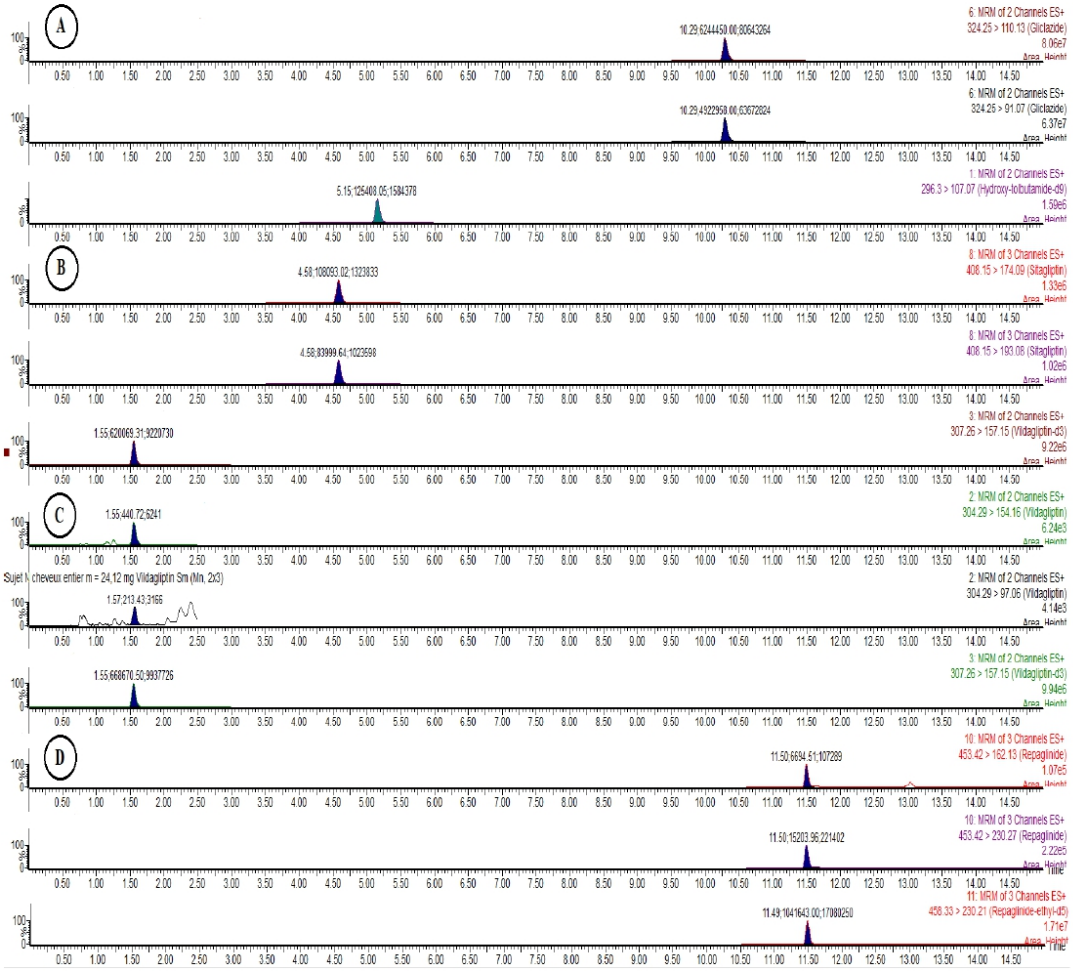

Table 1. Antidiabetics and IS specific transitions.

Drug class	Analyte	RT (min)	MRM transition	Cone (V)	CE (eV)	ESI polarity
Sulfonylureas	Gliclazide	10.18	324.25 > 110.13	32	20	+

	Glibenclamide	11.26	494.14 > 168.86	48	30	+
	Glipizide	8.87	446.09 >320.96	28	14	+
	Glibornuride	10.98	365.02 > 169.9	42	20	-
	Glimepiride	11.4	489.12 > 224.88	58	32	-
	Gliquidone	11.58	528.41 > 403.28	2	10	+
	Hydroxy tolbutamide-d9	5.02	296.3 > 83.2	24	14	+
Glinides	Repaglinide	11.42	453.42 > 230.27	32	28	+
	Nateglinide	11.35	318.33 > 125.14	28	16	+
	Repaglinide-ethyl-d5	11.5	458.33 > 330.1	46	30	+
Dipeptidyl peptidase-4 inhibitors	Vildagliptin	1.48	304.29 > 154.16	30	14	+
	Sitagliptin	4.47	408.15 > 174.09	38	28	+
	Saxagliptin	2.66	316.3 > 180.23	22	20	+
	Alogliptin	2.98	340.29 > 116.11	36	32	+
	Linagliptin	5.3	473.38 > 420.35	30	22	+
	Vildagliptin-d3	1.52	307.5 > 157.2	48	16	+

Table 2. Validation parameters of antidiabetics in hair.

Analyte	Linearity range	LOD (pg/mg)	LOQ (pg/mg)	Spiked concentration (pg/mg)	Intraday precision relative standard deviation (%)	Interday precision relative standard deviation (%)

Gliclazide	1-50000	0.5	1	10	10.4	14.5
				1000	13.2	13.8
Glibenclamide	10-5000	5	10	100	11.8	17.6
				1000	9.5	12.1
Glipizide	1-5000	0.5	1	10	15.9	18.3
				1000	13.6	12.1
Glibornuride	1-5000	0.5	1	10	14.1	13.2
				1000	11.9	15.4
Glimepiride	10-5000	5	10	100	19.5	16.3
				1000	14.2	18.7
Gliquidone	10-5000	5	10	100	15.8	11.4
				1000	13.9	14.6
Repaglinide	1-5000	0.5	1	10	5.5	4.7
				1000	3.8	6.9
Nateglinide	10-5000	5	10	100	10.5	11.7
				1000	8.3	10.9
Vildagliptin	1-5000	0.5	1	10	16.8	18.7
				1000	17.4	18.3
Sitagliptin	1-5000	0.5	1	10	8.5	13.5
				1000	9.6	14.7
Saxagliptin	1-5000	0.5	1	10	6.8	8.9
				1000	3.5	9.7
Alogliptin	10-5000	5	10	100	10.3	12.9
				1000	8.6	11.4
Linagliptin	1-5000	0.5	1	100	8.4	6.6
				1000	7.9	10.6

Table 3. Gain or loss of areas (%) for method 2 and 3 relative to method 1.

ND = not detected

	Method 2	Method 3
Vildagliptin	- 135 %	ND
Alogliptin	- 196.6 %	- 196.8 %
Saxagliptin	- 506.04	- 504.6 %
Sitagliptin	- 45.9 %	- 40.4 %
Linagliptin	- 35.7 %	- 41.9 %
Gliquidone	- 9.36 %	- 18.9 %
Gliclazide	- 443.5 %	- 338.5 %
Glipizide	- 20.1 %	- 9.6 %
Glibenclamide	+ 9%	+ 33.3 %
Glibornuride	- 1208.9 %	- 1133.2 %
Glimepiride	+ 8%	+ 6.8 %
Repaglinide	- 1370.2 %	- 1307.1 %
Nateglinide	+ 107. 8 %	+ 108.9 %

Table 4. Results of hair analysis.

Analyte	Subject	Dose (mg/daily)	Color	Concentration (pg/mg)
Gliclazide	1 (0-2 cm)	60	White	70
	1 (2-4 cm)	60	White	48
	1 (4-6 cm)	60	White	64
Gliclazide	2	80	Dark	21400
Gliclazide	3	80	Dark	132
Gliclazide	4	80	Dark	388
Gliclazide	5 (0-2 cm)	60	White	7
	5 (2-4 cm)	60	White	3
Sitagliptin	6	50	Dark	1
Sitagliptin	7	50	Brown	2
Vildagliptin	8	50	White	14
Vildagliptin	9	100	White	1300
Vildagliptin	10 (0-2 cm)	100	Brown	49
	10 (2-4 cm)	100	Brown	109
	10 (4-6 cm)	100	Brown	91
Vildagliptin	11	50	White	3
Vildagliptin	12	50	Brown	161
Vildagliptin	13	100	White	339
Vildagliptin	14	100	Blond	945
Vildagliptin	15	100	White	79
Vildagliptin	16	100	Brown	1740
Vildagliptin	17	100	Brown	10
Repaglinide	18	1	white	14

3.4 Développement d'une méthode d'identification et de quantification des gliflozines dans le sang

Bien que les gliflozines ne semblent pas être associées à des effets indésirables graves, étant donné qu'elles n'ont été autorisées que récemment, il existe peu de recul concernant leur toxicité.
Ce que l'on sait, en revanche, c'est que, étant donné qu'ils sont souvent associés à d'autres médicaments antidiabétiques, le risque d'hypoglycémie sévère est toujours présent.
En outre, en raison de la capacité de ces médicaments à faire perdre du poids, leur utilisation peut être détournée par les sportifs et d'autres personnes qui ne nécessitent pas de traitement antidiabétique.
Il m'a donc semblé important de mettre au point une méthode permettant d'identifier et de doser également les gliflozines. L'identification et la quantification des gliflozines sont réalisées par un système UHPLC-MS/MS (Waters Acquity UPLC (Waters Corporation, Milford, MA, USA)).
La décision de séparer ces molécules des autres médicaments antidiabétiques oraux est due au fait qu'en présence d'ammonium formiate (utilisé comme phase mobile pour la plupart des antidiabétiques), des adduits se forment, ce qui réduit la sensibilité de la détection.
Afin d'éviter la formation d'adduits, il a été décidé d'utiliser uniquement l'eau et l'acétonitrile comme phases mobiles.
En raison de la facilité de formation d'adduits, il faut veiller à éliminer le formiate ou l'acétate du système si d'autres essais utilisant des phases mobiles contenant de l'acide formique ou acétique sont effectués sur le même instrument afin d'éviter une perte de sensibilité.
Seuls 100 μL sont nécessaires pour l'identification des 3 gliflozines dans le sang (dapagliflozine, canagliflozine, empagliflozine). La liste des molécules, les temps de rétention, les transitions et les limites de détection et quantification sont décrits dans les tableaux 4 et 5.

Protocole d'extraction

L'extraction choisie est une précipitation de protéines. 20 ng de dapagliflozine-d5 (S.I.) et 2 ml d'acétonitrile sont ajoutés à 100 μL de sang. Les échantillons de sang sont mélangés au vortex pendant 1 minute puis centrifugés pendant 15 minutes à 3000 rpm. L'extrait est ensuite évaporé et le résidu est dissous dans 50 μL d'acétonitrile.
5 μL sont ensuite injectés dans un système UHPLC-MS/MS.

Paramètres du système UHPLC-MS/MS

La séparation LC a été réalisée à l'aide d'une colonne C18 HSS Acquity de Waters (150 x 2,1 x 1,8 μm). Le compartiment de la colonne était régulé à 50 °C. Une injection de 5 μL avec un débit de 0.4 mL/min de H_2O (A) et d'acétonitrile (B) a été utilisée. L'élution en gradient est décrite dans le tableau 3 Les temps d'élution des gliflozines et des étalons internes sont décrits dans le tableau 4.

Temps (min)	Eau (%)	Acétonitrile (%)
0	60	40
4	20	80
7	20	80
8	60	40
10	60	40

Tableau 3. Gradient UHPLC pour l'analyse des gliflozines

Un spectromètre de masse triple quadrupole XEVO TM TQS-μ (Waters Corporation, Milford, MA, USA) équipé d'une source d'ionisation électrospray (ESI) Z-sprayTM utilisée en mode d'ionisation positive et négative (ES+/-) a été utilisé pour l'analyse des composés.

Les conditions suivantes se sont avérées optimales pour l'analyse des gliflozines et de l'étalon interne : tension capillaire de 1 kV ; température du bloc source de 150 °C ; azote gazeux de désolvatation chauffé à 600 °C et délivré à un débit de 1 000 L/h. Afin d'établir une condition appropriée de surveillance des réactions multiples (MRM), la tension du cône a été ajustée pour maximiser l'intensité de l'ion moléculaire protoné et une dissociation induite par collision a été réalisée. La tension du cône et l'énergie de collision ont été ajustées pour optimiser le signal des ions plus abondants produits des antidiabétiques et des standards internes. La tension du cône, l'énergie de collision et les transitions *m/z* sont décrites dans le tableau 4.

Le logiciel MassLynx V 4.2 a été utilisé pour la quantification.

Analyte	Transitions MRM	T_R (min)	Cône (eV)	EC (eV)	Polarité ESI
Dapagliflozine	407,1 > 329,08 407,1> 259,06	2,07	62	12 18	-
Empagliflozine	451,05 >70,58 451,05 > 43,02	1,59	34	20 40	+
Canagliflozine	445,11 > 340,57 445,11 > 147,04	2,68	52	16 34	+
Dapagliflozine-d5	415,26 > 155,08 415,26 > 108,04	2,03	56	32 48	+

Tableau 4. Liste des gliflozines, transitions, temps de rétention, voltage du cône, énergie de collision et polarité électrospray

Analyte	Gamme de linéarité (ng/mL)	LD (ng/mL)	LQ (ng/mL)	Concentration (ng/mL)	CV répétabilité (%)	CV fidelité intermédiaire (%)
Dapagliflozine	1-1000	0,5	1	10 100	11,7 8,8	15 8
Empagliflozine	5-1000	1	5	10 100	14,1 5,6	19,5 7,9
Canagliflozine	5-1000	1	5	10 100	18,6 12,3	14,5 14,4

Tableau 5. Paramètres de validation des gliflozines dans le sang

3.5 Développement d'une méthode d'identification et de quantification des gliflozines dans les cheveux

Il n'existe aucune méthode dans la littérature pour l'identification des gliflozines dans les cheveux. Étant donné le grand intérêt des cheveux comme matrice analytique et compte tenu de l'absence de méthode pour leur identification dans la littérature, j'ai voulu développer une méthode pour la détection et le dosage des gliflozines et en particulier de la dapagliflozine, de l'empagliflozine et de la canagliflozine.
La méthode, comme celle du sang, a été développée et validée sur un système UHPLC-MS/MS.

Protocole d'extraction

20 mg de cheveux (coupés finement à l'aide d'une paire de ciseaux) sont pesés. Les cheveux sont incubés dans 2 mL d'acétonitrile pendant 2 heures dans un bain à ultrasons en présence de 1 ng de dapagliflozine-d5 (S.I) afin d'extraire les gliflozines.
Après incubation, les échantillons sont centrifugés pendant 15 minutes à 3000 rpm et la phase organique est recueillie et évaporée sous un flux d'azote. Après évaporation l'extrait sec est solubilisé dans 50 μL d'acétonitrile.
5 μL sont ensuite injectés sur le système LC-MS/MS.

Paramètres du système UHPLC-MS/MS

Les paramètres du système d'analyse sont les mêmes que ceux utilisés pour le dosage des gliflozines dans le sang (voir partie 3.4).
Les paramètres de validation de la méthode d'analyse des gliflozines dans les cheveux sont présentés dans le tableau 6.
La figure 11 montre les chromatogrammes obtenus après extraction de 100 pg/mg des gliflozines dans des cheveux.

Analyte	Gamme de linéarité (pg/mg)	LD (pg/mg)	LQ (pg/mg)	Concentration (pg/mg)	CV répétabilité (%)	CV fidelité intermédiaire (%)
Dapagliflozine	10-10000	5	10	50 100	16,5 11,8	19,3 15
Empagliflozine	10-10000	5	10	50 100	18,7 7,5	18,3 10,1
Canagliflozine	10-10000	5	10	50 100	19,5 13,7	18,5 17,2

Tableau 6. Paramètres de validation des gliflozines dans les cheveux

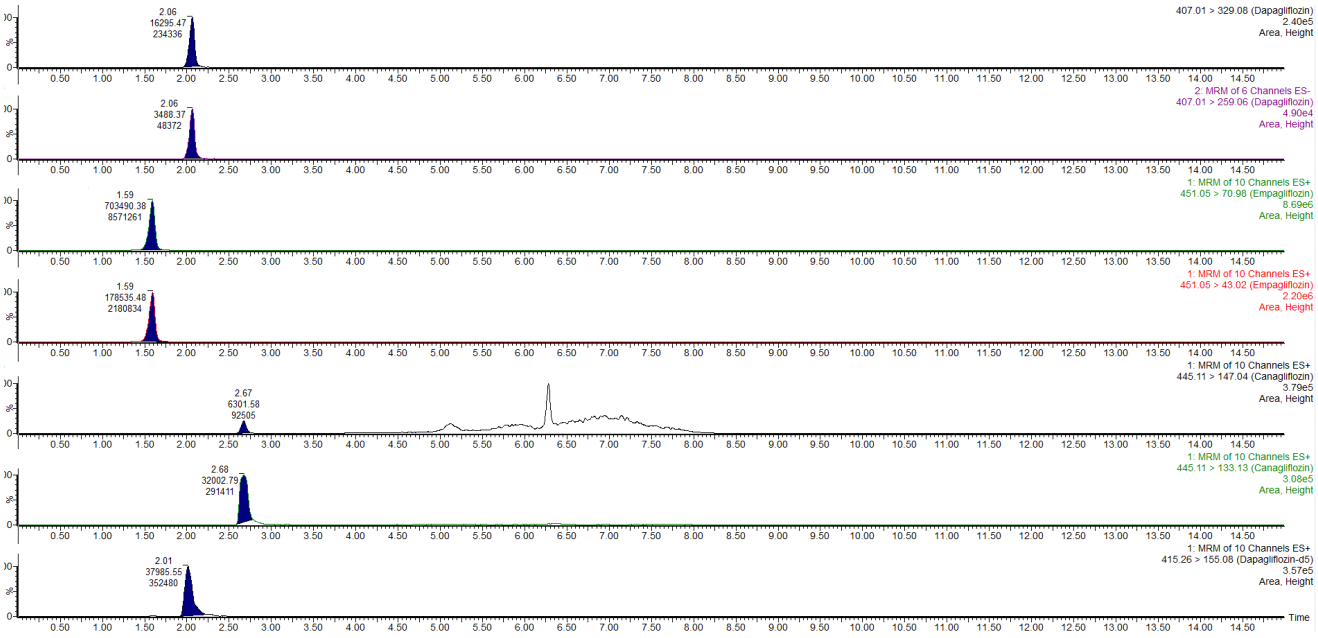

Figure 11. Chromatogrammes des transitions quantitatives et qualitatives pour un échantillon de sang contenant 100 pg/mg de chaque analyte

3.6 Applications à des cas d'expertises médico-judiciaires

Les méthodes développées pour l'identification et le dosage des antidiabétiques dans le sang et les cheveux ont été appliquées à des cas médico-légaux dont le décès était soupçonné être lié à un surdosage d'antidiabétiques et les cas où l'origine du décès était inconnue.

La méthode développée dans la matrice sanguine est également appliquée de manière routinière à des demandes hospitalières du service d'endocrinologie pour l'adaptation de la posologie et en cas d'hypoglycémie inexpliquée de la part du service des urgences.

Ces travaux ont donné lieu à une publication scientifique.

Article 6 : Une femme de 46 ans, présentant un diabète de type II, est retrouvée sans vie à son domicile. Le procureur a demandé une autopsie suivie d'investigations toxicologiques. Les résultats de l'autopsie (ecchymoses labiales, congestion multiviscérale, syndrome d'asphyxie, œdème cérébral modéré) étaient conformes à un possible décès par hypoglycémie. Les analyses toxicologiques ont révélé la présence de gliclazide dans le sang fémoral à 2,2 mg/L, ce qui est dans la fourchette des concentrations thérapeutiques publiées. Trois explications possibles ont été discutés avec le médecin légiste : 1. cause inconnue de la mort (autre qu'une mort toxique), 2. mort due au gliclazide qui aurait pu être dégradé en raison d'une instabilité chimique pendant l'intervalle de 6 semaines entre la mort et l'analyse (au moment de la mort, la concentration sanguine aurait pu être beaucoup plus élevée), et 3. la concentration sanguine était suffisante pour produire une hypoglycémie fatale chez un patient, qui ne suivait pas correctement son traitement. Selon le médecin légiste, aucune cause traumatique de la mort n'a pu être établie. Les tests de stabilité du gliclazide dans le sang total sur une période correspondant au délai des analyses toxicologiques ont démontré une instabilité (perte d'environ 65 % après 6 semaines de stockage à +4 ◦ C), mais cela a été jugé non pertinent pour un surdosage massif. Les cheveux ont été testés positifs au gliclazide dans les segments de 3 × 2 cm, à 7, 8 et 3 pg/mg, et étaient très indicatifs d'une utilisation non conforme du médicament. Le médecin légiste a donc conclu que la cause du décès du sujet était probablement due à une utilisation inappropriée du gliclazide, pouvant être responsable d'un trouble glycémique fatal.

En raison de la nouveauté de la famille des gliflozines (commercialisation à partir de 2020 en France), la dapagliflozine a été identifiée, seulement une fois, au cours des trois années de thèse. Le cas est décrit ci-dessous :

Il s'agissait d'une femme de 60 ans retrouvée décédée après un by-pass gastrique suite à un surdosage de metformine. La femme était en effet sous traitement avec Forxiga® (dapagliflozine, 10 mg).

La concentration de dapagliflozine mesurée dans le sang été de 2,5 ng/mL . La concentration retrouvée dans le sang se situe dans la fourchette thérapeutique

Les cheveux étaient négatifs pour la dapagliflozine. Aucune information ne permet de savoir depuis combien de temps le sujet était sous traitement. Mais, compte tenu de la commercialisation assez récente, elle n'a certainement pas été sous traitement depuis longtemps. Aucune information n'est disponible sur l'incorporation de cette famille dans les cheveux. Aucune méthode pour le dosage des gliflozines dans les cheveux. Aucune méthode pour le dosage des gliflozines dans les cheveux n'est disponible dans la littérature et, par conséquent, aucune donnée n'est disponible sur les concentrations dans les cheveux.

Aujourd'hui, une méthode a été développée dans ce laboratoire et il sera intéressant d'étudier les cas futurs afin de fournir plus d'informations sur les concentrations de gliflozines dans les cheveux.

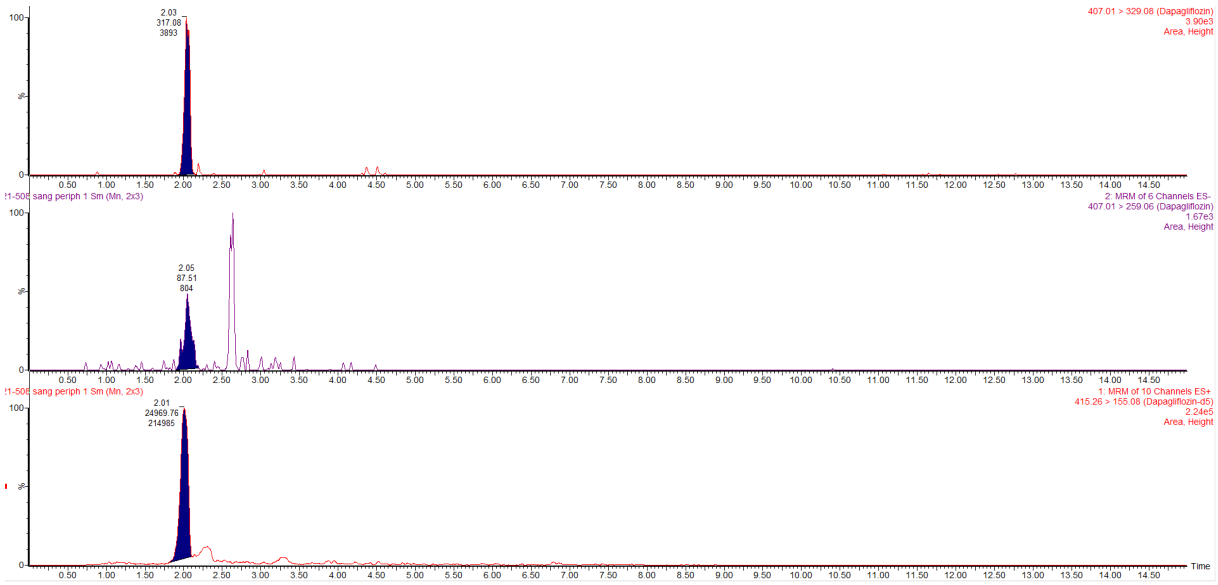

Figure 12. Chromatogrammes des transitions ioniques de l'échantillon de sang *postmortem* contenant (du haut vers le bas) 2 transitions de dapagliflozine et 1 transition de l'IS (dapagliflozine-d5), la concentration était de 2,5 ng/mL

Article 7: Is a "toxic" death possible with gliclazide, an oral hypoglycemic drug, found at therapeutic concentration?

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CASE REPORT

Is a ''toxic'' death possible with gliclazide, an oral hypoglycemic drug, found at therapeutic concentration?

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KEYWORDS
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Hair

Summary An unexpected death of a 46-year-old woman presenting a type II diabetes was observed and the Prosecutor requested an autopsy followed by toxicological investigations. Autopsy findings (labial ecchymosis, multiviscera congestion, asphyxia syndrome, moderate cerebral edema) were in accordance with a possible hypoglycemia death. Toxicological analyses revealed the presence of gliclazide in femoral blood at 2.2 mg/L, which is in the range of published therapeutic concentrations. During a meeting with the pathologist, 3 possible explanations were discussed: 1. unknown cause of death (other than a toxic death), 2. death due to gliclazide which could have been degraded due to chemical instability during the 6-weeks interval between death and analysis (at the time of death the blood concentration could have been much higher), and 3. blood concentration was enough to produce fatal hypoglycemia in a non-compliant patient. According to the pathologist, no traumatic cause of death could be established. Testing for gliclazide stability in whole blood over a period that matches the delay of the toxicological analyses demonstrated instability (loss of about 65% after 6 weeks storage at +4 °C), but this was found irrelevant for a massive overdose. Hair tested positive for gliclazide in the 3 × 2 cm segments, at 7, 8 and 3 pg/mg, and were highly indicative of non-compliant use of the medicine. It was therefore concluded by the pathologist that the cause of death of the subject was more likely than not an inappropriate use of gliclazide, which can be responsible of a fatal glycemic disorder.

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Introduction

Diabetes mellitus type 2 (non-insulin dependent diabetes) is a chronic metabolic syndrome, sometimes called insulin resistance, although it can be a mix of relative lack of insulin and insulin resistance. Major symptoms include hyperglycemia and aberrant metabolism of proteins, fats and carbohydrates. As a consequence, subjects with insulin resistance often have a group of symptoms including high blood glucose levels, high blood pressure and elevated triglycerides and cholesterol [1]. Control of blood glucose concentrations is possible by appropriate medication. Several classes of oral anti-diabetics are available, including sulphonylureas, thiazolidinediones, alpha-glucosidase inhibitors and metformin [2]. The group of sulphonylureas typically includes gliclazide, glibornuride, glimepiride, glipizide and glibenclamide, which differ mainly in their potency and their duration of action. Gliclazide, a member of this class, is on the WHO list of essential medicines.

Gliclazide is used for treatment of diabetes mellitus through inhibition of pancreatic ß cells ATP-dependent potassium channels, producing insulin release. This is reflected in a reduction in blood glucose levels, which is maintained during both short and long-term administration. It is given orally in a range 30 to 320 mg daily. Due to its duration of action of 12 to 24 hours, it can be administered in one or two divided portions.

Treatment with sulphonylureas can be potentially life threatening due to hypoglycemia [3]. In addition, sulfonylureas are associated with a higher risk of cardiovascular events compared with other anti-diabetic drugs, but within this class, gliclazide is considered as the drug with the least cardiovascular-related mortality [4].

Although it is used by millions of patients all around the world, very little forensic data are available for gliclazide. The drug has been observed in the context of Munchausen syndrome by proxy [5], but a comprehensive literature review on Pubmed, Scopus and Web of science did not find any citation of a fatal case report. Although gliclazide overdose can be observed [6], it seems that there is no reported blood concentration in the literature, so it is not possible to establish a toxic range of the drug. However, it is difficult to establish ''toxic'' ranges of these kinds of drugs since usually the biological duration of action of sulfonylureas does not correlate with their half-life and neither correlates with the blood concentration. It is established that the risk of hypoglycemia is higher with the long acting sulphonylureas compared to the short acting [3].

Hypoglycemia, a clinical and biological syndrome caused by an abnormal decrease in blood glucose levels to below 0.55 g/L is difficult to document in post-mortem specimens. In 1969 [7], Traub recommended to collect cerebrospinal and vitreous fluids to test for glucose, in order to compensate post-mortem alterations of blood glucose. The measurement of lactates, which increase post-mortem, could be established more accurately when using the Traub formula. However, the use of this formula is still under debate and Palmiere et al. [8] have demonstrated that vitreous glucose concentration appears to be the most reliable marker to estimate ante mortem blood glucose concentration. Other standard biochemical markers, such as glycated haemoglobin, acetone and 3-ß-hydroxybutyrate in blood or glucose in blood have been proposed to document glycemia disorders [8,9]. However, these tests have to be performed within hours after death and the results can be influenced by various diseases, including alcoholism, chronic inflammation, hepatic or renal dysfunction, and pancreatitis. Insulin, C-peptide, proinsulin or even insulin antibodies have also been proposed [10,11], but, again, the analytical investigations have to be performed as soon as possible to prevent hydrolysis.

The objective of this publication is to present the toxicological strategy to establish a toxic death involving gliclazide and to discuss possible factors influencing the final interpretation, in a situation where intelligence did not indicate, at the early time of death, to the forensic team a possible involvement of gycemic disorder.

Case history

A 46-year-old white woman (1.54 m tall, 84 kg) was found unconscious by her husband, at home. The Prosecutor in charge of the case requested an autopsy followed by toxicological investigations. External body examination and autopsy, performed three days after the death, revealed generalized organ congestion and the lack of any traumatic injury (confirmed by radiology). The heart was normal (353 g) and limited atheromatous plaque (not associated with calcification) was identified in the coronaries. Anatomic pathology tests did not evidence malformations. Pulmonary oedema was also observed during the histology investigations. Non-specific congestion of liver and kidney was also noticed. During the autopsy, cardiac blood, femoral blood, vitreous fluid, gastric content and hair (6 cm, brown) were collected for toxicological investigations. These specimens were immediately sealed and stored at +4 °C, except for hair, stored at ambient temperature. Two days after the autopsy (so it was too late to collect specific specimens and perform immediate analyses), the medical staff learned that she had been previously diagnosed with a type II diabetes, and intelligence revealed that she was being prescribed gliclazide (Diamicron), but the dosage and the possible use of modified release tablets remained unknown. Autopsy findings (labial ecchymosis, multi viscera congestion, asphyxia syndrome, moderate cerebral oedema) were in accordance with a possible hypoglycemia death.

However, due to administrative issues (in France, there is a need to obtain the validation of a quote before starting the analyses) a very long delay between collection of the specimens and toxicological analyses (6 weeks) occurred. Therefore, there was no attempt to test for glucose, insulin or other biochemical markers of hypoglycaemia [8,9], particularly because the vitreous fluid was not immediately centrifuged and contained numerous particles. Because the specimens have to be stored sealed until final approval of the Prosecutor, it was not possible to perform a glucose test in vitreous fluid immediately after knowing it was a possible hypoglycemic death. After, it was too late. According to the hypothesis of Traub, also known as the 'formula of Traub', post-mortem values of glucose and lactate found in the cerebrospinal fluid (not collected in this case) or vitreous fluid are considered indicators of ante mortem blood glucose levels. However, because the lactate concentration increases

in the vitreous fluid after death, again, it was not possible to measure the concentration. A delay of 6 weeks was found irrelevant to perform, or even attempt to perform insulin, peptide C, glucose and lactate determinations.

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 femoral blood and vitreous fluid by headspace 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 using NeoGen kits using the recommendations of the manufacturer. GHB was tested in femoral blood by GC/MS on an Aglilent 5971 using a standard validated procedure. A comprehensive screening for pharmaceuticals and drugs of abuse, including NPS was performed on blood using ultra-performance liquid chromatography-diode array detection (Waters UPLC-PDA) and ultra-performance liquid chromatography tandem mass spectrometry (Waters UPLC-MS/MS). Due to the nature of the case, gliclazide was specifically tested by a MRM method using LC/MS-MS. During the initial screening tests (ELISA and liquid chromatography), cardiac blood was used as it was available in larger quantities compared to femoral blood.

Gliclazide analysis

Gliclazide was extracted from 1 mL of fluid (cardiac and femoral blood, vitreous liquid, gastric content) in presence of 2 μg of prazepam used as internal standard, with 1 mL 1 M ammonium acetate buffer pH 5.2 and 5 mL of a mixture of ether/dichloromethane (80/20, v/v). Although gliclazide and prazepam are chemically very different, the rationale for choosing prazepam is its use during the initial screening of each sample and therefore the possibility of direct confirmation of the extract with a more specific method (i.e. this proposed method). 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 gliclazide was performed after decontamination with dichloromethane. Twenty milligrams of cut hair were incubated overnight at 50 °C in 1 mL 1 M ammonium acetate buffer pH 5.2, in presence of 1 μg of prazepam. 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 gliclazide 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 gliclazide: *m/z* 323.9 > 90.8 (CV: 42 V, CE: 36 eV) and 323.9 > 109.9 (CV: 42 V, CE: 20 eV), and the most abundant product ion of internal *standard* *m/z* 325.1 > 271.2 (CV: 40 V, CE: 22 eV). Transition *m/z* 323.9 > 109.9 was used for quantification of gliclazide. MassLynx 4.1 software was used for quantification.

Linearity was observed in blood for gliclazide concentrations ranging from 10 to 5000 ng/mL, with a correlation coefficient of 0.9996. The limit of detection was estimated to be 0.5 ng/mL, with a S/N ratio of 3. QC samples (50 ng/mL and 2000 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%. With respect to matrix effect, 9 different blood blank specimens were extracted and 50 μL of standard solution of gliclazide (1 mg/L) was added at the end of extraction and compared to 50 μL of standard solution of gliclazide in methanol (1 mg/L). The matrix effect was lower than 20%. Vitreous fluid and gastric content were tested using the blood calibration.

Linearity was observed in hair for gliclazide concentrations ranging from 2 to 100 pg/mg, with a correlation coefficient of 0.9986. The limit of detection was estimated to be 0.5 pg/mg, with a 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%. The matrix effect was 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 gliclazide for both blood (at 1000 ng/mL) and hair (at 50 pg/mg) were about 70%, 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.

Stability study procedure

A 25 mL pool of blank blood containing 10 mg/L of gliclazide was prepared and then aliquoted into 20 tubes of 1.2 mL. The first set of tubes (reference tubes) were immediately tested in triplicate, then the others were stored at +4 °C or −20 °C until analysis. The blood was regularly tested over 3 months.

Hair specimens from subjects under treatment

Hair specimens were collected in a local hospital from 6 volunteers (after obtaining their verbal consent) under

Table 1 Distribution of gliclazide in the biological specimens collected during autopsy.

Specimen	Gliclazide concentration
Femoral blood	2.20 mg/L
Cardiac blood	1.95 mg/L
Vitreous fluid	36 ng/mL
Gastric content	31 ng/mL
Hair, 0—2 cm	7 pg/mg
Hair, 2—4 cm	8 pg/mg
Hair, 4—6 cm	3 pg/mg

gliclazide therapy since at least 6 months, with a daily dosage in the range 60 to 240 mg. Only the proximal (0 to 3 cm) segment was tested for gliclazide.

Results and discussion

Ethanol tested negative in blood and vitreous fluid. GHB was considered normal (5.1 mg/L) in post-mortem blood. ELISA screenings were negative for pharmaceuticals and drugs of abuse. HbCO was 0.6% and cyanides were at physiological concentrations (< 80 ng/mL). The screening of blood by LC/DAD and LC/MS-MS was negative, except for gliclazide. Although not the best pH for gliclazide, extraction at pH 9.5 (borate buffer) during the screening, allowed a suitable chromatographic signal. When testing the blood under acidic condition (pH 5.2) by a specific MRM method, gliclazide was determined at 1.95 and 2.20 mg/L in cardiac and femoral blood, respectively (Table 1). Fig. 1 is the chromatogram obtained after extraction of the femoral blood.

Gliclazide is an old oral antidiabetic agent, commonly prescribed. It has been published [12] that the medium gliclazide blood concentration for patients taking 30 mg was 0.58 ± 0.12 mg/L. For those taking 60 mg Modified Release, the medium gliclazide blood concentration was 2.03 ± 1.19 mg/L. In another study, after a single administration of 30 mg (as a sustained release tablet) to 18 volunteers, the Cmax was 0.93 ± 0.31 mg/L [13]. The administration of 80 mg to 12 healthy volunteers was consistent with a Cmax at 2.68 ± 0.46 mg/L [14].

Based on the blood concentrations measured in the victim, there is no evidence of gliclazide post-mortem redistribution. However, this is not confirmed by literature citation, due to a lack of data. The measured concentrations in blood are in the range of published therapeutic concentrations (see supra). The drug was also identified in vitreous fluid, but at much lower concentration, as the drug only tested positive at 36 ng/mL. Again, given the lack of any post-mortem distribution data in the literature, this cannot be compared. Finally, the very low concentration measured in the gastric content is in accordance with drug administration back several hours before death with complete absorption and supports limited blood diffusion.

During a meeting with the pathologist, 3 possible causes of death were discussed:

- natural or traumatic cause of death (other than a toxic death);
- death due to gliclazide overdose which could have been degraded due to chemical instability as the gliclazide tests were performed 6 weeks after death (at the time of death the blood concentration could have been much higher);
- and gliclazide has produced a fatal hypoglycemia in a non-compliant patient.

Hypothesis 1 was ruled out, as the experienced pathologist did not observe signs of trauma and violence or organ failure during the autopsy.

Literature survey about gliclazide stability in biological material did not contribute in interpretation as there is no data dealing with storage + 4 °C for about 6 weeks before quantitative analysis. However, it is indicated that gliclazide is stable for at least 12 hours at ambient temperature [14], for 30 days at −50 °C [15], for up to 2 months at −20 °C [14] and for at least 3 months when stored at −70 °C [16]. In order to obtain more information about stability of gliclazide, a stability study was performed at the laboratory. Blank blood was spiked with gliclazide at 10 mg/L, stored at +4 °C and −20 °C and regularly tested over 3 months. Results are presented in Fig. 2. A degradation of gliclazide was observed, with a quicker degradation when stored at +4 °C. The percentage of concentration remaining from the initial concentration of gliclazide stored for about 6 weeks at +4 °C was about 35%. In view of these results, one can anticipate that the subject was exposed to higher concentrations of gliclazide that those measured during the analyses, but not enough to be far higher than the literature-based therapeutic concentrations. However, this cannot be put into a mathematical equation to back-calculate the concentration at the time of death, as numerous factors can influence the final concentration.

The last hypothesis was a toxic death, due to fatal hypoglycaemia even at therapeutic concentration, in a non-observant subject. This was considered possible by the pathologist, as some signs observed during the autopsy (labial ecchymosis, cerebral oedema) were consistent with seizures, which, in fine, can turn to death. At this stage, it was excluded that the toxicity was due to a drug-drug interaction on CYP2C9 which is the major enzyme involved in sulfonylurea drug metabolism [17] because no other drug was identified during the comprehensive toxicological screening, particularly those known to interfere with gliclazide, such as antifungals, phenylbutazone and ethanol [18]. Compliance to a long-term treatment can be documented by hair testing [19]. By providing information on exposure to a drug 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. During post-mortem investigations, a drug user is no more able to describe his/her pattern of use but hair will allow getting it as hair analysis can 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. The hair must be cut as close as possible to the scalp and particular care is also required to ensure that the individual hairs in the cut-off tuft retain their originally orientation. Based on an average of growth rate of 1 cm per month, each cm will represent the exposure to drug(s)

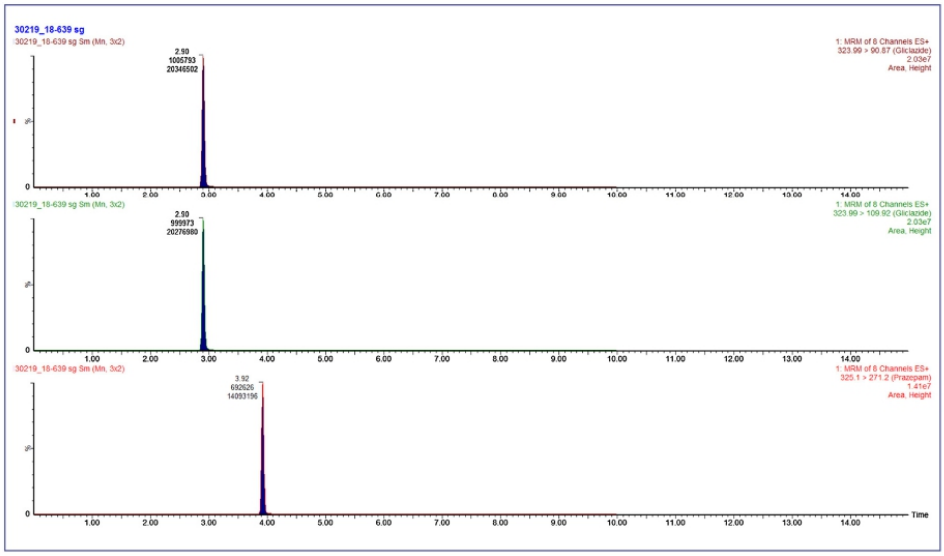

Figure 1. Chromatogram obtained after extraction of the femoral blood of the victim. Gliclazide concentration was 2.20 mg/L. From top to bottom: 2 transitions for gliclazide and 1 transition for prazepam (IS).

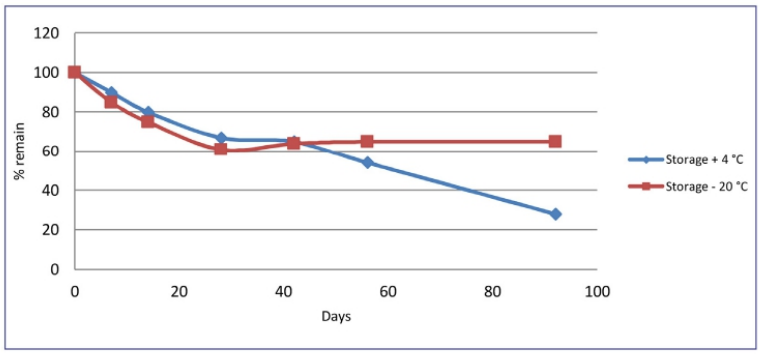

Figure 2. Percentage of concentration remaining from the initial 10 mg/L of gliclazide when stored at +4 °C and −20 °C for 3 months.

Table 2 Concentrations of gliclazide in the hair (proximal 0 to 3 cm segment) of 6 subjects under long-term therapy.

Subject	Daily dosage (mg)	Hair colour and length (cm)	Gliclazide concentration (pg/mg)
1, M	60	Black, 4	550
2, M	60	Grey, 3	650
3, F	60	Brown, 6	720
4, F	60	Light blonde, 8	580
5, F	120	Black, 4	870
6, F	240	Brown, 6	1200

M: male, F: female.

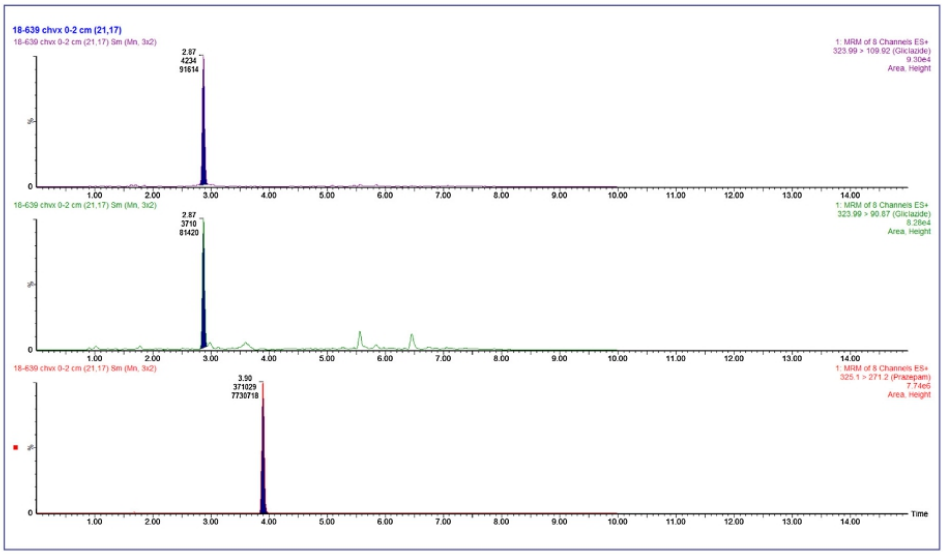

Figure 3. Chromatogram obtained after extraction of the 0 to 2 cm proximal hair segment of the victim. Gliclazide concentration was 7 pg/mg. From top to bottom: 2 transitions for gliclazide and 1 transition for prazepam (IS).

during one month. The hair specimen (6 cm, brown) of the victim tested positive for gliclazide at 7, 8 and 3 pg/mg in the 3 × 2 cm sections. There was no evidence of recent cosmetic treatment that could have lowered the concentrations [19]. The chromatogram obtained after the extraction of the proximal segment is presented Fig. 3. The toxicological significance of the measured concentrations is difficult to establish because this is an unusual drug, not reported in the medical literature, excepted by Binz et al. [20], who indicated concentrations in the range 32 to 101 pg/mg in the hair of 4 subjects, with no apparent dose-concentration correlation. Also, there is no controlled study about pharmacokinetic parameters of gliclazide incorporation into hair. It is therefore not possible to interpret the data in terms of dosage and frequency of use. In order to get some additional data, the laboratory collected 6 hair specimens from subjects under long-term therapy (more than 2 years) and submitted them to the analytical procedure. These subjects were in a local hospital and gave their oral consent with the aim to document exposure to their treatment. In addition, in France, collection of a hair specimen is considered as a non-invasive procedure. The results are presented in Table 2. Hair concentrations of daily users were in the range 550—1200 pg/mg, much higher than what was measured in the hair of the victim. It was therefore concluded that the victim was not compliant to her gliclazide treatment. The measured concentrations in the 3 segments are very close to the one measured in the hair of a subject receiving a single dose of glibenclamide, i.e. 5 pg/mg, which is the unique controlled data in the literature [21]. Stability of gliclazide was verified in hair by comparing the concentrations of 3 positive hair specimens obtained from subjects under long-term therapy measured after collection and after 2 months. Variations were less than 10%, which was found consistent with the precision of the method.

It was therefore concluded by the pathologist that the cause of death of the subject was an inappropriate use of gliclazide, a drug that could be responsible of a fatal hypoglycemia, even at normal therapeutic concentration. This was based on the autopsy findings (with indications of seizures), the histological tests and the toxicological analyses results and is totally different from drug accumulation due to drug-drug interaction or acute renal failure.

Conclusion

Due to a lack of reference values for blood concentration of sulfonylurea drugs in the literature, the forensic evaluation of gliclazide findings in blood samples is difficult. Interpretations with regard to the assessment of blood concentrations as well as an estimation of the ingested gliclazide amounts are very difficult to establish. However, toxicologists and forensic pathologists should be aware of possible death when dealing with hypoglycemic drugs. A comprehensive toxicological screening, targeting the drugs from this pharmacological class is of paramount importance when the death is unexpected, as sulphonyureas are not always screened.

This fatal case demonstrates the absolute need of collaboration between the active investigators, including Police, forensic pathologists (autopsy + histology tests) and toxicologists. This is of great importance as the literature seems extremely poor about oral hypoglycemic drugs, although they are used by millions of subjects.

Finally, the cause of death remains a hypothesis that could not definitively be proven. Other papers of that type with the analysis of biochemical parameters should be encouraged.

Ethical statement

Compliance with Ethical Standards: yes in accordance with regulations dealing with dead bodies.

Ethical approval: not needed.

Informed consent: not applicable.

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None.

Disclosure of interest

The authors declare that they have no competing interest.

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Quatrième Partie – L’Insuline

4.1 Présentation

L'insuline est une hormone peptidique composée de 51 acides aminés disposés en deux chaînes, une chaîne alpha (21 acides aminés) et une chaîne bêta (30 acides aminés) qui sont reliées par deux ponts disulfures. Sa structure a été déterminée pour la première fois par Frederick Sanger, ce qui lui a valu le prix Nobel en 1958.

L'insuline est produite dans les cellules β du pancréas sous forme de pro-insuline, un peptide à chaîne unique de 86 acides aminés, dans le réticulum endoplasmique, puis transformé et rassemblé en granules dans l'appareil de Golgi. Sa sécrétion se produit en réponse à une augmentation du niveau plasmatique de glucose (ou d'autres nutriments tels que les acides aminés et les acides gras libres).

Le glucose qui entre dans la cellule β est rapidement phosphorylé en glucose-6-phosphate et subit une oxydation dans les mitochondries, conduisant à la production d'adénosine triphosphate (ATP). L'augmentation du rapport ATP/adénosine diphosphate dans les cellules β entraîne la fermeture des canaux potassiques sensibles à l'ATP (K^+ ATP), ce qui provoque une dépolarisation de la membrane cellulaire et permet l'entrée du Ca2+ par l'ouverture des canaux calciques de type L dépendants du voltage. L'élévation des niveaux de Ca2+ intracellulaire induit l'exocytose des granules contenant la proinsuline de la cellule β pancréatique.

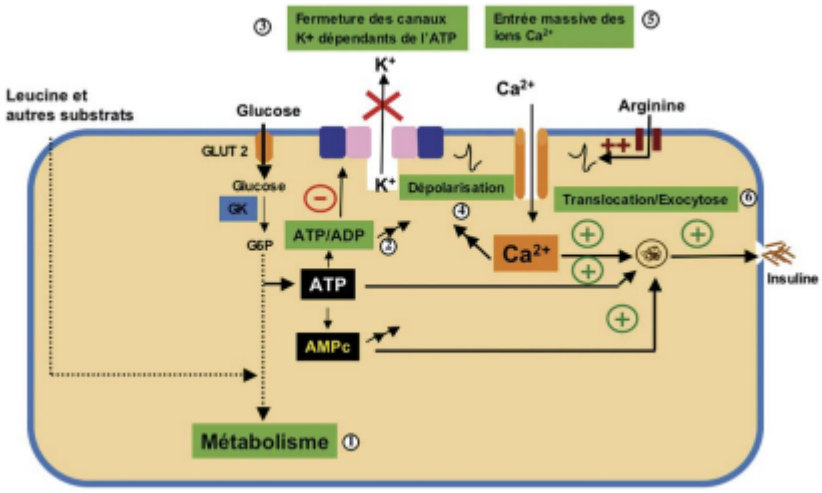

Figure 13. Mécanisme de sécrétion de l'insuline [43]

Bien que de nombreux types de cellules somatiques expriment des récepteurs de l'insuline, le rôle de l'insuline dans l'homéostasie du glucose est caractérisé par les effets directs de l'insuline sur les muscles squelettiques, le foie et les adipocytes blancs. Ces tissus nécessitent des voies de signalisation de l'insuline spécifiques aux tissus. Dans le muscle squelettique, un tissu représentatif de l'action de l'insuline, l'insuline favorise l'utilisation et le stockage du glucose en augmentant le

transport du glucose et la synthèse nette du glycogène. Dans le foie, l'insuline active la synthèse du glycogène, augmente l'expression des gènes adipogènes et inhibe la gluconéogenèse en diminuant l'expression des gènes gluconéogènes. Dans le tissu adipocytaire blanc, l'insuline inhibe la lipolyse et augmente le transport du glucose et l'adipogenèse. Bien que les effets de l'insuline varient d'un tissu à l'autre, les composants proximaux impliqués dans la signalisation de l'insuline sont très similaires dans toutes les cellules sensibles à l'insuline. En outre, l'insuline supprime la sécrétion de glucagon par les cellules α du pancréas, qui est elle-même un puissant inducteur d'hyperglycémie.
L'insuline exerce tous ses effets physiologiques connus en se liant au récepteur de l'insuline (INSR) sur la membrane plasmique des cellules cibles [25-26].

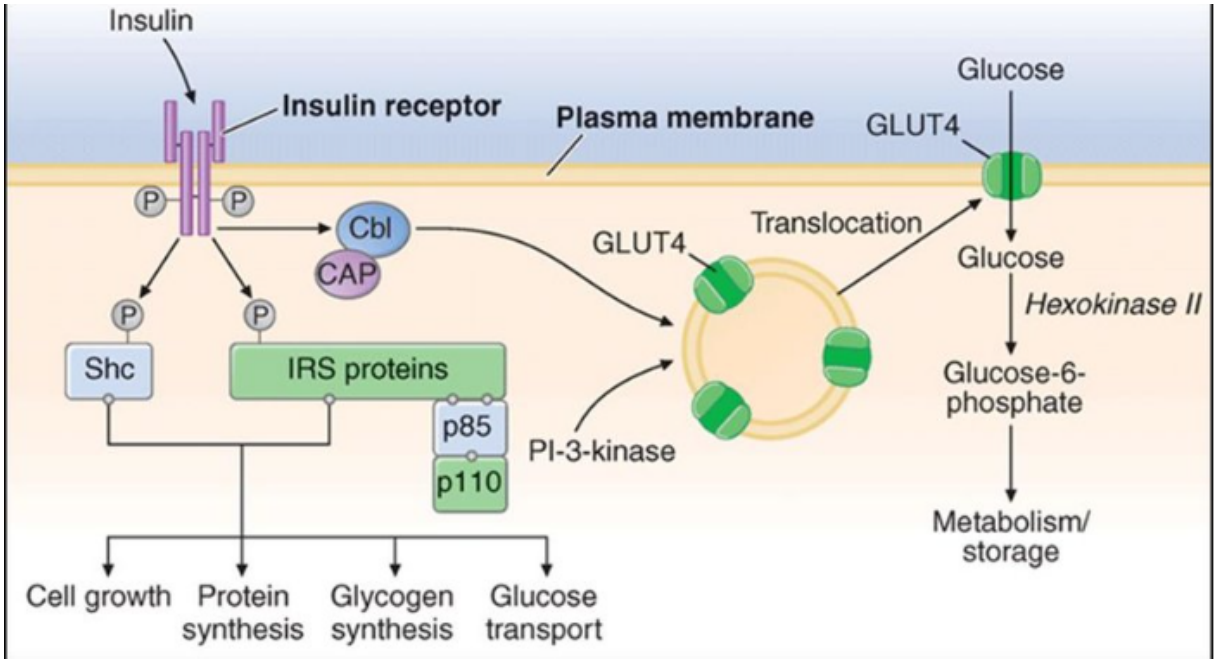

Figure 14. Mécanisme d'action de l'insuline [44]

Lorsque des taux d'insuline circulante plus élevés sont nécessaires pour obtenir la réponse intégrée de réduction du glucose décrite ci-dessus, un sujet est considéré comme résistant à l'insuline.
A un taux d'insuline plasmatique normal, les tissus cibles ne sont pas en mesure de mettre en place une réponse hypoglycémique coordonnée normale impliquant la suppression de la production endogène de glucose, la suppression de la lipolyse, l'absorption cellulaire du glucose plasmatique disponible et la synthèse nette de glycogène.
La résistance à l'insuline nécessite une sécrétion accrue d'insuline pour compenser, de sorte que les taux d'insuline plasmatique à jeun augmentent.
Cette charge de travail accrue pour le système endocrinien et le pancréas, et la décompensation cellulaire qui en résulte, est un mécanisme majeur du développement du diabète de type II [25, 27].

Le rôle du pancréas dans le diabète a été découvert pour la première fois par Von Mering et Minkowski en 1889 à Strasbourg lorsqu'ils ont reproduit le diabète en pancréactomisant des chiens. Le lendemain, ils se sont rendu compte que les chiens avaient très soif et que leur urine attirait les mouches.
Partant de cette observation, de nombreux scientifiques ont, dans les années suivantes, étudié comment le pancréas produisait quelque chose qui lui permettait de réguler la glycémie.
En 1921, Frederick Banting et son assistant, Charles Best, ont réussi à isoler un extrait de pancréas d'un chien qui avait subi une résection du pancréas, et ont constaté que lorsqu'ils réintroduisaient l'extrait dans le chien, sa glycémie s'améliorait rapidement.
Cet extrait a été appelé insuline, du mot latin "insula", en référence aux îlots de Langerhans.
En 1922, l'insuline a été administrée pour la première fois à un jeune diabétique de 14 ans, lui sauvant la vie [28].

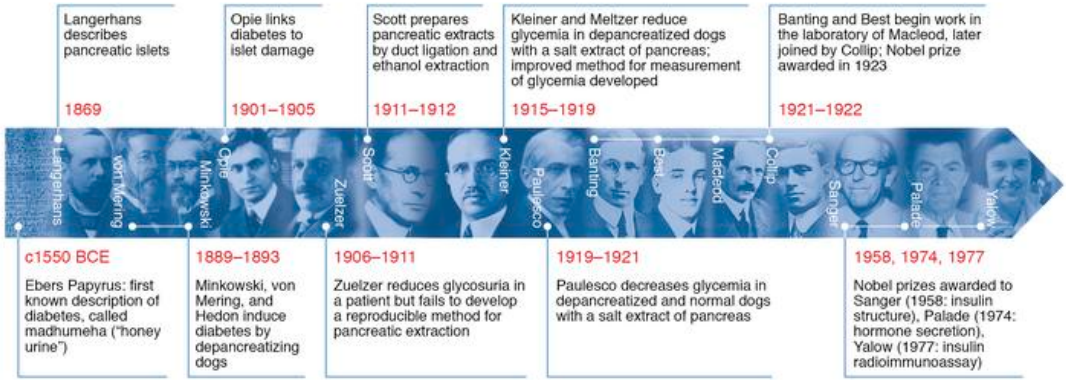

Figure 15. Histoire de la découverte de l'insuline au premier séquençage de l'insuline [28]

Jusqu'en 1980, les insulines administrées aux patients diabétiques étaient purifiées à partir de pancréas de porc et de bœuf. Par la suite, en raison du nombre élevé de réactions allergiques qui leur étaient associées, leur utilisation a progressivement diminué.

L'avènement des techniques de recombinaison de l'ADN a permis le développement et la production d'analogues de l'insuline humaine.
La structure de l'insuline est légèrement modifiée pour altérer les propriétés pharmacocinétiques de l'insuline, principalement en affectant son absorption à partir du tissu sous-cutané.
La région B26-B30 de l'insuline n'est pas critique pour la reconnaissance du récepteur de l'insuline, c'est pourquoi c'est généralement dans cette région que les acides aminés sont substitués.
Les insulines synthétiques sont divisées en deux catégories : les insulines à action rapide (aspart, lispro et glulisine) et les insulines à action lente (glargine, detemir et degludec).
Les insulines à action rapide sont conçues pour imiter la sécrétion physiologique d'insuline après une augmentation postprandiale du glucose. Pour cette raison les insulines rapides synthétiques présentent

des substitutions ou des inversions d'acides aminés qui tendent à ralentir ou à bloquer la tendance de l'insuline à s'associer en dimères et en hexamères.

Cela implique une formation plus rapide des monomères actifs après injection sous-cutanée et une absorption immédiate dans la circulation sanguine.

Dans l'insuline aspart (Novolog®) , l'acide aminé aspartique prend la place de la proline en position B28. L'insuline lispro (Humalog®) possède les acides aminés proline (B28) et lysine (B29) inversés. Et enfin, la substitution de l'asparagine en position B3 par la lysine et de la lysine en position B29 par l'acide glutamique génère la glulisine (Apidra®).

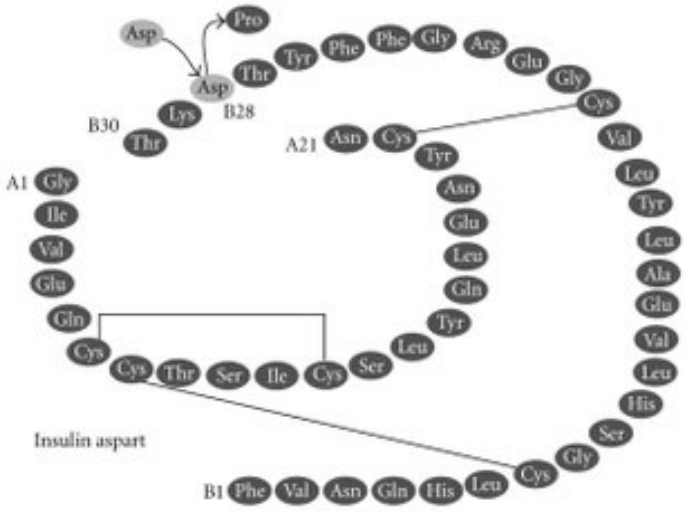

Figure 16. Structure de l'insuline aspart, insuline à action rapide [45]

Les insulines à action lente, quant à elles, sont conçues pour imiter la sécrétion d'insuline basale.

L'insuline glargine (Lantus®) présente l'ajout de deux arginines en position C-terminale de la chaîne β et le remplacement de l'asparagine par la glycine en position 21 de la chaîne α. Ces modifications déplacent le point isoélectrique de l'insuline glargine, ce qui la rend peu soluble à pH neutre mais totalement soluble au pH acide de la solution injectable (pH 4). Après injection dans le tissu sous-cutané (pH 7,4), la solution acide est neutralisée, ce qui entraîne la formation de micro-précipités qui représentent un "dépôt" à partir duquel de petites quantités d'insuline glargine sont libérées en continu. L'ajout de zinc à la préparation stabilise davantage le produit en retardant la libération des monomères et des dimères d'insuline glargine, ce qui prolonge sa durée d'action. Par conséquent, la courbe concentration/temps est régulière, sans pic, et la durée de son action est prolongée. Après injection sous-cutanée de Lantus®, l'insuline glargine est rapidement dégradée à l'extrémité carboxyle de la β -chaîne en deux métabolites actifs, M1 (21A-Gly-insuline) et M2 (21A-Gly-des-30B-Thr-insuline). Dans le plasma, le principal métabolite circulant est le métabolite M1 et sa concentration augmente avec la dose

administrée de Lantus®. Les effets de l'injection sous-cutanée de Lantus® sont principalement dus à l'exposition au métabolite M1.

La différence entre l'insuline detemir (Levemir®) et l'insuline humaine réside dans l'élimination de l'acide aminé B30 et la liaison de la lysine en position B29 à un acide gras à 14 carbones (acide myristique). Cette insuline modifiée sera plus encline à former des multi-hexamères au niveau sous-cutanée. Ce qui retarde encore plus sa distribution dans les tissus périphériques, c'est sa tendance à se lier à l'albumine présente à la fois dans les tissus sous-cutanés et dans la circulation sanguine par l'intermédiaire de l'acide myristique lié en position B29. Son action commence après environ deux heures et dure jusqu'à 24 heures.

L'insuline degludec (Tresiba®) est une insuline humaine modifiée à action ultra-longue dans laquelle l'acide aminé B30 est supprimé et elle est conjuguée à l'acide hexadécanedioïque au niveau de l'acide aminé lysine en position B29. Ces modifications confèrent à l'insuline dégludec la capacité de former des multihexamères après injection sous-cutanée. Des dépôts filamenteux se forment, à partir desquels les monomères d'insuline sont lentement libérés dans la circulation sanguine.

Son action commence environ 30 à 90 minutes après l'injection mais dure plus de 24 heures [26,29].

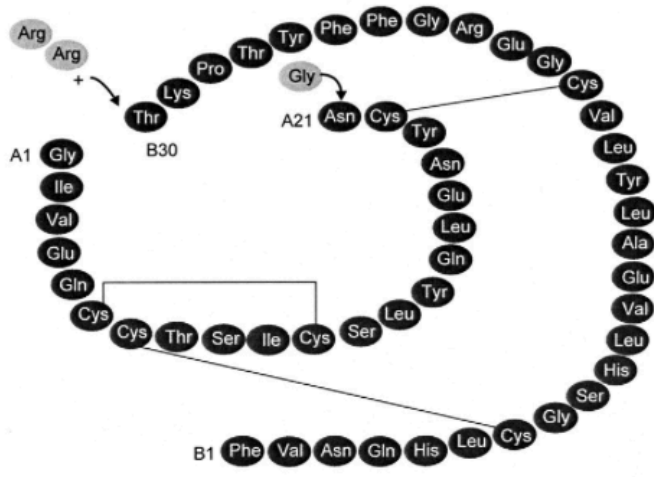

Figure 17. Structure de l'insuline glargine, insuline à durée prolongée [46]

4.2. Développement d'une méthode d'identification et de quantification de l'insuline humaine et des analogues synthétiques dans le sang

La méthode d'identification de l'insuline et de ses analogues synthétiques dans le sang a été réalisée sur un système de chromatographie liquide couplé à une spectrométrie à haute résolution, notamment un spectromètre de masse à temps de vol (UHPLC-XEVO G2-XS, Waters, Milford, Massachusetts, Etats-Unis). La méthode inclut l'insuline humaine, lispro, aspart, glulisine, glargine et detemir.

La difficulté initiale rencontrée lors de la mise au point de cette méthode est liée au fait que l'insuline est une protéine ayant un poids moléculaire assez élevé (> 5000 Da).

L'utilisation de méthodes d'ionisation classiques telles que l'électrospray entraîne la formation, pour les molécules de masse élevée, d'ions à charges multiples, c'est-à-dire qu'il s'agit de molécules polyprotonées. La formation d'ions à charges multiples est très importante car elle réduit le rapport masse/charge des ions, ce qui permet l'analyse de molécules très lourdes comme les protéines. Le spectre observé sera donc plus complexe que celui des molécules à charges unitaires.

Le rapport m/z des molécules sortant de l'électrospray est souvent compris entre 500 et 2000. Le nombre de charges absorbées par la molécule dépend à la fois de la basicité des différents groupes ionisables et du pH du solvant (un pH faible augmente le nombre de charges). L'analyse de l'insuline est possible grâce à la présence de résidus d'histidine dans la chaîne peptidique, qui sont des résidus basiques protonables.

En présence d'ions à charges multiples, tous les ions n'ont pas le même nombre de charges, mais on observe plutôt une série de pics dus à des ions ayant un nombre croissant de charges, de forme à peu près gaussienne :

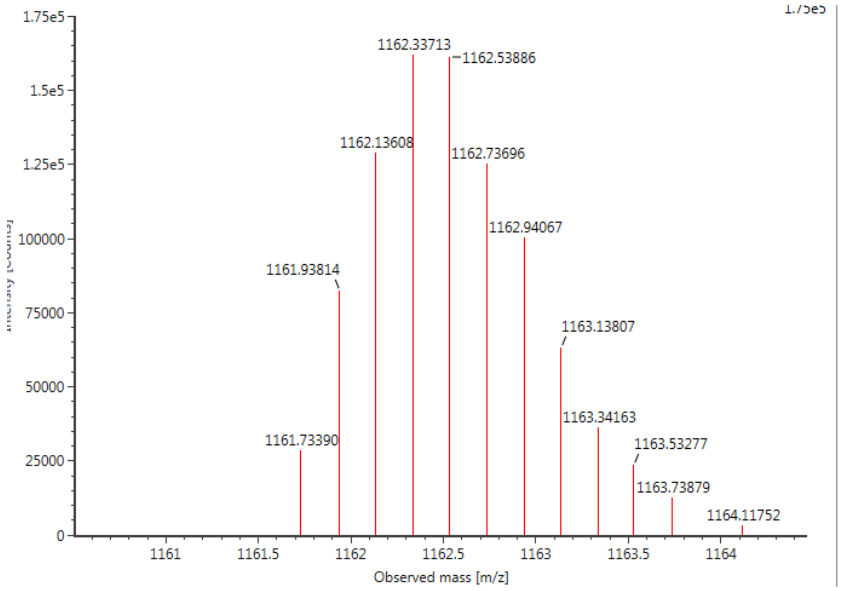

Figure 18. Spectre de l'ion pseudo-moléculaire m/z $[M+5]^{5+}$ 1162.33713 de l'insuline humaine

La distance entre les rapports m/z des différents ions [qui sont du type (M+H)/H, où H est le nombre de charges] diminue lorsque le nombre de charges augmente, ce qui permet d'établir le nombre de charges de chaque ion, et donc la masse réelle de la molécule (algorithme de déconvolution).
L'identification des insulines s'effectue à partir d'ions précurseurs 4 à 7 fois chargés représentant des ions pseudo-moléculaires de 800 à 1500 m/z.
Dans cette étude, la cellule de collision (CID) du spectromètre de masse à temps de vol a été utilisé pour obtenir des fragmentations qui peuvent nous donner des informations supplémentaires sur la séquence d'acides aminés des insulines.
Le schéma de fragmentation observé en CID est la fragmentation typique des terminaisons N et C décrite par Biemann et Roerpstorff. Les fragmentations peuvent produire des fragments N-terminal et C-terminal.
Si la charge est portée par le fragment N-terminal, les ions produits sont indiqués comme b, si elle est portée par le fragment C-terminal, les ions produits sont indiqués comme y.
Par conséquent, la structure des acides aminés est déterminée par la reconnaissance des ions b et y.
Dans le cas des insulines, la fragmentation se produit au niveau de l'extrémité C-terminale de la chaîne bêta, car c'est là que se trouvent la plupart des modifications par rapport à l'insuline humaine [30].

Plusieurs approches ont été testées avant d'établir la méthode définitive.
Toutes les méthodes testées jusqu'à la méthode finale étaient des adaptations de méthodes déjà publiées [31-33].

Dans un premier temps, une technique d'immunopurification inspirée d'une méthode publiée par Thevis et al. a été essayée.
Cette technique exploite les réactions antigène-anticorps en utilisant des billes magnétiques (Dynabeads ™, Thermofisher, Waltham, Massachusetts, Etats-Unis) recouvertes d'anticorps et un anticorps monoclonal anti-insuline. L'extraction est ainsi beaucoup plus ciblée, ce qui permet d'éliminer toutes les énormes interférences présentes dans le sang.
Ce test échoue, et dans un premier temps on a pensé que cet échec était dû à l'utilisation de tubes à essai inadaptés. L'un des problèmes de l'insuline, en effet, est qu'elle est soumise aux phénomènes d'adsorption et agrégation. Lorsque l'insuline est présente dans une solution à une faible concentration, elle se trouve principalement sous forme de monomères. Les monomères de l'insuline possèdent des groupes hydrophobes orientés vers l'extérieur qui ont tendance à interagir avec les groupes hydrophobes des parois du matériel utilisé (flacons ou tubes en polypropylène, en verre ou en borosilicate) pour former des liaisons. Une fois adsorbés à la paroi, ces monomères interagissent avec d'autres monomères en solution pour former des agrégats [34].

En effet, en utilisant des flacons en verre classique, nous avons constaté une perte progressive du signal déjà pendant l'infusion des standards dans le spectromètre.
Par conséquent, afin de limiter ce phénomène, des tubes Eppendorf ® LoBind protein (SigmaAldrich, Saint-Louis, Missouri, Etats-Unis) ont été testés. Le matériau de ces tubes est constitué d'un mélange de polymères qui contribuent à former une surface hydrophile empêchant la liaison protéine-surface.

Malgré l'utilisation de tubes à essai spécifiques, ce test ne fournit toujours pas la sensibilité requise et, après une étude approfondie de la littérature, l'utilisation de billes magnétiques pour le sang post-mortem n'est pas recommandée. Pour le sang *postmortem*, il est nécessaire d'effectuer une précipitation initiale des protéines avant l'immunopurification pour éliminer une grande partie des interférences présentes dans le sang. Dans cette méthode, j'avais utilisé un mélange d'acétonitrile et de méthanol, qui semble avoir un effet agglutinant sur les billes.

Pour cette raison, l'immunopurification est mise de côté pendant un certain temps et je commence à faire des essais en utilisant l'extraction en phase solide (SPE).
Malheureusement, cette méthode comporte également des inconvénients. Le principal problème concernant la matrice.
Le sang total étant l'échantillon le plus couramment utilisé en toxicologie médico-légale, il nécessite un traitement spécial avant l'extraction SPE pour éliminer les interférences des protéines endogènes et en particulier de l'albumine. Une précipitation des protéines est nécessaire dans des solvants tels que l'acétonitrile et le méthanol (avec lesquels l'insuline ne précipite pas) en présence d'un acide (dans notre cas l'acide formique) pour stabiliser l'insuline.
Cependant, après avoir agité le sang avec les solvants acidifiés, on a constaté la formation d'une seule agglomération, quelle que soit la proportion de solvant choisie.
C'est pourquoi l'ajout de ZnSO4 a été choisi pour permettre une séparation adéquate. Le ZnSO4 ne précipite que les protéines de très haut poids moléculaire (telles que les immunoglobulines) et favorise la lyse des cellules, ce qui permet d'obtenir des surnageants plus propres qui facilitent l'extraction subséquente en SPE.
Avec cette méthode, il est possible pour la première fois d'extraire l'insuline et ses analogues du sang humain *postmortem* avec une bonne sensibilité (20 ng/mL) lors des différents tests.
Cette sensibilité n'était pas encore suffisante.
Dans une tentative d'améliorer la méthode et d'éliminer le background endogène trop important à de faibles concentrations d'insuline, j'ai décidé d'essayer une étape ultérieure d'immunopurification avec des billes magnétiques. Malheureusement, le problème d'agglutination était toujours présent car le mélange d'élution SPE contient un pourcentage de méthanol (qui est essentiel pour l'élution).
Par la suite, un certain nombre d'ajustements de la méthodes ont permis de faire passer la LOD de 20 à 1 ng/mL.

Tout d'abord, des vials qui réduisent les pertes de peptides et de protéines dues aux interactions ioniques et aux liaisons non spécifiques ont été achetés (QuanRecovery Vials, Waters, Massachusetts, Etats-Unis), ce qui a considérablement augmenté la sensibilité en empêchant l'absorption de l'extrait aux parois du flacon.
Plus tard, l'utilisation d'une nouvelle colonne de séparation chromatographique a été introduite.
La colonne utilisée est une colonne recommandée par le fabricant de notre équipement analytique (Waters) pour la séparation des protéines. Il s'agit d'une colonne CORTECS C18 de 1,6 uM (Waters, Massachusetts, Etats-Unis). Cette colonne a été choisi parce que contrairement aux petites molécules, les protéines souffrent d'un mauvais transfert de masse dans et hors des particules entièrement poreuses. Les colonnes avec des particules à noyau solide permet d'obtenir des formes de pic plus nettes à des débits plus élevés. En ce qui concerne spécifiquement les insulines, le fournisseur soutien que l'utilisation d'une colonne remplie de particules contenant une faible charge de surface positive donne une forme de pic et une résolution supérieure à celles des autres colonnes.
L'utilisation de cette colonne nous apporte une légère amélioration, mais cela est négligeable par rapport au coût de la colonne et au changement continuel de colonne, qui doit être fait sur un équipement qui utilise toujours la même colonne pour d'autres analyses de routine. Cette colonne n'est donc pas utilisée.
Le protocole suivant a été validé pour l'insuline humaine et ses analogues synthétiques. Les transitions utilisées, le temps de rétention et les limites de quantification et de détection sont résumés dans l'***Article 8***.

Enfin, bien que la méthode présentait de bons résultats, elle était trop laborieuse et le temps de préparation et d'extraction était trop long pour être mise en place en routine. Pour cette raison, une méthode d'extraction publiée d'abord par Judak et al. [35] dans l'urine et puis appliquée par Bottinelli et al. [33] au sang *postmortem*, a été adaptée. Cette méthode prévoit une étape de précipitation des protéines, leur concentration par filtration et une étape d'immunopurification avec l'utilisation des puits coatés d'une plaque ELISA.

Protocole d'extraction n #1

Après avoir mélangé 1 ml de sang total avec 1 ml d'une solution aqueuse de ZnSO4 0,1 M, l'échantillon est soumis à une étape supplémentaire de précipitation des protéines avec 2 mL d'un mélange d'acétonitrile/méthanol + 1% d'acide formique en présence de 20 ng/mL d'insuline bovine comme standard interne. Après agitation et centrifugation, le surnageant est transféré dans un autre tube. Dans ce tube, 2 mL de solution d'ammoniaque à 5% sont ajoutés et l'échantillon est mélangé au vortex.

L'extraction en phase solide est réalisée avec des cartouches mixtes échangeuses d'anions (Waters MAX, 3 mL, 60 mg), qui sont préconditionnées avec 1 mL de méthanol et 1 mL d'eau avant de procéder au transfert du surnageant.
Les échantillons sont lavés avec 1 ml de solution d'ammoniaque à 5% et 1 ml de méthanol/acide acétique (60 :10). Enfin, l'échantillon est élué dans un vial avec 250 µL d'un mélange de méthanol/acide acétique (5:1) et 5 µL ont été injectés dans le système LC-HRMS.

Protocole d'extraction n #2

250 μL de sang total sont soumis à une précipitation des protéines avec un mélange d'acétonitrile et méthanol en rapport 1 :1 en présence de 12 μL de Tween 20 à 0,05% et 20 ng/mL d'insuline bovine utilisée comme standard interne.
Ensuite, une ultrafiltration est réalisée à l'aide des filtres Amicon® Ultra-0.5 centrifugal filter (avec un cutoff d'un poids moléculaire de 3,000 Dalton). Après 2 lavages avec PBS, le rétentat est déposé sur les puits de la plaque ELISA (Iso-Insulin, Mercodia) pour l'immunopurification. Après 1 heure d'agitation dans une plaque d'agitation (800 rpm), les puits sont lavés manuellement avec 300 μL de PBS et l'insuline est éluée avec une solution de H2O/ACN (80/20) et 2% d'acide formique. 5 µL sont ensuite injectés dans le système LC-HRMS.

Paramètres du système UHPLC-Q-TOF

Les analogues de l'insulines ont été rajoutés à la bibliothèque après infusion des solutions des standards à 1 mg/mL dans la source et optimisation manuelle des paramètres HRMS.
La séparation LC est réalisée à l'aide d'une colonne C18 Acquity HSS de Waters (150 x 2,1 x 1,8 μm) avec une température contrôlée maintenue à 50 °C. Une injection de 5 μL avec un débit de 0.25 mL/min d'eau avec 0.1 % d'acide formique (solvant A) et d'acétonitrile avec 0.1 % d'acide formique (solvant B) a été utilisée. L'élution en gradient était la suivante : le pourcentage initial de B de 20 % a été porté à 65 % en 5,4 minutes, de 65 à 98 % en 0,3 minute et le retour aux conditions initiales en 4,2 minutes. Le temps total d'exécution est de 9,90 minutes.
La séparation chromatographique n'a pas été possible pour les insulines en raison de leurs propriétés physico-chimiques presque identiques (sauf pour l'insuline detemir qui a un temps de rétention plus tardif que les autres).
Un spectromètre de masse haute résolution Xevo G2-XS Q-TOF (Waters corporation, Milford, MA, USA) a été utilisé, fonctionnant en mode ion positif et en mode sensibilité.

Le débit de gaz de désolvatation a été réglé à 1 000 L/h à une température de 600 °C, le gaz du cône à 50 L/h et la température de la source à 120 °C. La tension du capillaire et la tension du cône ont été réglées à 3 et 15 V, respectivement. Le mode de détection utilisé était le Full Scan, qui permet d'enregistrer tous les ions arrivant au détecteur. Les données ont été acquises de 100 à 2000 m/z.
Pour obtenir de nombreux ions fragmentés, une rampe d'énergie de collision a été utilisée dans la cellule de collision. L'énergie de collision était comprise entre 20 et 80 eV.
Le logiciel UNIFI a été utilisé pour l'acquisition des données, des chromatogrammes et des spectres.
En ce qui concerne la détection, les principales difficultés concernent l'insuline lispro et l'insuline humaine, et l'insuline aspart et l'insuline glulisine.
L'insuline humaine et la lispro ont la même masse moléculaire (5807 Da), ce qui se traduit par des temps de rétention et des spectres identiques.
Elles peuvent être distingués via un fragment spécifique pour le lispro qui est m/z 217.
Et pour l'insuline aspart et la glulisine, malgré des masses moléculaires différentes, le spectre est identique. Dans ce cas, le fragment distinctif est m/z 219 appartenant à la glulisine.

4.3. Applications à des cas d'expertises médico-judiciaires

Les overdoses d'insuline exogène peuvent s'avérer fatales en raison de l'hypoglycémie que l'insuline peut provoquer en cas de surdosage chez les patients diabétiques et même à des concentrations thérapeutiques chez les individus sains.
Au cours de mes deux dernières années de thèse, un certain nombre de cas de suicide, de tentative de suicide et de meurtre ont été portés à notre attention.
Une fois la méthode d'identification et de quantification de l'insuline humaine et de ses analogues synthétiques mise au point, elle a été appliquée aux cas suivants.
Trois cas ont donné lieu à des publications scientifiques :

Article 8 : Dans cet article, nous présentons le cas d'une infirmière qui a tenté de tuer sa fille de 10 ans en lui injectant de l'insuline asparte et qui, plus tard, a tenté de se suicider en s'injectant la même hormone. Deux seringues vides et un stylo FIASP® Flextouch ont été trouvés dans l'appartement de la femme. Une méthode de chromatographie liquide couplée à la spectrométrie de masse à haute résolution (LC-HRMS) a été mise au point afin d'identifier et de distinguer l'insuline asparte de l'insuline humaine dans les échantillons de sang ainsi que dans les seringues et le stylo, tandis qu'une méthode de chromatographie liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS) a été mise au point pour la quantification de l'insuline dans les échantillons de sang. L'insuline asparte a été testée positive à 5,7 et 2,4 ng/mL dans les échantillons de sang de la mère et de l'enfant, respectivement. La substance contenue dans les seringues et le stylo correspondait également à de l'insuline asparte. Bien

que la mère affirme avoir injecté une surdose d'insuline asparte, les concentrations trouvées se situaient dans la fourchette thérapeutique pour des sujets sous traitement. En raison de la grande instabilité de l'insuline et du long délai écoulé entre le prélèvement et l'analyse médico-légale (8 mois) pour des raisons administratives, la concentration au moment du prélèvement était probablement beaucoup plus élevée. Dans ce cas, il a été possible d'identifier l'insuline asparte et de la discriminer de l'insuline humaine dans un contexte de tentative de meurtre puis de tentative de suicide en utilisant la spectrométrie de masse à haute résolution, ce qui est d'une importance capitale en médecine légale.

Article 9 : L'insuline glargine est un analogue de l'insuline à action prolongée qui exerce son activité hypoglycémiante par l'intermédiaire de son principal métabolite M1 (21A-Gly-insuline), produit à la suite du clivage enzymatique de la paire d'arginine de la chaîne β.
Dans tous les cas de surdosage décrits dans la littérature, seules les concentrations de M1 ont été rapportées, alors que l'insuline glargine était toujours absente ou en dessous de la limite de quantification.
Dans cette étude, nous avons décrit un cas de suicide d'une jeune infirmière par injection d'insuline glargine dans lequel la molécule mère a été retrouvée à une concentration toxique dans le sang.
La détermination et la discrimination de l'insuline glargine de l'insuline humaine et d'autres analogues synthétiques dans l'échantillon sanguin ont été effectuées par chromatographie liquide couplée à la spectrométrie de masse à haute résolution (Waters XEVO G2-XS QToF). L'insuline glargine a été testée hautement positive dans le sang avec une concentration de 1,06 mg/L. En raison de la difficulté d'obtenir un standard pur de M1, les aires de M1 et d'insuline glargine ont été comparées, révélant une aire de métabolite environ 15 fois plus grande que celle de l'insuline glargine. Cette présence unique de la molécule mère, rapportée pour la première fois, peut être expliquée par la variabilité interindividuelle du taux de conversion en métabolite. L'injection intraveineuse versus l'injection sous-cutanée peut également expliquer la présence de l'insuline glargine. Enfin, la dose injectée peut avoir été si élevée au point de provoquer une saturation des enzymes protéolytiques responsables de la conversion en M1.

<u>Application à un cas de suicide à l'insuline asparte et étude de stabilité :</u>

Dans un cas de suicide, nous avons eu la possibilité d'analyser du sang fémoral collecté dans deux types de tubes différents (un contenant du NaF et un autre sec). La constatation de concentrations différentes dans les deux tubes m'a conduit à tester la stabilité de l'insuline asparte dans des tubes contenant différents types de conservateurs. Ce cas et l'étude qui a suivi ont donné lieu à une publication scientifique :

Article 10 :

Dans cet article, nous avons décrit un cas de suicide d'un homme de 64 ans qui est décédé après l'injection d'insuline aspart. Il souffrait d'un cancer du poumon en phase terminale et a laissé une lettre expliquant les raisons de son suicide. Quatre stylos Novorapid® vides ont été retrouvés près du corps. L'examen externe du cadavre était sans particularité et du sang fémoral a été prélevé dans 2 tubes Vacutainer secs (bouchon rouge) et 2 tubes de fluorure de sodium (bouchon gris).

Une méthode LC-HRMS a été utilisée pour identifier et discriminer l'insuline asparte de l'insuline humaine . L'insuline asparte a été testée positive à 36 et 37 ng/mL dans les tubes secs, et à 58 et 71 ng/mL dans les tubes contenant du fluorure de sodium après immunopurification dans les échantillons de sang et dans les stylos. La recherche de l'insuline asparte dans le sang a été effectué environ 3 semaines après le prélèvement des échantillons. Le contenu des stylos correspondait également à l'insuline asparte.

La stabilité de l'insuline dans le sang est un point critique dans l'interprétation des concentrations en raison de leur diminution rapide causée par l'activité des protéases dans le sang. Lors d'une étude de dégradation mise en place pour comparer 3 conservateurs et des tubes secs, une stabilité convenable de l'insuline asparte a été observée avec l'EDTA et le NaF. Étant donné que le NaF est un standard en toxicologie médico-légale pour la mesure des concentrations d'alcool dans le sang, les auteurs suggèrent son utilisation pour les prélèvements sanguins en cas de suspicion d'intoxication à l'insuline.

Article 8: Attempted murder of a young child followed by an attempted suicide of the mother by injection of insulin aspart: identification and quantification of insulin by LC-HRMS and UPLC-MS/MS in blood of the two cases

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Case Report

Attempted Murder of a Young Child Followed by an Attempted Suicide of the Mother by Injection of Insulin Aspart: Identification of Quantification of Insulin by LC–HRMS and UPLC–MS-MS in Blood of the Two Cases

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Abstract

The identification and quantification of insulin and its analogs have always been a challenge in the forensic field. Murder, suicide attempts and induced hypoglycemia in the context of factitious disorders have been described with the use of synthetic analogs of human insulin. There is very less information in the literature about aspart insulin concentrations in overdose cases. In this paper, we present a case of a nurse who tried to murder her 10-year-old daughter by injecting her aspart insulin and who, later, tried to commit suicide by injecting herself the same hormone. Two empty syringes and a FIASP® Flextouch pen were found in the woman's apartment. A liquid chromatography coupled to high resolution mass spectrometry (LC–HRMS) method was developed in order to identify and discriminate aspart insulin from human insulin in blood samples as well as in syringes and pen, while an liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) method was developed for the quantification of insulin in blood samples. Aspart insulin tested positive at 5.7 and 2.4 ng/mL in the blood specimens of the mother and the child, respectively. The substance contained in the syringes and pen also corresponded to aspart insulin. Although the mother claims to have injected an overdose of aspart insulin, the concentrations found were in the therapeutic range for subjects under therapy. Due to the high instability of insulin and the long time elapsed between sampling and forensic analysis (8 months) due to administrative reasons, the concentration at the time of collection was probably much higher. In this case, it was possible to identify aspart insulin and discriminate it from human insulin in a context of attempted murder and subsequent attempted suicide using high-resolution mass spectrometry, which is of paramount importance in forensic medicine.

Introduction

Aspart insulin (Novolog®) is a synthetic analog of insulin, which belongs to the class of fast-acting insulin (such as lispro and glulisine) indicated in the treatment of type I and type II diabetes. This type of insulin is generally administered subcutaneously shortly before meals as it is designed to accurately mimic physiological insulin secretion following a post-prandial blood glucose rise. Since insulin is stored as a hexamer but the active form is the monomer, the amino acid sequence of aspart has been modified by replacing the amino acid proline in position 28 of the β-chain, with aspartic acid. This structural change leads to a reduction in the interaction forces between dimers and a more rapid formation of monomers following subcutaneous injection, resulting in a more rapid onset of action. In order to improve the pharmacokinetic and pharmacodynamic profile, aspart insulin faster FIASP® was developed.

FIASP® insulin is a formulation marketed either in the form of a pre-filled pen (FlexTouch™) that allows injections from 1 to 80 units or in the form of 100-IU/mL bottles. Two excipients, nicotinamide (vitamin B3) and arginine, have been added to increase the monomeric fraction by ∼35% compared with classic aspart insulin, which promotes faster absorption after subcutaneous injection. In fact, FIASP® insulin appears in the blood after ∼4 minutes, which is twice as fast as classical aspart insulin (1).

Although the discovery and synthesis of synthetic insulins has saved many lives over the years, its misuse has been fairly described, as insulin intoxications are a serious health problem. Since 1922, when insulin was first isolated from the pancreas, various non-therapeutic uses of this hormone have been described, ranging from its deviation for doping purposes in sports (particularly bodybuilding) (2, 3) to its use for criminal intentions of murder and/or suicide (4). The toxic effects of insulin are related to the state of hypoglycemia it causes, which induces neurological damage to the point of becoming even fatal. Although it is clear that hypoglycemia can lead to death, the exact mechanism remains a mystery.

Due to the elusive pathological changes, the lack of morphological evidence and the lack of specific analytical methods routinely used in forensic toxicology laboratories, it is difficult to diagnose insulin intoxication. Neuroanatomical changes generally occur if the state of hypoglycemia lasts for more than 12 hours. Since the time of the psychiatrist Manfred Sakel,

who tried to cure certain mental diseases by inducing repeated states of hypoglycemia for more than 6 hours (5), it has been well recognized that hypoglycemia can be tolerated for many hours without causing permanent brain damage or death. In addition to the absence of pathological changes, there is limited morphological evidence, due to the use of fine needles that make it quite impossible to find the injection site (6).

All these factors have led over the years to underestimate the cases of hypoglycemia caused by voluntary insulin administration. Cases of insulin intoxication are generally not accidental but, in 90% of cases, are suicide attempts and seem to be more common among people who are familiar with the effects of this molecule: diabetics, people closely related to diabetics or health professionals. In fact, the percentage of accidental cases is mainly related to administration errors in hospitals (4). Diagnosis at a medico-legal level is therefore fairly complicated. As hypoglycemia can be tolerated for several hours before hypoglycemic syndrome is produced or death occurs, depending on the type of insulin used, the blood concentration may be very low when this specimen is collected. In addition, insulin misuse is difficult to diagnose because of the concomitant endogenous production by the pancreas.

Over time, the commonly used analytical methods for insulin assessment have been radioimmunoassay and enzyme immunoassay techniques, which have some drawbacks, particularly with regard to their specificity (7–9). These methods have the disadvantage of cross-reacting with other molecules such as insulin analogs, C-peptide, proinsulin and insulin metabolites. Furthermore, they tend to give a measure of total insulin activity rather than a specific concentration (10).

Given that the concentrations to be measured are often very low, it seems important to use very specific and sensitive analytical methods in order to be able to find even traces of insulin in blood samples. In recent years, due to the rising incidence of diabetes and the wider use of exogenous insulin, cases of its inappropriate use are increasing for doping purposes and for criminal purposes, for example, in the context of factitious disorders. In 2005, Giurgea et al. (11) reported a case of a nurse mother suffering from Munchausen by proxy syndrome who repeatedly induced hypoglycemic episodes in her 8-year-old daughter by parenteral insulin injection. Unable to determine the origin of the hyperinsulinemia, a partial pancreatectomy was performed. In another case (12), a diabetic mother, with the same psychiatric problem, injected her own insulin to her 6-year-old child while he was in hospital. Despite the increase in the misuse of this hormone, there are not so many cases described in the literature. In addition, this hormone is not detectable with the standard toxicological screening(s) usually performed in forensic laboratories. For this reason, administration is rarely known, and clinical signs are rarely described, pointing the importance of anamnestic assessment and circumstantial elements. It seems necessary, in order to prove voluntary intoxication, to identify and quantify the concerned insulin by specific and sensitive analytical methods.

Aspart insulin misuse has been described in some cases of suicide attempts (13, 14). However, insulin has been quantified by specific methods such as mass spectrometry in only two cases (15). Due to the high instability of insulin in blood samples and the long time period between collection and analysis in forensic practice, insulin is rarely identified and quantified. Therefore, not much information is available on insulin concentrations following severe hypoglycemia induced by exogenous insulin administration.

In the present study, the authors developed a LC–HRMS and a LC–MS-MS method for the detection and quantitation of aspart insulin in human blood specimens obtained from two cases of insulin intoxication in a context of attempted murder followed by an attempted suicide.

Case Report

A 48-year-old woman, with a history of depression, attempted to murder her 10-year-old daughter by administering her an unknown amount of aspart insulin (NovoRapid®). During police investigations, she indicated that she has attempted to commit suicide by injecting the same type of insulin. She confessed having done that quite immediately after injecting her daughter. Mother and daughter were not diabetics, but as a nurse, the mother was in possession of insulin. The woman reported that, repented of her action, she gave some pieces of sugar to the child and she called the Emergency Services. Upon the arrival at the emergency department of the local hospital, ~4 hours after insulin injection, the two subjects had normal vital signs and the mother's blood glucose was at 89 mg/dL. However, there is no information about the child's blood glucose level. In their apartment, two empty syringes and an insulin pen FIASP® FlexTouch were found. Later, these items were confiscated and sent to the forensic toxicology laboratory to analyze the contents. Blood samples were collected from the child (Vacutainer™ tube with a gray top) and the mother (Vacutainer™ tube with a green top) in the hospital and transferred to the laboratory for extensive toxicological screening. The samples were stored at +4°C until analysis, without centrifugation.

Materials and Methods

Chemicals and reagents

Human, aspart, lispro, glargine, detemir, bovine insulin and zinc sulfate were supplied by Merck (Saint Quentin Fallavier, France). Methanol, acetonitrile, glacial acetic acid and ammonia solution 28% for high performance liquid chromatography (HPLC) isocratic grade were obtained from V.W.R. Chemicals ProLabo (Fontenay-Sous-Bois, France). Formic acid for liquid chromatography coupled to mass spectrometry (LC–MS) analysis was obtained from Carlo Erba (Chaussée du Vexin, France). Solid-phase extraction cartridges OASIS MAX (60 mg, 3 mL) were supplied by Waters (Eschborn, Germany). For all aqueous buffers and solutions, ultrapure water was used.

Toxicological screening

A complete toxicological screening was performed on the blood samples of the two cases.

First of all, an immunoenzymatic screening test was performed using a NEOGEN® ELISA kit (Lansing, MI, USA) for the detection of a wide range of drugs and metabolites in blood.

Blood samples were tested for ethyl alcohol by gas chromatography, following injection into a head-space system and specific detection by flame ionization (HS-GC–FID, Perkin Elmer, Waltham, MA, USA).

A liquid chromatography method coupled to a diode array detector (UPLC–DAD, Waters Corporation, Milford, MA, USA) was used to test for pharmaceuticals (about 400 compounds).

Two other screenings were performed to test for narcotics and new psychoactive substances using a liquid chromatography method coupled with tandem mass spectrometry (UPLC–MS-MS Xevo TQD, Waters Corporation, Milford, MA, USA).

Insulin analysis

Syringes and pen analysis

In order to identify aspart insulin in the empty syringes and pen that were seized, LC–HRMS was used. After rinsing the syringes and the pen with 200 µL of 2% acetic acid solution, 10 µL was injected into the LC–HRMS system.

Blood analysis

Sample preparation

A 6-point calibration curve was prepared using the following concentrations: 0, 1, 5, 10, 50 and 100 ng/mL of aspart insulin in blood.

After mixing 1 mL of whole blood with 1 mL of aqueous 0.1 M $ZnSO_4$, the sample was subjected to further protein precipitation with 2 mL of a mix of acetonitrile/methanol + 1% of formic acid in the presence of 20 ng/mL of bovine insulin as the internal standard. After agitation and centrifugation, the supernatant was transferred to another tube. To this tube, 2 mL of 5% ammonia solution was added and vortexed. Solid-phase extraction was performed with mixed anion-exchange cartridges (Waters MAX, 3 mL, 60 mg), which were preconditioned with 1 mL of methanol and 1 mL of water prior to loading the supernatant. The samples were washed with 1 mL of 5% ammonia solution and 1 mL of methanol/acetic acid (60:10). Finally, the sample was eluted to a vial with 250 µL of a mixture of methanol/acetic acid (5:1) and 5 µL was injected into the LC–HRMS system, while in the LC–MS-MS, 10 µL was injected.

Validation procedure

The blood test method was validated for linearity, repeatability, reproducibility and detection and quantitation limits according to the SFTA (Societé Française de Toxicologie Analytique) guidelines (16).

Three calibration curves, which included 6 points (concentrations ranging from 1 to 100 ng/mL) and obtained over a 3-day period, were established for the study of linearity.

Quantification was achieved by plotting the peak area ratios of aspart to bovine insulin versus concentration followed by linear regression analysis. Repeatability and reproducibility were determined for two quality control (QC) levels, 1 and 10 ng/mL. For repeatability, six replicates of each QC level were processed the same day. For reproducibility, each QC level was processed three times for three different days over a period of 2 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 target and qualifier transitions. The limit of quantification (LOQ) was defined as the first point of the calibration curve.

LC–HRMS conditions

In order to identify aspart insulin in the blood samples, in the syringes and in the pen and to distinguish it from other insulin analogs, a LC–HRMS system was used.

LC separation was achieved using a Waters Acquity CORTECS C18+ column (150 × 2.1 × 1.6 µm) with a controlled temperature maintained at 50°C. A 5-µL injection with a 0.25 mL/minute flow of water with 0.1% of formic acid (solvent A) and acetonitrile with 0.1% (solvent B) of formic acid was used. The gradient elution was as follows: the initial 20% B was increased to 65% over 5.4 minutes, 65% to 98% over 0.3 minutes and returned to initial conditions over 4.2 minutes. The total run time was 9.9 minutes, and aspart insulin was eluted at 2.77 minutes.

A Xevo G2-XS Q-TOF high-resolution mass spectrometer (Waters Corporation, Milford, MA, USA) was used, operating in positive ion mode and in sensitivity mode.

Desolvation gas flow was set at 1,000 L/h at a temperature of 600°C, the cone gas to 50 L/h and the source temperature set to 120°C. The capillary voltage and the cone voltage were set to 3 and 15 V, respectively. In MS scanning, data were acquired from 100 to 2,000 *m/z*. Collision energy ranged from 20 to 80 V. HRMS parameters were optimized by infusing 1 mg/mL solution of the analytes into the ion source and manually optimizing the parameters. In addition to aspart, the presence of other analogs (lispro, glargine, detemir and glargine) and human insulin were tested. For aspart insulin, the 5-fold charged molecule was observed at *m/z* 1,165.94242 (which deconvolutes to 5,824.7121), while for human and bovine insulin, the 5-fold charged ions were observed at *m/z* 1,162.34172 and 1,147.53228, respectively. In addition, the 6-fold charged 971.95242 and 968.79023 were used to discriminate aspart from human insulin. Figure 1 shows the chromatograms and spectral information for the other insulin analogs included in the method.

UNIFI software was used for data, chromatograms and spectra acquisition.

LC–MS-MS conditions

In order to quantify aspart insulin in blood samples, given the higher sensitivity of the triple quadrupole system when compared to high-resolution mass spectrometry, a LC–MS-MS system was used.

Chromatographic separation was performed with the same equipment and parameters of the ones of the LC–HRMS system, except that the volume injected into this system was 10 µL.

A Xevo TQS micro triple-quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) provided with a Z-spray electrospray ionization source (ESI) used in the positive ionization mode (ESI+) was used for the analysis of the compounds.

The following conditions were found to be optimal for the analysis of aspart insulin: 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 1,000 L/h. The cone voltage and collision energy were adjusted to

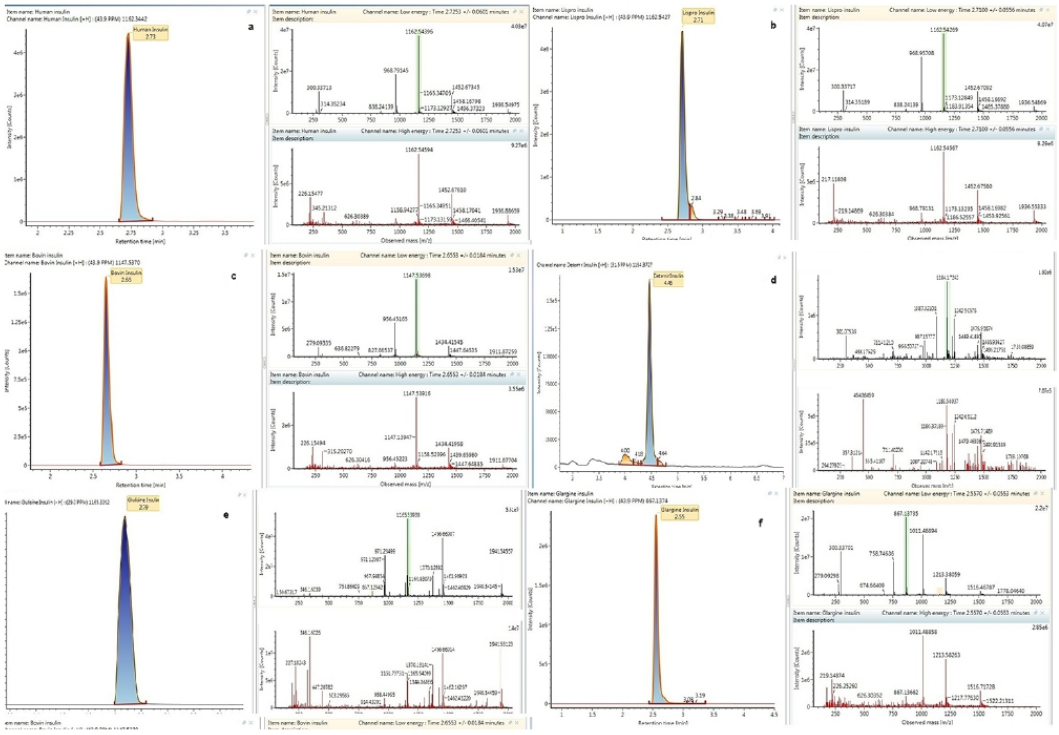

Figure 1. Spectral data information for insulin analogs by liquid chromatography coupled to high-resolution mass spectrometry (LC–HRMS). (a) Human insulin: *m/z* $[M+5H]^{5+}$ 1162.54396; (b) lispro insulin: *m/z* $[M+5H]^{5+}$ 1162.54265; (c) bovine insulin: *m/z* $[M+5H]^{5+}$ 1147.53698; (d) detemir insulin: *m/z* $[M+5H]^{5+}$ 1184.17242; (e) glulisine insulin: *m/z* $[M+5H]^{5+}$ 1165.53988; (f) glargine insulin: *m/z* $[M+7H]^{7+}$ 867.13735.

maximize the intensity of the protonated ion and to optimize the signal of the 2 most abundant ion products of aspart insulin: *m/z* 1,166.33 > 110.12 and *m/z* 120.13, and for the internal standard bovine insulin 1,147.57 > 136.00. Transition 1,166.33 > 110.12 was used for the quantification of aspart insulin. MassLynx 4.1 software was used for quantification.

Results and Discussion

Syringes and FlexTouch pen analyses by LC–HRMS confirmed the presence of aspart insulin in both products. Figure 2 shows the chromatograms obtained after injection of the syringes and pen's solutions.

For the method validation, the 6-point calibration curve showed a good linearity in the range 0–100 ng/mL with a correlation coefficient ranging from 0.9994 to 0.9999 during three tests. The LOD and the lower LOQ are 0.5 and 1 ng/mL, respectively. Repeatability was 18.5% and 10.2% at 1 and 10 ng/mL, respectively, and reproducibility was 17.2% and 8.3% at 1 and 10 ng/mL, respectively.

Mother's and daughter's blood specimens also tested positive for aspart insulin with concentrations of 2.4 and 5.7 ng/mL, respectively. Figure 3 shows the chromatograms obtained after the extraction of mother's and daughter's blood specimens. The chromatogram obtained after the extraction of a blank blood sample shows the absence of interference at aspart insulin retention time (Figure 4).

Further analysis, achieved during a complete toxicological screening, revealed the presence of ethanol (1.72 g/L), bromazepam (1.60 mg/L) and venlafaxine (265 ng/mL) in the blood collected from the mother. The simultaneous administration of large quantities of ethanol and bromazepam, an anxiolytic benzodiazepine found at a toxic concentration, could have induced severe impairment of the mother. Venlafaxine, an antidepressant, was at the upper limit of the therapeutic range. In the child's blood, no xenobiotics were identified.

The critical outcome associated with an insulin overdose is severe hypoglycemia. Factitious hypoglycemia induced by the injection of exogenous insulin is quite common. Nevertheless, the cases reported in the literature are uncommon. As a consequence, the total number of authentic cases appears to be underestimated as many cases are classified as accidental hypoglycemia or because they are obscured by a laboratory's inability to identify hypoglycemic drugs.

Therefore, cases of insulin intoxication are generally limited to case reports in which insulin is often not dosed. Only recently, the number of reported cases has increased, probably due to the greater availability of specific analytical methods. In 2004, Tofade et al. (13) described a case of a nondiabetic subject with history of depression who intentionally

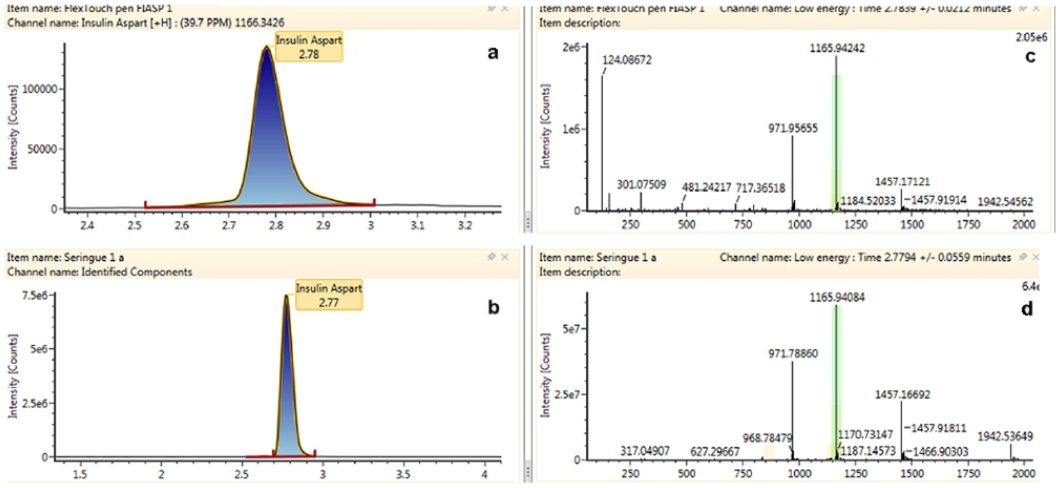

Figure 2. (a, b) Extracted chromatograms for aspart insulin from the pen and syringe solutions (top: pen, bottom: syringe). (c, d) Spectra from 5-fold charged ions of aspart insulin in the pen and syringe solutions (top: pen, bottom: syringe).

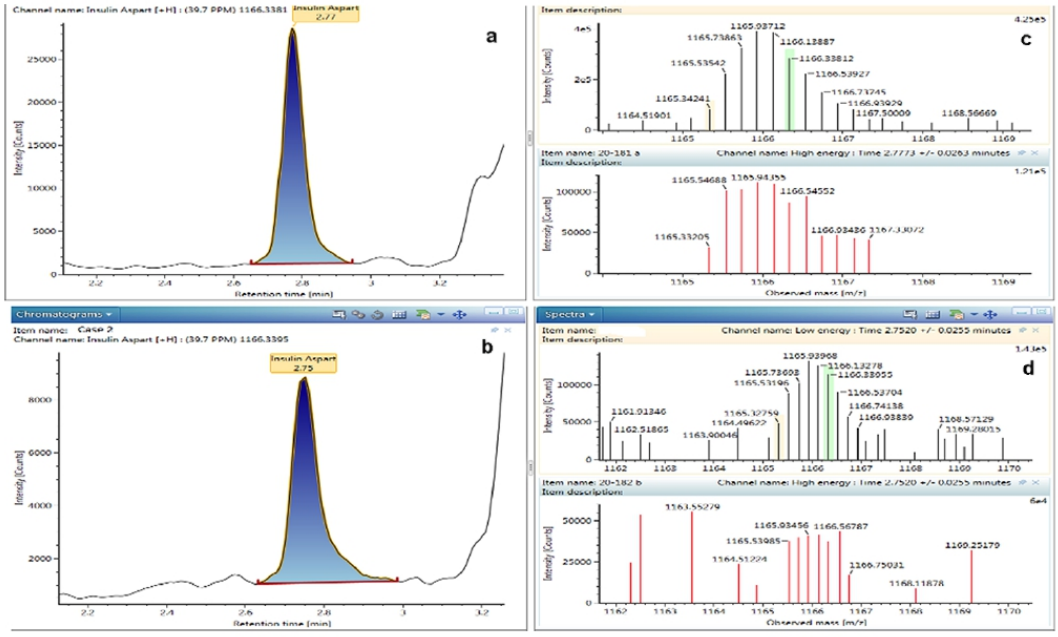

Figure 3. (a, b) Extracted chromatograms for aspart insulin from mother and daughter blood specimens (top: mother, bottom: daughter). (c, d) Spectra from 5-fold charged ions of aspart insulin from mother and daughter blood specimens (top: mother, bottom: daughter). Concentrations were, from top to the bottom, 5.7 and 2.4 ng/mL.

self-administered an overdose of insulin aspart and glargine that belonged to her husband; she was treated with dextrose infusion 15 hours after her suicide attempt and she survived. Dewaal et al. in 2017 (14) presented a case of attempted suicide of a non-diabetic woman who injected 300 units of NovoRapid (brand name of aspart insulin); she survived after a treatment with octreotide 40 minutes after the injection. As in our two cases, subjects were non-diabetic patients. A possible danger when exogenous insulin is administered to a non-diabetic subject (whose pancreas functions are normal) is that, in treating hypoglycemia with sugar solution, there is a risk of incurring to subsequent episodes of hypoglycemia due to the physiological endogenous production of insulin following the stimulus to the increase of blood sugar.

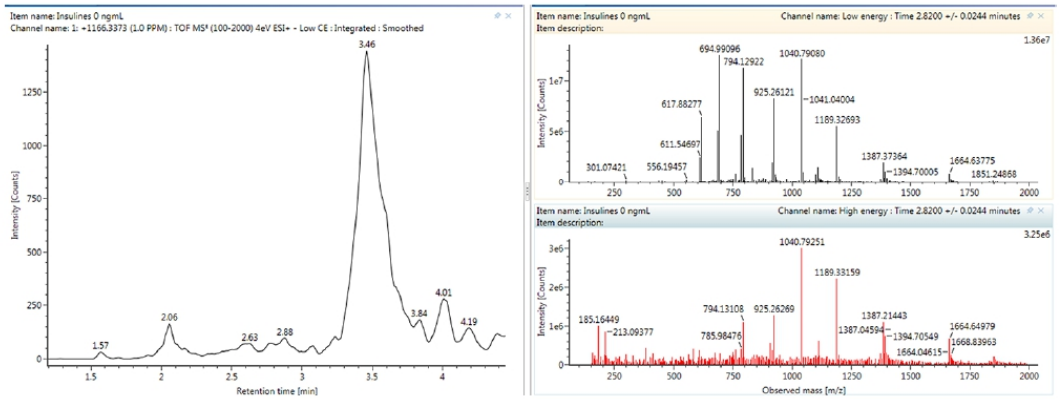

Figure 4. Chromatogram obtained after extraction of blank blood sample.

The reported cases in the medical literature mainly describe the treatment performed after hypoglycemia when the etiology is a possible insulin intoxication. From a clinical point of view, it is important to understand the cause of the patient's illness and to act to treat it in the most efficient way.

In forensic medicine, doubts and ambiguities are not accepted and it is therefore necessary to identify and quantify the type of insulin in question, in order to be able to prove with certainty that the intake of exogenous insulin contributed to intoxication or death.

In most of the published cases in the medical literature, the concept of insulin as the cause of hypoglycemia is confirmed by quite convincing circumstantial evidences, including suicide note, eyewitnesses, presence of empty syringes and/or insulin bottles next to the victim. Nevertheless, the only concrete evidence of exogenous insulin administration remains the identification and quantification of the molecule itself.

It is only since the 1960s that it was possible to document a hypoglycemia due to insulin overdose with the introduction of radioimmunological tests and immunoassays that allowed to test for insulin and C-peptide levels.

Thevis et al. in 2012 (17) described a case of a non-diabetic woman who died from an insulin overdose. Insulin was determined with immunoassays at 194 ng/mL, while C-peptide concentration was in a normal range (1.7–4.8 ng/mL). Beastall et al. in 2016 (18) reported the death of a non-diabetic woman, who had a history of alcohol and drug abuse, after self-administration of insulin. Insulin was quantified with a radioimmunoassay test at 4.8 ng/mL.

In 1992, Patel et al. (19) provided a table with normal (0.2–3 ng/mL) and fatal insulin concentrations (3.2–129 ng/mL). More recently, Sato et al. (20) reported a case of attempted suicide of a non-diabetic subject who self-injected 600 units of aspart insulin (NovoRapid). Following the detection and quantification of insulin by immunoassays, aspart insulin was at 62.9 ng/mL 4 hours after the injection. Although the development of both radioimmunoassay and enzyme linked immunosorbent assay (ELISA) assays has enabled the rapid quantitation of insulins, these assays also possess some disadvantages, most notably in regard to their specificity.

These tests are not very specific, and therefore, cross-reactivity with insulin precursors and degradation products is quite common. Furthermore, they tend to give a measure of total insulin activity rather than an individual measure of concentration (10).

Considering the very low insulin concentrations to be determined, an analytical method such as a liquid chromatography coupled to mass spectrometry seems necessary.

Unlike immunological methods, mass spectrometry allows to differentiate endogenous human insulin from its synthetic analogs, each of which has a different aminoacidic sequence that results in a different dissociation pattern after activation of the collision.

Aspart insulin has been used, identified and quantified in two documented suicide attempts. In a case described by Kim et al. in 2016 (15), aspart insulin was measured by LC–MS-MS at 76.46 ng/mL, 7 hours after the injection. The same year, Sunderland et al. (21) reported a case of suicide with aspart insulin, which was determined by an immunocapture LC–MS-MS assay at 161.8 ng/mL.

The therapeutic reference range for NovoRapid aspart insulin has not yet been identified, but according to the European Medicines Agency (22), when aspart insulin is administered at a therapeutic concentration of 0.15 unit/kg, the maximum plasma concentration is 4.4 ng/mL after 40 minutes from the subcutaneous injection. In our two cases, although the mother claims to have injected an overdose of aspart insulin, the concentrations found (2.4 and 5.7 ng/mL) were close to this 'therapeutic' concentration.

Insulin levels reported in the literature, in fatal and non-fatal cases, are highly variable. The variations in the values depend mainly on the time between injection and sampling and the time between sampling and analysis.

Aspart insulin NovoRapid has a duration of action from 1 to 3 hours and plasma levels measurable for up to 6 hours after a therapeutic dose injection. Considering that the time elapsed between the injection and the collection of the blood sample by the emergency staff was approximately 4 hours, the concentration at the time of collection was far from the peak concentration.

It should also be said that insulin determination in blood samples is influenced by the high chemical instability of insulin in blood samples. It has been published that spiking insulin in serum does not produce a decrease in insulin concentration,

while spiking it in whole blood results in a 20% loss of the initial concentration after 5 hours at 37°C (23).

Rapid separation of serum from whole blood and storage at –20°C has always been recommended for blood samples planed for insulin analysis in order to limit loss, but in forensic practice, it is not always possible. Since insulin degradation is mainly due to the activity of enzymes proteolytic released from the cells following hemolysis, storage at low temperature would slow down the activity of these insulinases (24). In our cases, blood samples were not centrifuged and were stored at +4°C for a period of 8 months. Almost certainly, the concentration at the time of sampling was higher. This long, unusual delay, was due to administrative reasons and the time necessary to develop the analytical strategy.

Due to the high instability of insulin and the different delays between blood collection and analysis, the few values reported in the literature in cases of insulin overdoses vary over a wide range. Therefore, the concentrations found by the authors are not comparable with what has been reported in the literature. Insulin analysis is a special assay not routinely performed by toxicology laboratories. When exogenous insulin administration is suspected, immunoassays are used in most laboratories. In forensic medicine, the delay between the collection of the sample and analysis is often very long, and therefore, identified concentrations are often very low. In addition, there are many insulin analogs available in the market, all with a very similar structure and molecular weight. Therefore, very specific and sensitive methods are required. In the literature, most of the HRMS methods for the identification of insulin analogs have not been applied to real cases of intoxication. In this work, the authors have chosen to identify aspart insulin and thus distinguish it from all other analogs by LC–HRMS and to quantify it by LC–MS-MS. Although the quantification is also possible using HRMS alone, the latter is preferred for applications requiring specificity between insulins of the same mass, such as aspart insulin and glulisine insulin, where a high-resolution mass spectrometer is required to distinguish the isotopic envelope. Despite the specificity of an HRMS, its sensitivity does not exceed that of a triple quadrupole.

In this case report, a specific LC–HRMS method was developed and applied to two cases of aspart insulin intoxication in the context of an attempted murder, after 8 months of blood storage at +4°C. Since the two subjects were not diabetics, the mere presence of insulin of exogenous origin is an evidence of its administration.

Conclusion

Insulin is one of the most widely prescribed hypoglycemic molecules. Although its use in the clinical practice is targeted at the treatment of diabetes mellitus, its use in a forensic context has already been described. Due to the lack of specific analytical methods, the number of deliberate intoxications with insulin is probably underestimated. It seems necessary that a forensic toxicology laboratory should own highly specific and sensitive analytical methods to be able to discriminate between the various insulin analogs available in the market. The development of an analytical method using high-resolution mass spectrometry and tandem mass spectrometry technologies enabled to identify aspart insulin and to distinguish it from human insulin in the context of an attempted murder followed by a suicide attempt.

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Article 9: Intentional overdose of glargine insulin: first determination of the parent compound in postmortem blood by LC-HRMS

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Intentional overdose of glargine insulin: first determination of the parent compound in postmortem blood by LC-HRMS

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Abstract

Insulin glargine is a long-acting insulin analogue which is converted after enzymatic cleavage of the arginine pair of the β- chain into its main metabolite M1 (21^{A}-Gly-insulin), which is responsible for the hypoglycaemic activity.

In all the overdose cases described in the literature, only M1 concentrations have been reported, whereas insulin glargine was always absent or below the limit of quantitation.

In this study, we present a case of suicide of a young nurse by injection of insulin glargine in which the parent molecule was found at a toxic concentration in blood.

The determination and the discrimination of insulin glargine from human insulin and other synthetic analogs in the blood specimen was performed by liquid chromatography coupled to high resolution mass spectrometry (Waters XEVO G2-XS QToF). Glargine insulin tested highly positive in the blood with a concentration of 1.06 mg/L. Due to the difficulty in obtaining a M1 pure standard, the areas of M1 and insulin glargine were compared, revealing a metabolite area approximately 15 times larger than the area of insulin glargine. This unique presence of the parent molecule, reported for the first time, can be explained by inter-individual variability in the rate of conversion to metabolite. Intravenous injection versus subcutaneous injection can also explain the presence of insulin glargine. Finally, the dose injected may have been so high that saturation of the proteolytic enzymes responsible for conversion to M1 should have occurred.

Key words
Insulin glargine, suicide, postmortem blood, LC-HRMS, forensic toxicology, diabetes

Introduction

Insulin glargine (Lantus®) is a long-acting synthetic human insulin used for basal insulin therapy and available for subcutaneous injection as a pre-filled pen in dosages of 100 and 300 units/mL, once a day. It is only intended to be administered subcutaneously, as this is precisely the route of injection that allows its prolonged effect.

The addition of two arginines at the C-terminal position of the β -chain and the replacement of asparagine by glycine at position 21 of the α- chain shifts the isoelectric point of insulin glargine, making it poorly soluble at neutral pH but fully soluble at the acidic pH of the injection solution (pH 4). After injection into the subcutaneous tissue (pH 7.4), the acid solution is neutralised, resulting in the formation of micro-precipitates that represent a "depot" from which small quantities of insulin glargine are continuously released. The addition of zinc to the preparation further stabilises the product by delaying the release of insulin glargine monomers and dimers, extending its duration of action. As a result, the concentration/time curve is regular, with no peak, and the duration of its action is prolonged [1].

After subcutaneous injection of Lantus®, insulin glargine is rapidly degraded at the carboxyl end of the β -chain into two active metabolites, M1 (21^{A}-Gly-insulin) and M2 (21^{A}-Gly-des-30^{B}-Thr-insulin). In plasma, the major circulating metabolite is the M1 metabolite and its concentration increases with the the administered dose of Lantus®. The effects of subcutaneous injection of Lantus® are mainly due to exposure to the M1 metabolite, as insulin glargine and the M2 metabolite are almost never detectable after subcutaneous injection of insulin glargine [2].

Insulin overdoses are under-reported in the literature despite the large number of diabetics using this therapy. Indeed, diabetes is one of the most common chronic diseases in the world and in 2020, about 3.5 million people in France were treated with antidiabetic drugs, which represents 5.3% of the population [3]. As the prevalence of diabetes increases, cases of insulin misuse and overdose are also increasing. Compared to the cases described in the 20th century where less than 5% of cases were suicide cases [4], in the last 20 years, 90% of insulin glargine overdoses were suicide attempts [5-9, 13-16],

Although at therapeutic doses insulin glargine pharmacokinetics is well described, there is a lack of information in overdose situations due to the limited data on the literature.

In the cases described in the literature over the last 20 years, insulin glargine was seldom identified and quantified. When identification and dosage were reported, immunoassay methods were generally used. In a 2016 study performed by Kim et al. [9] involving insulin glargine overdose, the metabolite M1 was identified and quantified throughout the hypoglycemic state a using a LC-MS/MS technology. However, the authors did not detect the parent molecule.

In order to increase the specificity and sensitivity of the method and to avoid any interference with other analogues, it is suitable to use separative methods coupled to mass spectrometry or high-resolution mass spectrometry (HRMS) and to avoid immunoassay tests. To date, HRMS has only been used in a study

performed by Yong-Xi Li et al. [10] on the toxicokinetic of insulin glargine in dogs and was never applied to human biological specimens.

In this report, the authors present a case of suicide by insulin glargine overdose with the first identification of the parent molecule using a high-resolution mass spectrometry system (UHPLC/Q-TOF-MS).

Case Report

A 26-year-old woman (nurse by profession) was found dead at home lying on a sofa. The woman was found in her home by a friend after he received a letter mentioning her suicidal intention.

Several syringes corresponding to insulin injection devices were found next to the body, as well as loxapine capsules. According to what has been reported to the investigators, it seems that the young woman had attempted suicide twice before this fatal outcome.

The body examination and the post-mortem body scanner revealed a marked asphyxiation syndrome and nail cyanosis.

A tablet corresponding to a blood glucose sensor located on the right arm suggested that the young woman was diabetic. A venous catheter fixed on the left wrist of the corpse was also found.

As this was an external examination of the body and no autopsy was performed, no histological data are available. In addition, no biochemical tests were performed, in particular blood glucose, lactates and ketone bodies, as this type of analysis was not available given the long delay between autopsy and the request of the Prosecutor. However, by directly investigating the toxic molecule involved, indirect measurements of antemortem blood glucose are not necessary.

As the forensic pathologist was focused on a toxic death, samples of femoral blood, urine and hair were collected for toxicological tests.

Blood and urine were stored at +4° C until analysis, while head hair was stored in an envelope at room temperature.

Material and Methods

Chemicals and reagents

Human, aspart, lispro, glargine, detemir and bovine insulin and zinc sulfate were supplied by Merck (Saint Quentin Fallavier, France).

Methanol, acetonitrile, glacial acetic acid and ammonia solution 28% for high performance liquid chromatography (HPLC) isocratic grade were obtained from V.W.R. Chemicals ProLabo (Fontenay-Sous-Bois, France).

Formic acid for liquid chromatography coupled to mass spectrometry (LC-MS) analysis was obtained from Carlo Erba (Chaussée du Vexin, France). Solid-phase extraction cartridges OASIS MAX (60 mg, 3 mL) were supplied by Waters (Eschborn, Germany). For all aqueous buffers and solutions, ultrapure water was used.

Insulin blood analysis

For insulin blood analysis, a method previously developed and validated by the authors was used [11]. Briefly, 1 mL of whole blood was subjected to protein precipitation in the presence of 20 ng/mL of bovine insulin as the internal standard.
Subsequently, solid-phase extraction was performed with mixed anion-exchange cartridges (Waters MAX, 3 mL 60 mg), in which the sample was eluted to a vial with 250 µL of a mixture of methanol/acetic acid (5:1) and 5 µL was injected into the LC-HRMS.

LC-HRMS conditions

LC separation was achieved using a Waters Acquity HSS C18 column (150 x 2.1 x 1.8 µm) with a controlled temperature maintained at 50 °C. A 5- µL injection with a 0.25 mL/min flow of waters with 0.1 % of formic acid (solvent A) and acetonitrile with 0.1 % of formic acid (solvent B) was used. The gradient elution was as follows: the initial 20% B was increased to 65% over 5.4 minutes, 65 to 98% over 0.3 minutes and returned to initial conditions over 4.2 minutes. The total run time was 9.9 minutes, and glargine insulin was eluted at 2.69 minutes.
A Xevo G2-XS Q-TOF high-resolution mass spectrometer (Waters corporation, Milford, MA, USA) was used, operating in positive ion mode and in sensitivity mode.
Desolvation gas flow was set at 1,000 L/h at a temperature of 600 °C, the cone gas to 50 L/h and the source temperature set to 120 °C. The capillary voltage and the cone voltage were set to 3 and 15 V, respectively. In MS scanning, data were acquired from 100 to 2,000 m/z. Collision energy ranged from 20 to 80 eV. HRMS parameters were optimized by infusing 1 mg/mL solution of the analytes into the ion source and manually optimizing the parameters. In addition to glargine, the presence of other analogs (lispro, aspart, glulisine and detemir) and human insulin were tested. For glargine insulin, the 7-fold charged ion was observed at *m/z* 867,46288 (which deconvolutes to 6072,24016), while for human and bovine insulin, the 5-fold charged ions were observed at *m/z* 1,162.34172 and 1,147.53228, respectively. UNIFI software was used for data, chromatograms and spectra acquisition.

Results and Discussion

The method used for the analysis of the blood sample has been previously validated and published [11]. Insulin glargine was found in the femoral blood at the concentration of 1.06 mg/L. Figure 1 shows the chromatogram after the extraction of the blood specimen.

Further analyses, achieved during a complete toxicological screening, revealed the presence of tiapride (24.95 mg/L), loxapine (0.18 mg/L) and nordiazepam (0.16 mg/L). While tiapride was at a toxic level, loxapine and diazepam were considered at therapeutic levels.

The presence of other drugs (tiapride, loxapine and nordiazepam) may have contributed or aggravated the hypoglycemia caused by insulin glargine. In fact, the use of antipsychotics in diabetic patients has been shown to be associated with a higher risk of hypoglycemia [12].

In the literature, the cases of insulin glargine overdose are limited to case reports in which the drug is often not quantified [5-9, 13-16].

In forensic medicine, the cause of death can be based on circumstantial evidence such as the presence of empty syringes, testimonies, suicide letters or even insulin/peptide C ratio by means of immunoassays (when requested). However, immunoassays are not appropriate for *postmortem* specimens due to the lack of specificity of the antibodies which often fail to distinguish human insulin from its analogues, metabolites and insulins of animal origin.

In the scientific literature, only Kim et al. [9] investigated insulin glargine in an overdose case by mass spectrometry. The authors conducted an analysis of the pharmacokinetics of this type of insulin by establishing insulin levels several times. Only the M1 metabolite of insulin glargine was identified and quantified 7 hours (18 ng/mL), 30 hours (19.5 ng/mL), 54 hours (15.3 ng/mL) and 80 hours (9.6 ng/mL) after injection. The authors observed a multiphasic peak, probably due to the delayed release from the depot or the presence of several injection sites where the absorption rate was different.

Our results for insulin glargine cannot be compared with previous data as the parent compound was never identified after overdose. The therapeutic range for this type of insulin has not yet been defined, but according to a study by Bolli et al. [2] when insulin glargine was subcutaneously administered at a therapeutic dose of 0.3 units/kg, the M1 metabolite, mostly responsible for the glucodynamic effects, was found at a maximum concentration of 470 pg/mL. In this study, insulin glargine was never present in the blood circulation, irrespective of the injected dose. Kim et al. [9] also reported that insulin glargine is never found in circulation above the LOQ, unlike its major metabolite M1, suggesting that the parent molecule is not the main responsible for the hypoglycaemic activity.

Danne et al. [17] again performed a study in 2012 involving a group of children under 6 years of age treated with insulin glargine. They analysed blood samples taken 24 hours after subcutaneous injection of insulin glargine and only identified the M1 metabolite, which was listed as the main component mediating the hypoglycaemic effect. The limitation of this study was that analysis was performed on

specimens collected at +24 hours, and therefore the results do not necessarily reflect the values of the parent molecule at other times.

Since buying the reference standard of the M1 metabolite is very difficult (not proposed by any vendor), it was not possible to quantify it in our case. However, we investigated the M1 metabolite in our sample and the observed mass spectrum was compared with data in the literature. We have identified a peak whose spectrum corresponds to the 5-fold ion charged *m/z* 1150.3317 described in the paper of Yong-Xi et al [10]. In our case the 5-fold charged ion was observed at *m/z* 1150.89968. A comparison of the sub-peak areas of the parent molecule and the possible metabolite shows that the latter would be present at 15 times the concentration of the parent molecule. The M2 metabolite was also investigated by comparing the mass spectrum with the literature data, but it wasn't found. This is in agreement with the study published by Bolli et al. [2].

In order to be able to explain our findings, various hypotheses can be elaborated. First of all, according to the study by Bolli et al. [2] insulin glargine, when detectable, appears very early in blood. Therefore, one possibility could be that the time between injection and death was very short. As a hypoglycaemic syndrome can last for many hours before causing death, this hypothesis is not plausible. In another study conducted by Agin et al. in 2007 [18], it was shown that insulin kinetic shows some inter-individual variability which affects the rate of transformation of insulin glargine into M1 metabolite. This variability could be explained by genetic differences in the activity of the carboxypeptidases responsible for its metabolism. Indeed, in a study carried out on 69 individuals, the rate of conversion of glargine to M1 in 30 minutes varied between 46 and 98 %.

Another hypothesis is that the injection route in our case was not the standard subcutaneous administration route, as this is the unique fatal case of intravenous insulin glargine overdose described in the literature. It is possible that the metabolism of insulin glargine is different when injected intravenously. In a study by Werner et al. [19], the kinetics of insulin glargine was compared after intravenous and subcutaneous injection. When injected intravenously, insulin glargine accounts for 15-37 % of total circulating insulin while when injected subcutaneously, the parent molecule was almost never found. Thornton et al. in 2012 [20] presented a case of a patient accidentally receiving 100 units of insulin glargine intravenously instead of subcutaneously. As the long-lasting properties of insulin glargine are due to its absorption kinetic, intravenously it acts like regular human insulin. In fact, the patient had an unremarkable course and recovered without any parenteral glucose. Insulin glargine dosage was not performed in this case.

Finally, as proposed by Werner et al. [19], since insulin glargine was injected at very high dosage, its presence can be explained by a possible saturation of the proteolytic enzymes responsible for the conversion of insulin glargine into its metabolite. This phenomenon was also observed in a study published by Lucidi et al. [21]. The authors evaluated the metabolism of insulin glargine in diabetic patients, observing that while the parent molecule was not detectable after a therapeutic dose injection,

insulin glargine could be found after a slight overdose. These results suggest that at high dosages there is a greater opportunity to find the parent molecule.
As no injection site was found in our case but only a venous catheter at the left wrist (not submitted to analysis), the possibility of an intravenous administration cannot be eliminated.

Conclusion

In this article, the authors have presented a case of insulin glargine overdose. Insulin glargine has never been found in *postmortem* blood samples due to its rapid transformation into its major metabolite. Its presence at a toxic concentration could probably be due to the use of a route of administration different from the classic subcutaneous way, such as the intravenous route. The metabolism of this substance could indeed be different when injected intravenously. As this represents the first case of a death reported in the literature with such high insulin glargine concentration in blood, it will be necessary to document further cases to confirm the hypotheses.

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Figure 1. Extracted chromatogram and spectra for insulin glargine from blood specimen by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS). Insuline glargine: *m/z* $[M+7]^{7+}$ 867.46288. Concentration was 1.06 mg/L.

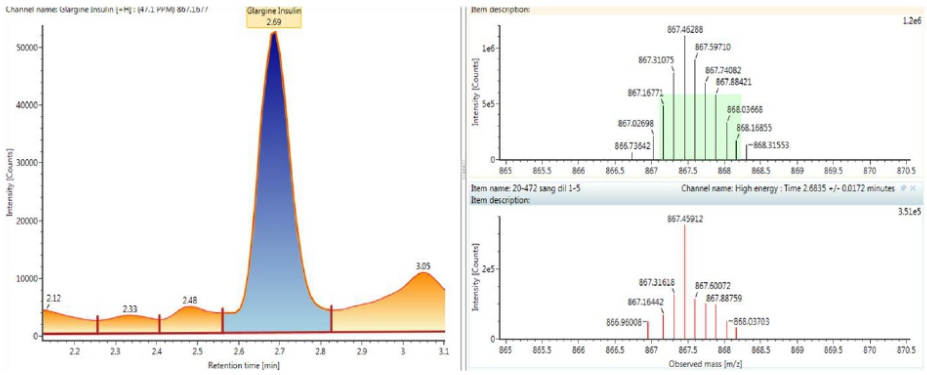
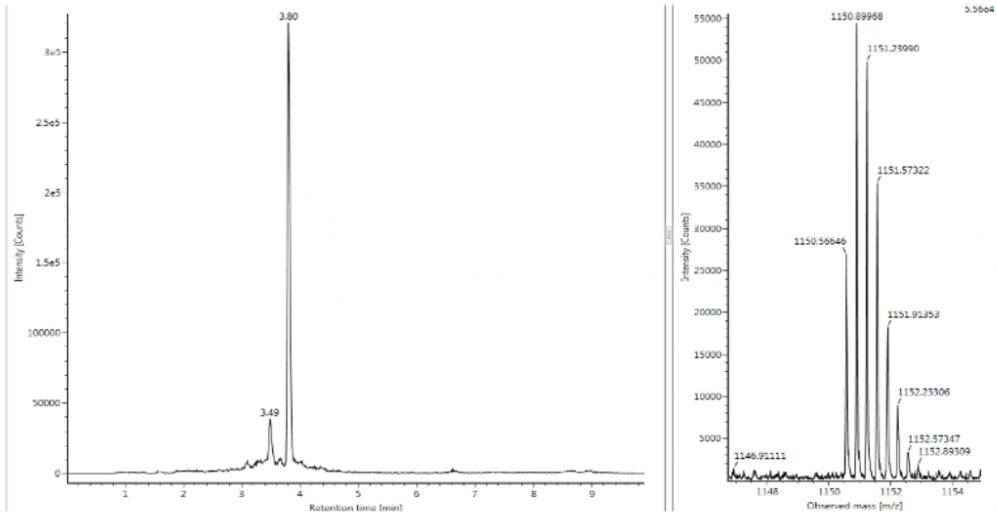


Figure 2. Extracted chromatogram and spectra information for insulin glargine metabolite M1 (21^{A}-Gly-insulin). M1 : *m/z* $[M+5]^{5+}$ 1150.899969.



Article 10: Influence of insulin collection preservatives in postmortem blood: application to a case of insulin aspart suicide.

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Influence of insulin collection preservatives in postmortem blood: application to a case of insulin aspart suicide

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Abstract

Insulin aspart (Novorapid ®) is a fast-acting analog of human insulin, indicated in the treatment of type I and II diabetes. It is administered before meals to mimic the physiological insulin secretion that follows a rise in blood glucose. Its misuse for the purposes of suicide, murder and in the context of factitious order has often been described. In forensic medicine, the identification of insulin in biological samples has always been complex.

In this paper, we present a case of suicide of a 64-year-old man who died after the injection of insulin aspart. He was suffering from terminal lung cancer and left a letter explaining the reasons of his suicide. Four empty Novorapid® pens were found near the body. Body

examination was unremarkable and femoral blood was collected in 2 dry Vacutainer tubes (red cap) and 2 sodium fluoride tubed (grey cap).

A LC-HRMS method was used to identify and discriminate aspart insulin from human insulin after immunopurification in the blood samples and in the pens. Blood aspart insulin tested positive at 36 and 37 ng/mL in dry tubes, and at 58 and 71 ng/mL in tubes containing sodium fluoride when tested about 3 weeks after collection of the specimens. The contents of the pens also matched with insulin aspart.

The stability of insulin in blood is a critical point in the interpretation of the concentrations due to their rapid decrease caused by the activity of proteases in blood. During a degradation study implemented to compare 3 preservatives and dry tubes, suitable insulin aspart stability was observed with EDTA and NaF. Given NaF is standard in forensic toxicology for measuring blood alcohol concentrations, the authors suggest its use for blood collection when insulin intoxication is suspected.

Keywords: LC-HRMS, insulin aspart, postmortem, NaF, whole blood, preservatives

Introduction

The increase in the incidence of diabetes in recent years has led the synthetic analogues of human insulin, used to treat type I and II diabetes, to become more widespread in the general population (1). As with any pharmaceutical, in addition to its therapeutic properties, its toxic effects can also be diverted for other purposes, such as criminal poisoning.

The use of synthetic insulin in a diabetic subject regulates blood glucose levels, whereas administration to a healthy subject can cause hypoglycaemia that can be fatal (2).

For this reason, its use for criminal murder or for suicide has often been described and the number of cases reported in the literature is increasing due to analytical advancements (3).

Insulin aspart, marketed under the name Novolog®, is a fast-acting insulin used to treat type I and type II diabetes. The structural difference from human insulin lies in the substitution of the amino acid proline at position 28 of the beta chain by aspartic acid. This substitution results in a more rapid conformational change in its active form and consequently a more rapid onset of action (4).

The ability to identify and measure blood insulin is of great importance for both clinical and forensic purposes. This allows discriminating between pathological excessive secretion of endogenous insulin (caused, for example, by an insulinoma) and unexplained hypoglycemia caused by exogenous insulin administration. Although the importance of this type of analysis

is quite clear, its diffusion is limited by some practical restrictions. In the forensic context, determining the diagnosis of insulin poisoning is quite complex. The difficulty lies in the absence of morphological evidence in the pathological changes, which are minimal, and in the absence of specific and reliable tests that can distinguish the different types of insulin. The analysis of insulin and its synthetic analogues is complicated by their high molecular weight (which requires the use of specific instruments), sampling difficulties and the chemical instability of insulin, particularly in postmortem blood (5).

The problem is mainly related to the decomposition phenomena that occur after death, especially hemolysis, which seems to play a major role in the degradation of insulin. This issue, not only means that the sample will not be suitable for analysis by enzymatic immunoassays, which are very sensitive to this phenomenon, but also that proteases released by the erythrocytes will rapidly degrade insulin, making its identification and much more its quantification very difficult even with the most sensitive and specific methods (6-7).

Because of all these issues, insulin testing is not systematically performed in all laboratories. Consequently, the total number of real cases seems to be underestimated as many of them are classified as accidental or are masked by the laboratory's inability to perform the analysis.

To this aim, advances have been made in recent years in sample preparation and methods of identifying synthetic insulin analogues (7). As a consequence, nowadays, the idea that insulin is considered as the perfect crime is devalued by analytical progresses. Modern methods allow insulin to be detected in complicated situations such as in postmortem specimens. To limit the problem of degradation, some pre-analytical practices can be used, such as immediate separation of the serum by centrifugation and its storage at – 20 ˚C to reduce protease activity (5). Nevertheless, these practices can seldom be implemented in forensic discipline.

The interesting point would be to understand the usefulness of preservatives in stabilizing the concentration of insulin at the moment of sampling.

Many studies published in the literature suggest that insulin is stable in serum and whole blood in the presence of EDTA at + 4 ˚C and -20 ˚C in the long term but also at room temperature for at least 24 hours (8-10). On the opposite, other studies suggest that the delay in separation from the corpuscular part causes an important decrease in the initial insulin concentration (11-12).

In this report, the authors present a case of suicide by insulin aspart in which blood concentrations of insulin aspart were different in blood collected in dry tubes when compared to a collection in tubes containing NaF, used as a preservative. In order to determine the

impact of the preservative type on insulin stability, tests of stability were performed using tubes containing different preservatives.

Case Report

A 64-year-old man was found dead at home by his two daughters, lying on a sofa. He was recognized diabetic for about a year and was suffering from lung cancer, for which he had refused surgery. Four pens of insulin injection devices (NovoRapid®) were found on a table next to the body, as well as a letter mentioning his suicidal intention.

The body examination revealed a marked cyanosis of the fingernails and lips and marked cervical-facial congestion. Ecchymosis areas located in the crease of the left elbow and in the anterior part of the left wrist presented recent punctiform lesions that would correspond to vascular injection points. There was also a blood glucose measuring device on the outside of the left overarm. The body was not in a putrefactive state and its death was declared between 15 and 48 hours before the external examination of the body. No autopsy was performed, and, as a consequence, no histology was requested by the local prosecutor.

Since the external examination revealed an asphyxial syndrome not specific to a cause of death and fresh injection sites, the forensic pathologist suggested a toxic death.

For this reason, femoral blood samples were collected using four Vacutainer™ tubes, two containing NaF with grey caps and two dry tubes with red caps, for toxicological tests.

Blood was stored at +4° C until analysis, which occurred about 3 weeks after collection.

Material and Methods

Chemical and reagents

Human, aspart, lispro, glargine, detemir and bovine insulin standards were supplied by Merck (Saint Quentin Fallavier, France). Methanol, acetonitrile for high performance liquid chromatography (HPLC) isocratic grade and glacial acetic acid were obtained from V.W.R. Chemicals ProLabo (Fontenay-Sous-Bois, France). Formic acid for liquid chromatography coupled to mass spectrometry (LC-MS) analysis was obtained from Carlo Erba (Chaussée du Vexin, France). Tween 20, Amicon Ultra Filters (3 kDa, 500 µL) and eppendorf (low-binding protein) were purchased by Merck (Fontenay-sous-bois, France). Mercodia Iso-Insulin ELISA immunopurification plates were obtained from Mercodia (Paris, France). For all aqueous buffers and solutions, ultrapure water was used.

Insulin blood analysis

Insulin blood analysis was performed using a method previously published and validated by Bottinelli et al. (13). Briefly, 250 µL of whole blood was subjected to protein precipitation in the presence of 20 ng/mL of bovine insulin used as internal standard. Subsequently, ultra-filtration was performed using centrifugal concentrators and after 2 washes, the retentate was deposited on the wells of the immunoassay plate for immunopurification. After 1 hour of agitation in a stirring plate (800 rpm), the wells were washed, and the insulin eluted with a 2 % formic acid solution of H_2O/ACN (80/20). 5 µL was injected into the LC-HRMS system.

A 6-point calibration curve was prepared using the following concentrations of insulin aspart: 0, 1, 5, 10, 50, 100 ng/mL.

Insulin stability study

A 30 mL pool of blank blood containing 100 ng/mL of insulin aspart was prepared and then separated into 48 tubes (12 dry tubes, 12 tubes containing NaF, 12 tubes containing lithium heparinate, 12 tubes containing EDTA). 1 mL of the pool was analyzed immediately in quadruplicate in order to obtain the initial reference concentration. The other tubes were

stored with half at + 4˚ C and half at – 20 ˚C until analysis. The stored blood was analysed in duplicate for each tube type after 24 hours, after 1 week and after 2 weeks.

Instrument

LC separation was achieved using a Waters Acquity HSS C18 column (150 x 2.1 x 1.8 µm) with a controlled temperature maintained at 50 ˚C. A 5- µL injection with a 0.25 mL/min flow of waters with 0.1 % of formic acid (solvent A) and acetonitrile with 0.1 % of formic acid (solvent B) was used. The gradient elution was as follows: the initial 20% B was increased to 65% over 5.4 minutes, 65 to 98% over 0.3 minutes and returned to initial conditions over 4.2 minutes. The total run time was 9.90 minutes, and aspart insulin was eluted at 2.70 minutes.

A Xevo G2-XS Q-TOF high-resolution mass spectrometer (Waters corporation, Milford, MA, USA) was used, operating in positive ion mode and in sensitivity mode.

Desolvation gas flow was set at 1,000 L/h at a temperature of 600 ˚C, the cone gas to 50 L/h and the source temperature set to 120 ˚C. The capillary voltage and the cone voltage were set to 3 and 15 V, respectively. In MS scanning, data were acquired from 100 to 2000 *m/z*. Collision energy ranged from 20 to 80 eV. HRMS parameters were optimized by infusing 1 mg/mL solution of the analytes into the ion source and manually optimizing the parameters.

In addition to aspart, the presence of other analogs (lispro, glargine, detemir, glargine) and human insulin were tested. For aspart insulin, the 5-fold charged molecule was observed at *m/z* 1165.9424 (which deconvolutes to 5824.7121), while for human and bovin insulin the 5-fold charged ions were observed at *m/z* 1162.3417 and 1147.5323 respectively. In addition, the 6-fold charged 971.9524 and 968.7902 were used to discriminate aspart from human insulin.

UNIFI software was used for data, chromatograms and spectra acquisition.

Results and Discussion

The analysis of the contents of the 4 pens NovoRapid® confirmed the presence of insulin aspart. Figure 1 shows the chromatogram obtained after injection of one of the pen solutions.

The blood specimens also tested positive for aspart insulin with concentrations of 36 and 37 ng/mL in dry tubes, and at 58 and 71 ng/mL in tubes containing NaF. Figure 2 shows

chromatograms obtained after the extraction of the blood contained in one dry tube and in one tube containing NaF.

Further analysis performed during a complete toxicological screening revealed the presence of paracetamol (33 mg/L), zopiclone (132 ng/mL), quinine (71 ng/mL), gliclazide (3 mg/L) and metformin (1,4 mg/L). All these concentrations were considered in the therapeutic ranges. No ethanol was detected.

Insulin was considered as the hypoglycemic agent mostly active in blood, when compared to gliclazide and metformin. Hypoglycemia caused by insulin can be fatal because the drop in blood glucose causes an alteration of the central nervous system that can lead to coma and neurological death if not treated in time. In addition to insulin, two oral antidiabetic agents, gliclazide (an insulin-secretagogue) and metformin (an insulin-sensitizer), were also present. The latter were present, however, at nontoxic concentrations. Moreover, quinine, an antimalarial agent, has been shown to be a potent stimulant of insulin release from pancreatic beta cells, so in some conditions its use may lead to hypoglycemia (14). Therefore, the presence of 3 other hypoglycemic substances could have contributed or aggravated the hypoglycemia caused by exogenous insulin.

Despite the undefined therapeutic range for NovoRapid® insulin aspart, according to the EMA (European Medicines Agency), following a subcutaneous administration of 0.15 units/kg of insulin aspart NovoRapid ®, a maximum plasma concentration of 4.4 ng/mL is reached after 40 minutes (4). Taking an average of the concentrations of insulin aspart found in the blood samples, it will be about 15 times higher than the standard therapeutic concentrations.

Cases in the literature of fatal and nonfatal insulin aspart overdoses report different plasma concentrations. In 2018, Sato et al. (15) reported the case of a suicide attempt following administration of insulin aspart, where plasma concentration 4 hours after injection was 62.9 ng/mL (dosage performed by enzyme immunoassay). The subject survived. Kim et al. (16) described a case of insulin aspart overdose whose plasma concentration measured after 7 hours was 77 ng/mL. The definitive decision of a fatal insulin aspart concentration is difficult to establish, as demonstrated by the 2 previous references. In addition, Bottinelli et al. (13) reported in 2021 two deaths due to insulin aspart intoxication where plasma concentrations (16 and 17 ng/mL) were lower than those found in the present case.

The insulinemia levels that can be measured vary depending on a range of factors such as the time between intake and death (often long), the time elapsed between death and collection and

the time between collection and analysis. In fact, the degradation of insulin continues even after collection in the tube (5). Insulin is rapidly degraded in vivo by a metalloprotease called IDE (Insulin degradation enzyme), which explains its very short half-life (4-6 minutes) (17).

In postmortem blood, autolysis of red blood cells caused by the release of hydrolytic enzymes by lysosomes results in the release of IDE present within the cells. This release causes the degradation of circulating insulin into inactive fragments (6). The chemical instability of insulin caused by this phenomenon represents the major analytical challenge related to its identification in *postmortem* specimens.

As it was observed a difference in insulin aspart concentration between blood stored in dry tubes and blood stored with NaF, it was decided to perform a stability test of insulin aspart at 100 ng/mL stored in 4 different types of tubes with different preservatives. The results of the stability tests are shown in Figure 3. As can be seen from the results, insulin aspart did not drop below 90% of the initial concentration in almost all samples stored at – 20 ˚C and + 4 ˚C in the first 24 hours following sampling. At + 4 ˚C, almost good stability was observed, except for samples stored in lithium heparin (64%) and samples stored in dry tubes (88%). Storage in lithium heparin appears to have the most negative effect on insulin, which is no longer detectable after 1 week at + 4 ˚C.

From these results, it would appear that EDTA and sodium fluoride are the most effective preservatives at both + 4 ˚C and – 20 ˚C degrees for insulin. This can be due to the ability of these salts to cause non-specific protein binding that affects peptide analysis (18).

Storage at -20 ˚C is known to limit the activity of insulin degradation enzymes. Often, immediate freezing is not possible, especially in forensic medicine, and for this reason it is interesting to know whether the freezing step can be avoided by using a common preservative. What was observed in the stability study seems to be in agreement with what has been reported in the literature. McDonald et al. (10) in 2012 perform a study on the stability of insulin in K^+-EDTA-containing tubes and observed that in addition to remaining stable at -20 ˚C for 5 days, insulin was also stable at room temperature for at least 24 hours. This was already suggested by Ellis et al. (9) in 2003, who investigated the stability of a group of hormones in unseparated whole blood and observed that insulin was stable at 4 ˚C for 24 hours when stored with EDTA. Only in a study realized by Evans et al. (8) the effect of NaF as a preservative was tested, demonstrating satisfactory results, as insulin remained stable for 5 days at 4 ˚C degrees without the need of freezing. Stability in the presence of EDTA appears to be due to its chelating properties for protecting peptides from proteolysis by limiting the activation of proteases. With respect to sodium fluoride, the preservative used for

blood alcohol measurements, its inhibitory enzymatic activity could also have a preservative property for insulin. However, when compared, this activity is less significant than the one of EDTA. This could be due to the fact that sodium fluoride at the same time also promotes erythrocyte hemolysis to which insulin is quite sensitive (18).

Conclusion

The instability of insulin, particularly in postmortem blood samples, the delay between autopsy and toxicological analysis, and the lack of adequate pre-analytical practices have led over time to an underestimation of cases of insulin poisoning. This is even complicated by the requirement of a sophisticated analytical equipment to be able to discriminate the different insulin forms available on the therapeutic market.

The case reported by the authors in this study allows highlighting a positive effect on insulin stability by NaF, used as preservative. The comparison of NaF and other preservatives has demonstrated good stability of insulin aspart in tubes containing NaF (routinely used in forensic medicine) and EDTA, even at + 4 °C without the need for immediate freezing of the

sample. As there are no similar results obtained on postmortem blood, further studies will be needed to confirm these initial findings.

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Figure 1. Left: extracted chromatogram for aspart insulin from the NovoRapid® pen solution.

Right: spectra from the 5-fold charged ions of aspart insulin in the pen solution.

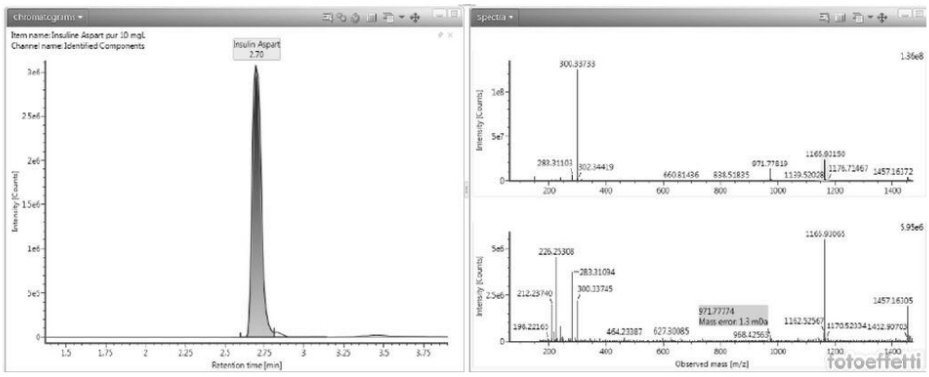


Figure 2. Extracted chromatograms for aspart insulin from the blood specimen collected in one of the tubes containing NaF (top of the figure) and blood sample collected in one of the dry tubes (bottom of the picture). Concentrations were, from top to the bottom, 71 and 36 ng/mL.

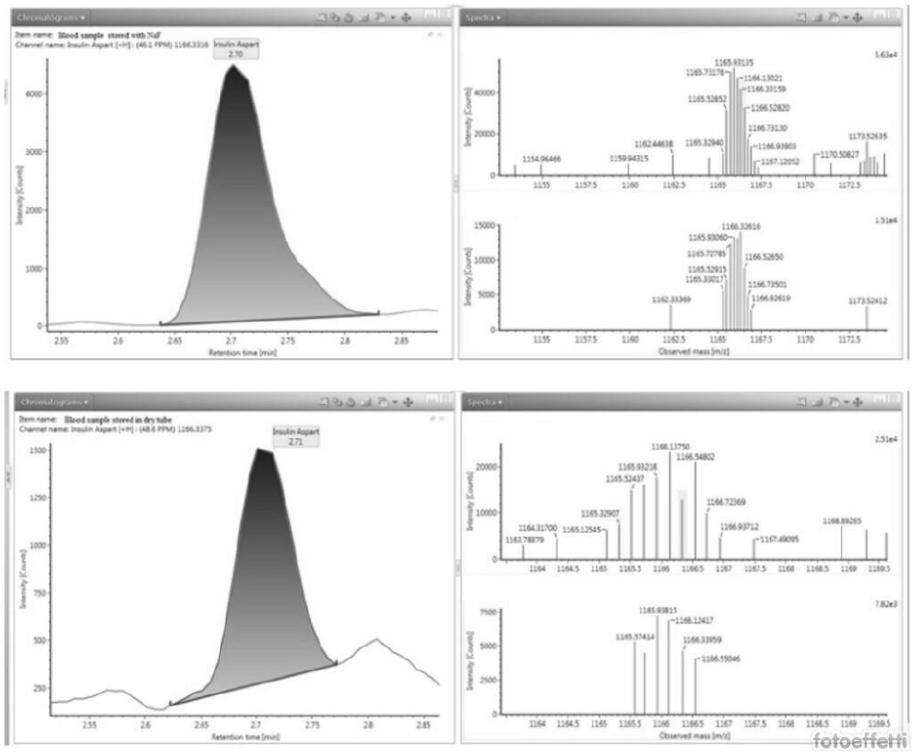
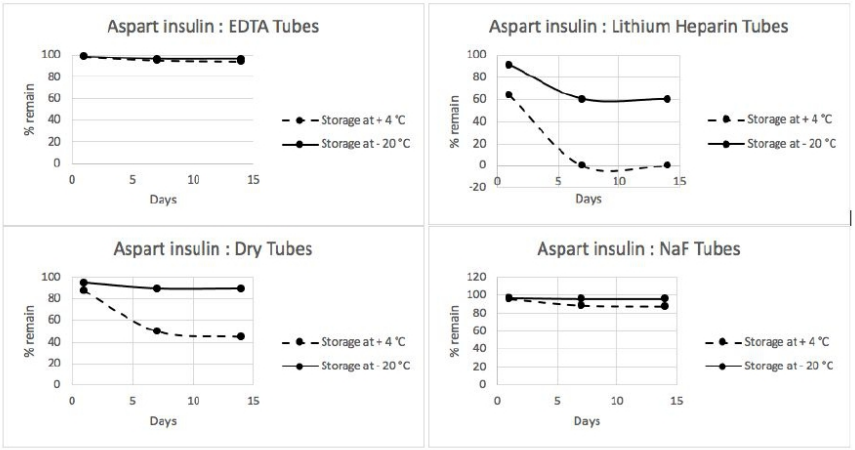


Figure 3. Percentages of concentration from initial 100 ng/mL of insulin aspart in blood when stored at + 4 ˚C and -20 ˚C for 2 weeks.



Conclusion

Les antidiabétiques oraux, malgré les avantages qu'ils présentent en ce qui concerne la réduction de la morbidité et de la mortalité des patients diabétiques, représentent un problème de santé en raison de leur utilisation abusive.

L'absence de méthode analytique spécifique pour le dépistage de ces substances a conduit au fil du temps à une sous-estimation de l'exposition toxique à ces substances et à des interventions drastiques inutiles.

Pour cette raison, il semble essentiel qu'un laboratoire de toxicologie soit capable d'identifier et de quantifier la plupart des médicaments antidiabétiques sur le marché afin d'expliquer les épisodes d'hypoglycémie peu clairs.

En outre, en raison de la fenêtre de détection limitée des échantillons de sang et d'urine, il est important de pouvoir identifier ces molécules dans les cheveux également. L'analyse des cheveux peut parfois être la seule preuve de l'exposition à un médicament, grâce à sa large fenêtre de détection et à la stabilité des molécules qui y sont incorporées.

Au début de cette thèse en 2019, l'objectif de ce travail était de créer des méthodes analytiques capables d'identifier et de quantifier les antidiabétiques dans les fluides biologiques et les cheveux (sauf l'insuline). A ce moment, il y avait peu de données sur les concentrations dans les cheveux des antidiabétiques sauf un article pour la metformine et deux pour les sulfamides.

Aujourd'hui, grâce aux travaux réalisés au cours de cette thèse, des méthodes sont disponibles pour l'identification et la quantification de 5 familles d'antidiabétiques oraux dans les cheveux (sulfanilurées, glinides, gliptines, gliflozines et biguanides).

L'application de ces méthodes à des sujets sous traitement et à des sujets décédés d'une overdose a permis de fournir les premières concentrations de ces substances dans les cheveux qui serviront de référence.

En ce qui concerne l'insuline, son utilisation dans un contexte médico-légal a déjà été décrite.

En raison de l'instabilité de l'insuline, en particulier dans les échantillons de sang *postmortem*, le délai entre l'autopsie et l'analyse toxicologique, et le manque de pratiques pré-analytiques adéquates ont conduit au fil du temps à une sous-estimation des cas d'empoisonnement à l'insuline.

Le développement d'une méthode analytique utilisant la technologie de la spectrométrie de masse haute résolution a permis de mettre en évidence plusieurs cas d'exposition souvent fatale à l'insuline dans cet Institut.

En 2020, le laboratoire de toxicologie de Strasbourg pourra, pour la première fois, rendre une expertise impliquant une analyse de l'insuline dans un cas de tentative d'homicide et de suicide.

Suite à ce développement, la méthode a été appliquée à différents cas, notamment des suicides.

L'étude de stabilité réalisée avec différents conservateurs nous permet également de recommander l'utilisation de tubes à essai contenant de l'EDTA et du NaF (déjà utilisés en médecine légale pour le dosage de l'alcool) en cas de suspicion de surdosage en insuline.
L'interprétation d'une concentration d'insuline reste toujours difficile et nécessite une approche complexe impliquant l'évaluation de nombreux facteurs tels que les antécédents médicaux du sujet, le mode et l'état de conservation de l'échantillon.

Avec le développement des méthodes, la recherche sur les antidiabétiques est appliquée à la fois aux demandes hospitalières pour le suivi thérapeutique et l'ajustement de la posologie et aux cas de surdosage provenant des services de réanimation et d'urgence. En médecine légale, la recherche d'antidiabétiques s'ajoute à la routine tant pour les cas de suspicion de surdosage que pour les cas de décès inexpliqués.

Les travaux réalisés pendant ces trois années de thèse ont donné lieu à 10 publications scientifiques, dont 8 internationales et 2 nationales.

Au cours de cette thèse de 3 ans, mon intérêt s'est porté sur les médicaments antidiabétiques les plus prescrits et donc les plus présents sur le marché. Malheureusement, il ne m'a pas été possible d'inclure tous les médicaments antidiabétiques existants, tels que les analogues du GLP-1.

Compte tenu de la gravité du problème, il est nécessaire que le plus grand nombre possible d'antidiabétiques soient dépistés dans notre laboratoire, raison pour laquelle mes projets futurs prévoient d'ajouter davantage d'antidiabétiques à nos bibliothèques MS.

De plus, en raison de l'ampleur du sujet (qui inclut d'autres antidiabétiques) je n'ai pas pu traiter la recherche de l'insuline dans les tissus (notamment musculaires et adipeux). Cette étude fera l'objet d'un projet futur.

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Aspects analytiques, cliniques et médico-judiciaires liés à l'identification des molécules à pouvoir hypoglycémiant dans les intoxications par antidiabétique (s)

Résumé

L'utilisation des médicaments antidiabétiques, malgré leurs fins thérapeutiques, n'est pas dénuée de risques. Le plus grand risque lié à l'utilisation de ces médicaments est l'hypoglycémie, qui peut être fatale en cas d'utilisation inappropriée.

L'utilisation détournée est en fait assez bien connue à des fins criminelles de suicide et de meurtre, comme substances dopantes dans le milieu sportif (dans le cas de l'insuline) et dans le cadre des hypoglycémies factices (syndrome de Münchhausen).

Dans le cadre de ma thèse, je me suis intéressé à 5 familles d'antidiabétiques oraux (sulfamides hypoglycémiants, glinides, gliptines, biguanides et gliflozines) et à l'insuline.

L'objectif principal était de développer des méthodes analytiques pour l'identification et la quantification de ces substances dans le sang et autres fluides biologiques et d'apporter un intérêt à la recherche de ces substances dans les cheveux afin de fournir des critères d'interprétation pour mieux expliquer les concentrations retrouvées dans les cheveux en raison du manque de données dans la littérature.

La première partie de ce travail a été consacrée au développement de méthodes analytiques pour détecter les antidiabétiques oraux dans le sang et les cheveux par chromatographie en phase gazeuse couplée à la spectrométrie de masse en tandem en ce qui concerne la metformine et à l'aide de la chromatographie liquide couplée à la spectrométrie de masse en tandem pour les autres quatre familles d'antidiabétiques oraux.

Après avoir développé des méthodes analytiques pour la recherche d'antidiabétiques oraux, mon travail s'est orienté vers le développement d'une méthode d'identification de l'insuline dans la matrice sanguine *postmortem* sur un système de chromatographie liquide couplé à la spectrométrie de masse à haute résolution (Q-TOF).

Aujourd'hui, les bibliothèques de MS contiennent six antidiabétiques appartenant à la famille des sulfamides comme agents hypoglycémiants, un biguanide (metformine), deux glinides, cinq gliptines et trois gliflozines . En outre, elles comptent désormais aussi les spectres de l'insuline humaine et de cinq de ses analogues synthétiques.

Ces méthodes ont été appliquées à la fois dans des contextes cliniques pour l'ajustement de la posologie et aux cas de surdosage provenant de la réanimation et des urgences, et dans des cas médico-légaux impliquant principalement des suicides et des surdosages dus à une mauvaise utilisation de ces médicaments.

Ces travaux ont donné lieu à 7 publications internationales et 3 publications nationales.

Mots-clés : antidiabétiques oraux, insuline, médecine légale, chromatographie liquide et gazeuse couplée à la spectrométrie de masse en tandem et haute résolution (UHPLC/GC - MS/MS et UHPLC-HRMS), cheveux.

Abstract

The use of anti-diabetic drugs, despite their therapeutic purposes, is not risk-free. The greatest risk associated with the use of these drugs is hypoglycaemia, which can be fatal if used inappropriately. Misuse is actually quite well known for criminal purposes of suicide and murder, for doping purposes in sports (in the case of insulin) and in the context of factitious hypoglycaemia (Munchausen syndrome). In my thesis, I was interested in 5 families of oral antidiabetic drugs (hypoglycaemic sulphonamides, glinides, gliptins, biguanides and gliflozins) and in insulin. The main objective was to develop analytical methods for the identification and quantification of these substances in blood and other biological fluids and to provide an interest in the research of these substances in hair in order to provide interpretation criteria to better explain the concentrations found in hair due to the lack of data in the literature. The first part of this work was devoted to the development of analytical methods to detect oral antidiabetic drugs in blood and hair by gas chromatography-tandem mass spectrometry for metformin and by liquid chromatography-tandem mass spectrometry for the other four families of oral antidiabetic drugs. After developing analytical methods for the investigation of oral antidiabetics, my work turned to the development of a method for the identification of insulin in the postmortem blood matrix on a liquid chromatography-high resolution mass spectrometry (Q-TOF) system. Today, the MS libraries contain six antidiabetic drugs belonging to the sulphonamide family as hypoglycaemic agents, one biguanide (metformin), two glinides, five gliptins and three gliflozins. In addition, they now also include the spectra of human insulin and five of its synthetic analogues. These methods have been applied both in clinical settings for dose adjustment and overdose cases from the intensive care unit and emergency room, and in forensic cases involving mainly suicides and overdoses due to incorrect use of these drugs. This work has led to 7 international and 3 national publications.

Keywords: antidiabetic drugs, insulin, forensic medicine, liquid and gas chromatography coupled with tandem mass spectrometry and high resolution mass spectrometry (UHPLC/GC - MS/MS and UHPLC-HRMS), hair.