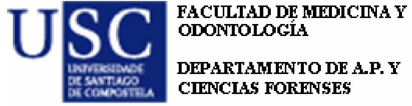

ANALYSIS OF FORENSICALLY RELEVANT DRUGS IN BLOOD AND URINE BY CONVENTIONAL AND ADVANCED LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHODS

Memoria presentada por

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Para optar al grado de Doctora por la Universidad de Santiago de Compostela

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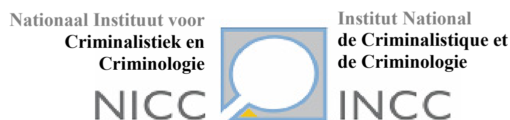

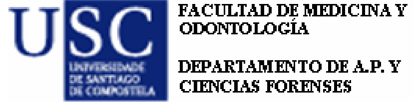

D. Manuel López-Rivadulla Lamas, Catedrático de Toxicología de la Universidad de Santiago de Compostela, y Dña Nele Samyn, Jefe de Laboratorio del Departamento de Toxicología del Instituto Nacional de Criminalística y Criminología,

Certifican:

Que la presente memoria, titulada "Análisis de drogas relevantes desde el punto de vista forense, en sangre y orina, mediante métodos de cromatografía líquida acoplada a un doble espectrómetro de masas en tandem convencionales y avanzados", que presenta la licenciada **María del Mar Ramírez Fernández** para optar al grado de Doctora ha sido realizada bajo su dirección en ambos departamentos y, a su juicio, reúne todos los requisitos exigidos por la normativa vigente para la elaboración y presentación de Tesis Doctorales.

Y para que así conste, firma el presente informe en Santiago de Compostela, a 21 septiembre de 2009.

M. López-Rivadulla N. Samyn

No digas que fue un sueño...

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List of abbreviations

AMD: Advanced Method Development

AMU: Atomic Mass Unit

APCI: Atmospheric Pressure Chemical Ionization)

API: Atmospheric Pressure Ionization

APLI: Atmospheric pressure Laser Ionization

APPI: Atmospheric Pressure Photo-Ionization

CI: Chemical Ionization

CID: Collision Induced Dissociation

CNS: Central Nervous System

CV: Coefficient of Variation

DC: Direct Current

DP: Particle size

DUID: Driving Under de Influence

EMCDDA: European Monitoring Centre for Drugs and Drug Addiction

ESI : ElectroSpray Ionisation

FAB: Fast Atom Bombardment

FIA: Flow Injection Analysis

FTICR: LIT-Fourier-Transform Ion Cyclotron Resonance

GC : Gas Chromatography

HPD: High Pressure Dispenser

ICR: Ion Cyclotron Resonance

ID: Internal Diameter

IDMS: Isotope Dilution Mass Spectrometry

IS: Internal Standard

IT: Ion trap

LC : Liquid Chromatography

LIT: Linear Ion trap

LLE: Liquid Liquid Extraction

LOD : Limit of Detection

LOQ : Limit of Quantification

LPME: Liquid-Phase MicroExtraction

LSD : Lysergic Acid Dietylamide

MALDI: Matrix-Assisted Laser Desorption Ionization

6-MAM: 6-MonoAcetylMorphine

MBDB: 3,4-MethylBenzoDioxolylButanamine

MDEA : 3,4-MethyleneDioxy-N-EhylAmphetamine

MDMA: 3,4-MethylenenDioxyMethAmphetamine

MDA : 3,4-MethylenenDioxyAmphetamine

MRM: Multiple-reaction monitoring

MS: Mass Spectrometry

MS/MS: tandem Mass Spectrometry

NMDA: N-Methyl-D-Aspartate

NP : Normal Phase

PBI: Particle Beam

PMA : 4-Para-MethoxyAmphetamine

PPT: Protein PrecipiTation

QC: Quality Control

Qi: Quadrupole

QqQ: Triple Quadrupole

Quad: Quadrupole

RAM: Restricted Access Materials

RP: Reverse Phase

RSD: Relative Standard Deviation

SIL: Stable Isotope Labeled

SIM: Selected Ion Monitoring

S/N: Signal to Noise

SPE: Solid Phase Extraction

SPME: Solid-Phase Micro-Extraction

SOFT: Society of Forensic Toxicologists

SRM: Selected Reaction Monitoring

SSI: Sonic Spray Ionization

TDM: Therapeutic Drug Monitoring

THC: Δ9-Tetrahydrocannabinol

11-OH-THC: 11- Hydroxy- Δ9-TetraHydroCannabinol

THC-COOH: 11-nor-9-Carboxy- Δ9-TetraHydroCannabinol

TIC: Total Ion Current

TQ: Triple Quadrupole

TOF: Time Of Flight

TSI: ThermoSpray Ionization

UPLC: Ultra-Performance Liquid Chromatography

XLC: On-line SPE

Summary

The present work intended to evaluate the analysis of forensically relevant drugs in blood and urine by conventional and advanced LC-MS/MS methods.

Chapter I gives a short review of the pharmacology of the drugs studied in this thesis (amphetamines, cannabis and hallucinogens) and their effects on human behavior and performance. Hallucinogens are psychoactive substances that powerfully alter perception, mood, and a host of cognitive processes. They are considered physiologically safe and do not produce dependence or addiction. Their origin predates written history, and they were employed by early cultures in a variety of sociocultural and ritual contexts. Nowadays, cannabis is still Europe's most commonly consumed illicit drug as nearly a quarter of all Europeans have tried cannabis in their lifetime. Relaxation, well-being and somnolence are one of the effects of cannabis. Moreover, in parts of Europe, use of amphetamines constitutes an important part of the drug problem. They stimulate the CNS, increasing the heart rates and blood pressure and decreasing appetite, among other effects.

LC-MS(/MS) is a widely used method in forensic laboratories, particularly in applications where non-volatile, labile or high-molecular-weight compounds are being analyzed. **Chapter II** aim to give an overview of the current LC-MS(/MS) applications in forensic laboratories and detailed information in relation to ionization, ion separation, and ion detection, together with tandem mass spectrometry. Suppression or enhancement of analyte ionization by coeluting compounds is a well known phenomenon in LC-MS(/MS) analysis mainly depending on the sample matrix, the sample preparation procedure, the quality of chromatographic separation, mobile phase additives, and ionization type. Therefore, common sample preparation procedures are protein PPT, LLE and SPE. Optimum sample preparation leads to enhanced selectivity and sensitivity. Detailed information about the recent applications of LC-MS(/MS) to the analysis of amphetamines, cannabis and hallucinogens in blood and urine are included at the end of this chapter.

However, sample preparation is often regarded as time-consuming, a laborious work. Recent developments in on-line SPE aspects of high-throughput quantitative bioanalysis of drug and metabolite in biological matrices are described in **Chapter III**. High-throughput analysis is becoming increasingly important in forensic laboratories. One commercial automated on-line SPE system is the Symbiosis system manufactured by Spark Holland. In on-line SPE sorbents of very small particles are packed in a miniature LC column (cartridge) and higher pressures are

applied to distribute the SPE solvents. Switching valves direct the flow to the LC column or waste, as appropriate. In contrast of traditional off-line SPE, several times consuming steps are eliminated. The extracted sample is directly injected to the analytical column by a simple valve switch. As a consequence no sample volume is lost during transfer, thus increasing the overall assay sensitivity. An extensive literature survey is given about the application of this instrument to the analysis of drugs in biological matrices.

Chapter IV presents the objectives of the thesis which are an evaluation of the conventional LC-MS/MS technique and the new trend, on-line SPE-LC-MS/MS (Symbiosis), for the analysis of:

a) multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid and metabolites in urine using off-line SPE,

b) THC and metabolites in blood with LLE as off-line sample preparation procedure,

c) THC-COOH (main metabolite of THC in urine) by on-line SPE, and

d) 7 amphetamines and metabolites in blood and urine also by on-line SPE.

Chapter V.I presents the development and validation of a LC-MS/MS method for the quantification of hallucinogens and other related compounds in urine. The method comprises an off-line SPE procedure, evaporation to dryness and reconstitution in mobile phase. The total run time was 20 min. External QCs containing LSD where analyzed within each series of analysis. The method was fully validated and applied to authentic urine samples (containing psilocin, ketamine, norketamine and chlorpheniramine). **Chapter V.II** describes the validation of the method for the analysis of THC and two of its main metabolites in blood. LLE with hexane: ethyl acetate was applied as clean-up procedure followed by centrifugation, complete evaporation, and reconstitution. The run time was 13 minutes. Two external QCs were used within each series of analysis. The method was completely validated in terms of precision, accuracy, specificity, recovery, matrix effects and stability. Finally the method was applied to authentic blood samples from forensic cases. **Chapter V.III** focuses on the development and validation of a method using the Symbiosis system for the analysis of THC-COOH in urine (500 µL). As the THC-COOH is glucoronized in urine, a previous hydrolysis was carried out using KOH 10 M. Then, the diluted urine was acidified in the LC vials for its direct injection. The method was fully validated and applied to authentic samples from cannabis users. Another application of the on-line-SPE-LC-MS/MS system is presented in **Chapter V.IV** for

the direct quantification of 7 amphetamines and metabolites in blood and urine. The method, completely validated following international guidelines, required a minimum sample handling: the dilution of 100 µL blood or 50 µL of urine samples in the aqueous mobile phase, spiked with the IS, directly in the LC vials, previous injection. HLB cartridges were used for sample clean-up. The method was applied for the analysis of blood and urine samples from a MDMA study, and from forensic cases.

The conclusions in **Chapter VI** show the advantages of LC-MS/MS and the new trend in on-line SPE. The results demonstrated that although conventional LC-MS/MS methods are still robust and confident, on-line SPE coupled to LC-MS/MS is highly effective in terms of time safe and high throughput.

Chapter VII is focused in the future perspectives in relation to LC-MS/MS applications. On-line SPE-LC-MS/MS and the UPLC-MS/MS are potent candidates for the analysis of drugs in conventional and alternative matrices (e.g. oral fluid), in such a way that the innovative chromatographic technologies are re-shaping the ways that separations and sample preparation are performed in high-throughput laboratories.

Résumé

L’objet de cette thèse est d’évaluer l’analyse de drogues, pertinentes du point de vue médicolégal, dans le sang et l’urine par le biais des méthodes LC-MS/MS conventionnelles et avancés.

Le **Chapitre I** présente un bref exposé de la pharmacologie des drogues traitées dans cette thèse (amphétamines, cannabis et hallucinogènes) et de leurs effets sur le comportement. Les hallucinogènes sont des substances psycoactives qui altèrent dans une large mesure la perception, l’humeur et la raison. Elles sont considérées physiologiquement sans risque parce qu’elles ne produisent pas de dépendance ou d’addiction. Leur origine remonte aux cultures ancestrales étaient utilisées dans les rituels sacrés et autres contextes socioculturels. Aujourd’hui le cannabis est la drogue illicite la plus consommée en Europe, de sorte que près d’un quart des européens l’ont goûté au moins une fois dans leur vie. Les effets du cannabis sont la relaxation, le bien-être et la somnolence. D’autres drogues problématiques en Europe sont les amphétamines. Elles stimulent le CNS en augmentant la pression artérielle et diminuant l’appétit, entre autres effets.

LC-MS/MS est une technique largement utilisée dans les laboratoires médico-légaux, en particulier dans l’analyse de substances volatiles, thermiquement instables ou de poids moléculaire élevé. Le **Chapitre II** fourni un exposé général des applications récentes avec LC-MS(/MS) dans les laboratoires médico-légaux ainsi qu’une information détaillée sur l’ionisation, la séparation et la détection des ions, en plus de la spectrométrie de masses en tandem. Le phénomène connu comme suppression ou augmentation de l’ionisation des ions dans les analyses avec LC-MS(/MS) dépend principalement de la nature de la matrice, du processus de préparation de l’échantillon, de la qualité de la séparation chromatographique, des additifs de la phase mobile et du type d’ionisation. Les procédures type de préparation de l’échantillon sont la PPT, la LLE, et la SPE. Une préparation optimale de l’échantillon entraîne une augmentation de la sensibilité et de la sélectivité. A la fin de ce chapitre, on trouvera également des information détaillées sur les applications récentes de LC-MS(/MS) pour l’analyse des amphétamines, cannabis et hallucinogènes dans le sang et l’urine.

Cependant, la préparation de l’échantillon est considérée comme une étape fastidieuse et nécessitante du temps. Le **Chapitre III** expose les récents développements en SPE on-line à propos de productivité dans les analyses quantitatives des drogues dans les matrices biologiques. Ainsi, les analyses de

haute production sont en passe de devenir essentiels dans les analyses médico-légales. Un des systèmes commercialisés avec SPE on-line est le système Symbiosis produit par Spark Holland. Dans le système SPE on-line, le matériau d'extraction est emballé avec des particules très petites dans des colonnes LC (cartouches) qui travaillent à d'haute pression et auxquelles on applique les solvants d'extraction. Les valves d'échange dirigent le flux vers la colonne ou l'évacuent, si nécessaire. A la différence du SPE off-line classique, le système on-line permet d'éviter plusieurs étapes intermédiaires. Ensuite, l'échantillon extrait est injectée directement dans la colonne analytique par le biais d'une simple valve d'échange. Par conséquent, il n'y a pas de perte de volume de l'échantillon et cela permet d'augmenter la sensitivité. Ce chapitre inclue également une révision bibliographique sur les applications actuelles de cet instrument pour l'analyse des drogues dans les fluides biologiques.

Le **Chapitre IV** présente les objectifs de la thèse qui sont l'évaluation de la technique LC-MS/MS conventionnelle ainsi que la nouvelle tendance SPE-LC-MS/MS on-line (Symbiosis), pour l'analyse de

a) nombreux hallucinogènes, chlorpheniramine, ketamine, acide ritalinique et métabolites dans l'urine avec SPE off-line :

b) THC et métabolites dans le sang au moyen de LLE off-line comme procédure de préparation de l'échantillon,

c) THC-COOH (principal métabolite du THC dans l'urine) par vu de SPE on-line et

d) 7 amphétamines et métabolites dans le sang et l'urine avec SPE on-line.

Le **Chapitre V.I.** présente le développement et la validation d'une méthode avec LC-MS/MS pour la quantification des hallucinogènes et d'autres composés analogues dans l'urine. La méthode consiste en une LLE off-line, une évaporation à sec et une reconstitution dans la phase mobile. La durée totale de l'analyse est de 20 min. Avec chaque série d'analyse nous avons analysé des QC externes qui contiennent le LSD. La méthode a été entièrement validée et appliquée à l'analyse d'urines authentiques (avec de la psilocin, ketamine, norketamine et chlorpheniramine). Le **Chapitre V.II** décrit la validation de la méthode pour l'analyse du THC et deux de ses métabolites dans le sang. Comme procédure de nettoyage de l'échantillon nous avons utilisé LLE avec hexane: acétate d'éthyle, ensuite une centrifugation, une évaporation complète (16 min) et une reconstitution. La durée de l'analyse a été de 13 min. Nous avons ajouté deux QC externes à chaque série d'analyse. La méthode

a été entièrement validée en termes de précision, d'exactitude, de spécificité, de récupération, d'effet matrice et de stabilité. Finalement la méthode a été appliquée aux analyses de sang de cas médico-légales. Le **Chapitre V.III** est centré sur le développement et la validation d'une méthode utilisant le système Symbiosis pour l'analyse de THC-COOH dans l'urine (500 µL). Etant donné le THC-COOH est glucuronidé dans l'urine, on a effectué une hydrolyse préalable avec KOH 10 M. Ensuite, l'urine diluée a été acidifiée directement dans les fioles LC en vue de son injection directe. La méthode a été entièrement validée et appliquée à l'analyse d'urine de consommateurs de cannabis. Une autre application du système SPE-LC-MS on-line est décrite dans le **Chapitre V.IV**. pour l'analyse de 7 amphétamines et métabolites dans le sang et l'urine. La méthode, qui a été entièrement validée selon les normes internationales, a nécessité moindre manipulations: la dilution en phase aqueuse, contenant l'IS, de 100µL sang ou de 50 µL d'urine directement dans les fioles LC, avant l'injection. Pour le nettoyage de l'échantillon nous avons utilisé des cartouches HLB. La méthode a été appliquée à l'analyse de sang et urine dans une étude avec MDMA, et à de cas médico-légaux.

Les conclusions du **Chapitre VI** mettent en évidence les avantages du LC-MS/MS et la nouvelle tendance SPE on-line. Les résultats montrent que même si les méthodes LC-MS/MS conventionnelles restent fiables, la SPE on-line couplée au LC-MS/MS est très efficace et permets de gains de temps et de productivité significatifs.

Le **Chapitre VII** est centré sur les perspectives futures liées aux applications de la LC-MS/MS. Le progrès en SPE on-line et la séparation chromatographique (UPLC) sont les candidats potentiels pour l'analyse des drogues dans les matrices conventionnelles et alternatives (comme le fluide oral), de sorte qu'on est en train de métamorphoser la méthode dans laquelle la séparation et le traitement des échantillons appliqués dans les laboratoires de grande productivité.

Resumen

El objetivo de esta tesis ha sido la evaluación del análisis de drogas, relevantes desde el punto de vista forense, en sangre y orina mediante métodos LC-MS/MS convencionales y avanzados.

El **Capítulo I**, presenta una breve revisión de la farmacología de las drogas incluidas en esta tesis (anfetaminas, canabis y alucinógenos) y sus efectos en la actitud y comportamiento humano. Comenzando por los alucinógenos, son sustancias psicoactivas que alteran en gran medida la percepción, el humor y la razón. Están considerados fisiológicamente seguros al no producir dependencia o adicción. Su origen se remonta a las culturas más ancestrales siendo empleados en rituales sagrados y otros contextos socioculturales. Hoy en día, el canabis es la droga ilícita más consumida en Europa, de tal modo que casi un cuarto de los europeos lo han probado alguna vez en su vida. Varios de los efectos del canabis son relajación, bienestar y somnolencia. Otras drogas que constituyen un problema importante en Europa son las anfetaminas. Estimulan el CNS, aumentando la presión arterial y disminuyen el apetito, entre otros efectos.

LC-MS(/MS) es una técnica ampliamente utilizada en los laboratorios forenses, especialmente en el análisis de sustancias no volátiles, térmicamente inestables o de alto peso molecular. El **Capítulo II** tiene como objetivo dar una idea general de las aplicaciones recientes con LC-MS(/MS) que existen en los laboratorios forenses y dar información detallada sobre la ionización, la separación y detección de iones, además de la espectrometría de masas en tandem. El fenómeno conocido como supresión o aumento de la ionización de los iones en el análisis mediante LC-MS(/MS) depende principalmente de la naturaleza de la matriz, del proceso de preparación de la muestra, de la calidad de la separación cromatográfica, de los aditivos de la fase móvil y del tipo de ionización. Por lo tanto, los procedimientos típicos de preparación de la muestra son PPT, LLE y SPE. Una óptima preparación de la muestra da lugar a un aumento de la sensibilidad y la selectividad. Al final de este capítulo se encuentra información detallada sobre las recientes aplicaciones de LC-MS(/MS) para el análisis de anfetaminas, canabis y alucinógenos en sangre y orina.

No obstante, la preparación de la muestra está considerada como un proceso laborioso que requiere mucho tiempo. En el **Capítulo III** se describen los desarrollos recientes en SPE on-line (en línea) respecto a la alta producción en los análisis cuantitativos de drogas en las matrices biológicas. Los análisis de alta producción

se están volviendo primordiales en los laboratorios forenses. Uno de los sistemas comercializados con SPE on-line es el sistema Symbiosis fabricado por Spark Holland. En el sistema SPE on-line el material de extracción está empaquetado con partículas muy pequeñas como las columnas LC (cartuchos) que trabajan a altas presiones a las que se les aplican los disolventes de extracción. Las válvulas de cambio que posee este sistema dirigen el flujo hacia la columna analítica LC o al deshecho, según convenga. A diferencia de la clásica SPE off-line, este sistema on-line permite ahorrarse varios etapas intermedias. Posteriormente, la muestra extraída es inyectada directamente a la columna analítica mediante una simple válvula de cambio. Por lo tanto, no hay pérdida de volumen de muestra, aumentado la sensibilidad. En este capitulo también se incluye una revisión bibliográfica que existe hasta el momento sobre las aplicaciones de este instrumento al análisis de drogas en fluidos biológicos.

El **Capítulo IV** presenta los objetivos del estudio que son la evaluación de la técnica LC-MS/MS convencional y la nueva tendencia, SPE-LC-MS/MS on-line (Symbiosis), para el análisis de:

a) numerosos alucinógenos, clorofeniramina, ketamina, ácido ritalínico y metabolitos en orina con SPE off-line,

b) THC y metabolitos en sangre mediante LLE off-line como proceso de preparación de la muestra,

c) THC-COOH (principal metabolito del THC en orina) mediante SPE on line y

d) 7 anfetaminas y metabolitos en sangre y orina mediante SPE on-line.

El **Capítulo V.I**. ofrece el desarrollo y la validación de un método con LC-MS/MS para la cuantificación de alucinógenos y otros compuestos relacionados en orina. El método consiste en una LLE off-line, evaporación a seco y reconstitución en la fase móvil. La duración total del análisis es de 20 min. Con cada serie se han analizado QC externos que contenían LSD. El método ha sido completamente validado y aplicado al análisis de orinas auténticas (que contenían psilocina, ketamina, norketamina y clorofeniramina). El **Capítulo V.II** describe la validación del método para el análisis de THC y dos de sus metabolitos en sangre. Como proceso de limpieza de la muestra se empleó LLE con hexano: acetato de etilo, seguida de centrifugación, evaporación completa y reconstitución. El tiempo de duración del análisis fue de 13 min. Se añadieron dos QC externos en cada serie de análisis. El método ha sido completamente validado en cuanto a precisión, exactitud,

especificidad, recuperación, efecto matriz y estabilidad. Finalmente el método se aplicó al análisis de muestras de sangre de auténticos casos forenses. El **Capítulo V.III** se centra en el desarrollo y validación de un método utilizando el sistema Symbiosis para el análisis de THC-COOH en orina (500 µL). Como el THC-COOH esta glucuronizado en orina, se realizó una hidrólisis previa con KOH 10 M. A continuación, la orina diluida fue acidificada directamente en los viales LC para su inyección inmediata. El método fue validado completamente y aplicado al análisis de auténticas muestras de consumidores de canabis. Otra aplicación del sistema SPE-LC-MS/MS on-line se presenta en el **Capítulo V.IV**. para la cuantificación directa de 7 anfetaminas y metabolitos en sangre y orina. El método, que ha sido enteramente validado según las normas internacionales, requirió una mínima manipulación: la dilución en fase móvil acuosa, conteniendo el IS, de 100 µL de sangre o 50 µL de orina, directamente en los viales LC, previa a la inyección. Para la limpieza de la muestra se emplearon cartuchos HLB. El método fue aplicado al análisis de sangre y orina de un estudio con MDMA y a casos forenses.

Las conclusiones del **Capítulo VI** exponen las ventajas del LC-MS/MS y la nueva tendencia en SPE online. Los resultados demuestran que aunque los métodos LC-MS/MS convencionales siguen siendo robustos y seguros, la SPE on-line acoplada al LC-MS/MS es altamente efectiva en cuanto a ahorro de tiempo y productividad.

El **Capítulo VII** se centra en las perspectivas futuras en relación a las aplicaciones con LC-MS/MS. El progreso en SPE on-line y la separación cromatográfica (UPLC) son los candidatos potenciales para el análisis de drogas en matrices convencionales y alternativas (como la saliva), de manera que está reformando el modo en el que la separación y tratamiento de la muestra estas siendo realizadas en los laboratorios con gran productividad.

I. INTRODUCTION:

PHARMACOLOGY OF AMPHETAMINES, CANNABINOIDS AND HALLUCINOGENS

1. 1. Introduction

One of the biggest problems facing society today is the abuse and misuse of drugs. There are numerous drugs available to the community at large. Many offenders charged with violent crimes, or victims of violent crime, may have been under the influence of psychoactive drugs at the time a crime was committed. Therefore, it is important to understand the possible pharmacological effects associated with the use of these drugs.

The principal drugs of concern can be divided into the categories shown in Table 1.

Table 1. Principal classes of psychoactive substances (1)

CNS depressants		
1	Ethanol	All alcoholic beverages (alcohol)
2	Benzodiazepines	Alprazolam, diazepam, flunitrazepam, oxazepam, temazepam, etc
3	Opioids	Codeine, heroin, methadone, morphine, oxycodone, pethidine, etc
4	Antipsychotics	Chlorpromazine, clozapine, fluphenazine, haloperidol, olanzapine, etc
5	Antidepressants	Amitriptyline, doxepin, dothiepin, fluoxetine, moclobemide, sertraline, etc
6	Marijuana	Various forms of Cannabis sativa containing tetrahydrocannabinol
7	Barbiturates	Amylobarbital, butobarbital, secobarbital, phenobarbital, thiopental, etc
CNS stimulants		
8	Amphetamines	Speed' (methamphetamine), 'ecstasy'
9	Cocaine	Free base 'crack' and hydrochloride
10	Other stimulants	Ephedrine, pseudoephedrine, phentermine, fenfluramine, etc
Other substances		
11	Inhalants	Petrol, solvents, propane (LPG), paint, butane lighter fluid, etc
12	Hallucinogens	LSD, ecstasy, plant-derived substance such as mescaline, psilocybin, etc
13	Phencyclidine	Usually abbreviated as PCP, and ketamine
14	Anabolic steroids	Testosterone, stanozolol, etc

Cannabis in its various forms is arguably the second most commonly abused drug after alcohol. Smoked, eaten, imbibed –or just talked about- it seems the world has a strong appetite for it. An estimated one in five European adults has tried cannabis, in such a way that globally, nearly 50 000 tones of cannabis herb or resin is produced each year. Little wonder, then, that cannabis has become a controversial cultural and commercial phenomenon (2).

On the other hand, drugs such as MDMA (3,4-MethylenenDioxyMethAmphetamine), ketamine and LSD (Lysergic Acid Dietylamide), are typically used by teenagers and young adults at bars, clubs, concerts, and parties, and the use of these drugs is reported to help maintain energy levels for dancing or to enhance an altered state of consciousness (3). The use of these called 'club drugs' has increased significantly over the past 2 decades. One reason for their augmented use is the easy availability and low cost.

In the following, the drugs studied in this thesis are described.

1.2. Amphetamines

In many European countries the second most commonly used illegal substance is some form of synthetically produced drug. The use of amphetamines and related drugs among the general population is typically low, but prevalence rates among younger age groups are significantly higher, and in some social settings or cultural groups the use of these drugs may be particularly high. The amphetamines are central nervous system (CNS) stimulants, with similarities to some naturally occurring weak stimulants like ephedrine, a weak stimulant that occurs naturally in the branches of a number of an Ephedra species in concentrations up to 1.2% (4). The amphetamines are related in structure to the legal stimulants. Substitutions on the nitrogen and the ring system account for most of the structural variations and differences in stimulating and euphoric effects. Due to the chiral carbon atom adjacent to the nitrogen and to which a methyl group is attached, stereoisomerism has some serious implications in analytical chemistry and forensic toxicology (Figure 1).

Figure 1. Structures of selected amphetamines

Amphetamine Methamphetamine MDMA

MDA MDEA

PMA Ephedrine

The abused amphetamine analogues are synthesized in clandestine laboratories by a variety of chemical processes from a number of precursor molecules i.e. from ephedrine or pseudoephedrine to d-methamphetamine. The presence of significant quantities of the starting product and impurities in the street samples allows for profiling of the source of drug. MDMA ('ecstasy') has become one of the most popular recreational drugs among young people visiting raves and mega house parties. Although the media often write about a very fast evolution in the choice of stimulants: MDEA (3,4-MethyleneDioxy-N-EhylAmphetamine) ('Eve'), MDA (3,4-MethylenenDioxyAmphetamine), MBDB (3,4-MethylBenzoDioxolylButanamine), PMA (4-Para-MethoxyAmphetamine), etc, our experience in the INCC in Belgium, is that amphetamine and MDMA are by far the most popular drugs, with other variants popping up only occasionally.

Physical effects can include reduced appetite, increased/distorted sensations, hyperactivity, tachycardia, increased blood pressure, sweating, etc. Psychological effects can include anxiety and/or general nervousness, euphoria, creative of philosophical thinking, perception of increased energy, increased sense of well being, feeling of power or superiority, talkativeness, etc (1).

The amphetamine-like stimulants have good oral and intranasal bioavailability and give maximum blood concentrations (0.1-0.4 µg/mL) within 1-3 h after a normal single dose. Proportionally higher concentrations are expected with higher doses, although non-linear kinetics of MDMA in humans has been described. The amphetamines are metabolized by similar pathways that involve a combination of hydroxylation of the ring and the side-chain carbon atom adjacent to the ring, and removal of the nitrogen. Drugs with alkyl groups on the nitrogen are dealkylated sometimes producing pharmacologically active metabolites: methamphetamine is metabolized to amphetamine, MDMA and MDEA are both metabolized to MDA. All metabolites with hydroxyl groups are excreted as conjugates (5,8).

The half-life of amphetamines varies from 3-6 h to more than a day. Drugs with relatively long elimination half-lives will often show an accumulation of blood concentration with repeated dosing (chronic administration). Their clearance is particularly sensitive to the pH of urine. This is due to the basic nature of amphetamines, which are excreted rapidly if urine is acidic (increases ionization of drug), but only relatively slowly if urine is basic. Detection times in urine range up to 1-2 days following usual doses. In contrast to many other drugs of abuse, substantial

amounts of unchanged drug are excreted in urine. Amphetamine is excreted almost completely unchanged when urine is kept acidic, while basic urine will retard elimination and allow substantial metabolism to occur. Similar phenomena occur with other amphetamine analogues, including methamphetamine and ephedrine. (1,9-12). Thus, under normal conditions, methamphetamine is excreted largely as the parent drug (40%) following smoked and intravenous single doses, while only 7% of the dose is excreted as its main metabolite, amphetamine (13,14). Abstinence from amphetamine can still result in detection in urine for 2 days. However, high-dose abusers can have urine positives for up to 9 days after last use (15,16). MDMA is metabolized to the demethylated active analogue, MDA. The MDA to MDMA urine concentrations are 0.15 or less following consumption of MDMA (17-19). Ephedrine is substantially excreted into urine unchanged (70% of dose). A single dose would be detectable in urine for 24-36 h (20).

1.3. Cannabis

Cannabis is the collective term for the psychoactive substances of the Cannabis sativa plant and one of the most frequently used illicit drug in the Western World. Cannabis preparations include marijuana plant material, hashish (resin of female flowering tops) and hashish oil (extra from the resin). Cannabis is consumed by inhalation by either smoking or using a bong or a water pipe, or by ingestion of baked cannabis products (21). Cannabis products are mostly smoked in combination with tobacco. Thus, Δ^9-tetrahydrocannabinol (THC), the primary psychoactive analyte, is found in the plant's flowering or fruity tops, leaves, and resin. There are over 421 different chemical compounds in cannabis, including 61 cannabinoids, chemical compounds containing 21 carbons atoms related to THC. During smoking more than 2000 compounds may be produced by pyrolysis; nitrogenous compounds, amino acids, hydrocarbons, sugars, terpenes, and simply fatty acids, are contributing to known pharmacological and toxicological properties of cannabis. The composition of cannabinoids varies depending on growing conditions, plant strain and the age of the sample. Smoking cannabis produces a much quicker absorption with maximum plasma concentrations occurring within a few minutes of smoking. The amount absorbed is also much higher than with ingestion; studies show a bioavailability of 14-50%, since THC is activated by a heating process through a decarboxylation reaction (22).

The acute behavioral and physiological effects of cannabis have been well described (23-28): euphoria, relaxation, well-being, somnolence, changes in visual and auditory perception, altered perception of time and space, short-term memory loss, impaired learning, reduced performance, anxiety and panic reactions, disphoria, hallucinations, flashbacks, cardiovascular symptoms, red conjuctiva, small increase in pupil size, and reduced lacrimation

There are three metabolites with significance: the 11-hydroxy (11-OH-THC), the 9-carboxy (THC-COOH) and the glucuronide of THC-COOH (Figure 2). Other metabolites found in blood are the 8- and 2-hydroxy-THC metabolites.

Figure 2. Major metabolic route for THC

THC —Hydroxylation→ 11-OH-THC —Oxydation→ THC-COOH

THC —Hydroxylation→ 8-OH-THC

11-OH-THC —Hydroxylation→ 2-OH-THC

THC-COOH —Glucuronidation→ THC-COOH-glucuronide

The oral availability of cannabis is about 6%, which means that only 6% of the ingested THC is absorbed into the blood stream. The remainder is metabolized prior to entry into the blood stream, or is not absorbed. THC is measurable in plasma within seconds after inhalation of the first puff of marijuana smoke. Concentrations continue to increase rapidly and peak concentrations occur at approximately 9 min. Whole blood cannabinoid concentrations are approximately one half of the concentrations found in plasma due to the low partition coefficient of drug into erythrocytes (THC is 97-99 % protein bound in plasma). Once absorption has taken place, THC is rapidly distributed to tissues, concentrations being highest in adipose (fat) tissue due to its low water solubility and high affinity for fatty tissues. This distribution phase results in a rapid decline in blood plasma THC concentrations. THC concentrations greater than 10 ng/mL are uncommon after 1h even after moderate to high doses of cannabis (29-35). The terminal elimination half-life of THC is reported as 4-5 days, with a range of 3-13 days, although a shorter half-life of about 1 day has also been quoted. At first glance, this relatively long half-life contradicts the well-known short action of cannabis. However, this half-life is

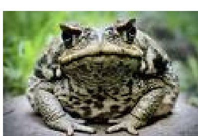
calculated after the process of distribution to tissues has taken place and represents the terminal phase of the elimination curve. In high-dose (3.5% cigarette) users of marijuana, concentrations of THC can be detected for up to 24h after last use (>0.5 ng/mL) (32,36-38).

The metabolite THC-COOH is also found in the blood of cannabis users (39). Because of difficulties in measuring THC accurately (because of the low concentrations), laboratories frequently measure also this inactive metabolite. Plasma concentrations of unconjugated THC-COOH following a 1.97% THC cannabis cigarette peak at 43 ng/mL at 20 min after smoking. By 6 h plasma concentrations fall to 13 ng/mL. 11-OH-THC is also produced from THC metabolism and also exhibits THC-like activity. Blood concentrations are rapidly detected following a 1.75% or 3.55% marijuana cigarette within 15-20 min. This metabolite is only detectable in blood for 12-24 h after normal-strength marijuana cigarettes (40,41).

The main metabolite of THC in urine is THC-COOH. However, interpretation of results in urine is not simple. The amount of time that THC-COOH remains detectable in urine depends of the following factors: amount and frequency of use of cannabis, the metabolic rage, the body mass, the age, the overall health, the drug tolerance and the urine pH. Some researchers have used urinary THC-COOH concentrations in excess of 80 ng/mL to imply impairment. However, others have found little predictive value in using urinary THC-COOH concentrations, especially since 24% of users would have blood THC concentrations of less than 1 ng/mL, a concentration that does not normally cause impairment. Many persons with impaired THC concentrations, would not be impaired based on urinary THC-COOH concentrations (42,43). Gustafson et al (44) demonstrated that the terminal urinary elimination of THC-COOH following oral administration was approximately two to three days for doses ranging from 0.39 to 14.8 mg/d.

1.4. Hallucinogens and related compounds

1.4. 1. Bufotenine



Bufotenine (Figure 3) is a hallucinogenic substance that exists naturally in some plants and in the cutaneous secretions of amphibians. (45-47). Although it is used as a therapeutic agent in China and other Asian countries, and as an aphrodisiac, 'love stone' in the West Indies, recent reports indicate its toxicity may carry a significant mortality rate (48,49). On the other hand, bufotenine has long been accepted as a naturally occurring component of human blood, brain and cerebral spinal fluid. However, while its biological presence at low concentrations is acknowledged, the biological function remains a mystery (50).

Figure 3. Structure of bufotenine

There is not so many information about the pharmacokinetics of bufotenine. Fuller et al (51) administered bufotenine subcutaneously in rats (1-100 µg/kg). It was distributed mainly to the lungs, heart and blood, and to much lesser extent, the brain and liver. It reached peak concentrations at 1 hour and it was nearly completely eliminated within 8 hours. In humans, bufotenine is rapidly absorbed following intravenous administration and it is excreted predominantly in the urine (70%).

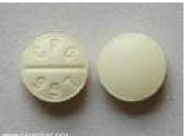
1.4.2. Cathinone

Khat (Catha edulis Fosk) is an evergreen plant that grows at high altitudes in East Africa and Arabian Peninsula, being used as a stimulant plant for centuries. Chewing its fresh leaves is a widespread habit in local populations, with several million people consuming khat regularly in social sessions that often last for hours. Its current use among particular migrant communities in Europe and elsewhere has caused alarm among policy makers and health care professionals (52,53). A typical dose of 100 g fresh khat leaves contains on average 36 mg cathinone (Figure 4), with a higher content of cathinone in young leaves that are still growing (54). Users of khat report increased levels of energy, alertness and self-esteem, a sensation of elation, enhanced imaginative ability and a higher capacity to associate ideas (55-57).

Figure 4. Structure of cathinone

Chewing results in a high extraction of the alkaloids with only 9% remaining in the leaf residues. A chewing dose of 45 g khat leaves i.e. 0.6 g/kg of body weight results in a mean absoption dose of 45 mg of cathinone. The euphoric effects of khat start after about 1h of chewing of 60 g fresh khat leaves per subject. Blood levels of cathinone start to rise within 1h and peak plasma levels are obtained 1.5-3.5h after the onset of chewing (58). Maximum plasma levels range from 40 to 140 ng/mL after 1 h chewing. In plasma, cathinone is detectable up to 24h. The elimination half life is some 260 min. Only 2% of administered cathinone was found unchanged in the urine (53,59).

1.4.3. Chlorpheniramine



Chlorpheniramine is a first generation alkylamine antihistamine used in the prevention of the symptoms of allergic conditions such as rhinitis and urticarian (Figure 5). Its worldwide popularity as an antihistamine is based on effectiveness, low cost (generic) and tolerability (60). Adverse side effects have been reported when used alone or in combination with other drugs. These side effects include hypertension, arrhythmias, seizures, hallucinations, agitation and anxiety, and psychosis (61,62). Many of these reported reactions occurred after ingestion of the drug over a prolonged period or in dosages in excess of recommended allowances. Ishigooka et al (63) described the mental disturbances of 44 abuses cases of “Bron”, an over-the-counter cough suppressant solution containing chlorpheniramine and other medicaments. Major psychiatric symptoms observed included hallucinatory paranoid state.

Figure 5. Structure of chlorpheniramine

Although its widespread use, surprinsingly little has been reported about its pharmacokinetics profiles in man. Chlorpheniramine after oral administration is strongly metabolized in the liver by demethylation. Two metabolites have been identified: monodesmethylchlorpheniramine and didesmethylchlorpheniramine. After a single therapeutic dose, the metabolites cannot be detected in blood. In urine, unchanged chlorpheniramine account for about 20% of the dose (64,65).

1.4.4. Kavain

Kava-kava is the name given by Pacific islanders to both a shrub Piper methylsticum G. Foster belonging to the pepper family and psychoactive beverage made from the rhizome (by extraction with coconut milk). Kava-kava may have first been domesticated less than 3000 years ago in Vanuatu (nowadays called the New Hebrides), a group of islands in eastern Melanesia. The use of kava-kava seems then to have spread both westwards to New Guinea and part of Micronesia and eastward into Fiji and the Polynesia. Locally the plant is known by a number of common names, including kawa-kawa, ava ava, awa awa, yati, and yagona. The beverage is used in social and ceremonial life because of the narcotic and soporific effects and it counteracts fatigue, reduces anxiety and generate state of well being. Extracts of kava-kava with 30-70% kavalactones have also been employed in the western medicine for the sedative, muscle relaxant, analgesic, anticonvulsive, anaesthetic, anti-arrythmic, anti-thrombosis, neuroprotective and anti-spasmodic (66-70). However, serious side effects, e.g. hepatitis and acute liver failure were observed recently (71). This led to its ban in many countries, which has prompted wide discussion in its relative benefits and risks as a social beverage and an herbal remedy.

Figure 6. Structure of kavain

Only a few methods have been applied for the detection of kava consumption or intoxication and are focused on the non-alkaloids kavalactones (72). Koeppel and Tenczer identified 10 metabolites of kavain in urine after oral consumption of the substance (73). Other more sensitive methods have been developed to investigate the pharmacokinetics in blood, urine and hair (74,75). Most metabolites were mainly excreted in the form of their conjugates. All kavain metabolites were detectable in serum and urine. Within 1 and 4 h after oral administration, the serum concentrations ranged between 40 and 10 ng/mL for kavain (Fiugre 6).

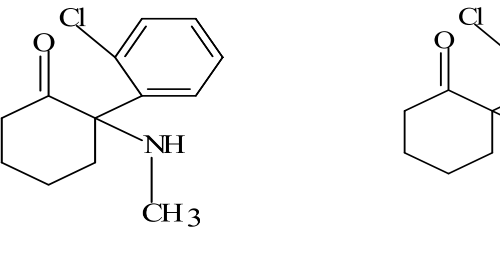
1.4.5. Ketamine

Ketamine was first synthesized by Calvin Stevens in 1962. Early clinical studies on ketamine with human volunteers found it to be more effective and shorter acting than phencyclidine, with fewer emergence symptoms and less toxicity. The drug was first manufactured in the United States in the 1960s as Ketalar. Use of ketamine as a surgical anesthetic escalated when it gained popularity on the battlefields of Vietnam (1). It was promoted as a dissociative anesthetic because of its ability to induce a lack of responsive awareness, not only to pain but also to the general environment.

By the early 1980s a wide range of unauthorized preparations were available in the US including capsules, powder, crystals, tablets and solutions, in addition to the authorized injectable forms. Solutions sold on the street have gone by names such as K, Kay, Jet, Super Acid, vitamin K and Special K (1,9,76,77).

At low doses ketamine induces distortion of time and space, hallucinations and mild dissociative effects. However, at large doses (i.e. over 150 mg) ketamine induces more severe dissociation commonly referred to as a ‘K-hole’, wherein the user experiences intense detachment to the point that their perceptions appear located deep within their consciousness, thus causing reality to appear far off in the distance (77,78). Since ketamine is odorless and tasteless, it can be added to beverages, without being detected, to induce amnesia. Because of such properties, the drug is sometimes misused in a sexual assault, referred as date-rape drug (79).

Figure 7 .Ketamine and norketamine

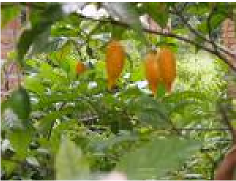


Ketamine Norketamine

Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist which interferes with the action of excitatory amino acids including glutamate and aspartate (80). Ketamine has a plasma half-life of 2-4h. It is highly lipid soluble and has a distribution half-life of approximately 7-11 min. Ketamine is metabolized to at least two compounds: first by N-demethylation, to norketamine, which has 1/3-1/2 of the potency of ketamine. Norketamine is further dehydrogenated to produce dehydronorketamine (81-83). The parent compound and both major metabolites are further transformed by hydroxylation and conjugation prior to elimination (Figure 7).

About 90% of a dose is excreted in the urine in 72h, with about 2% of the dose as unchanged drug, 2% as norketamine, 16% as dehydronorketamine and 80% as conjugates of hydroxylated metabolites.

1.4.6. Ibogaine



Ibogaine is a naturally occurring plant alkaloid with a history if use as a medicinal and ceremonial agent in West Central Africa. It is an indole alkaloid found in the root bark of the Apocynaceous shrub Tabernanthe iboga. It is used by native Africans to offset hunger and fatigue but when abused, ibogaine causes hallucinations (72,84). The onset after ingestion is within 1 to 3 hours, with a duration of action in the order of 4 to 8 hours. The predominant reported experiences appear to involve a panoramic readout of long-term memory, particularly in the visual modality, and “visions” or “walking dream” states featuring archetypal experiences such as contact with transcendent beings, passage along a lengthy path, or floating (85). Recent studies also reported the efficacy of this drug in the treatment of drug addiction. Thus, ibogaine attenuate both dependence and withdrawal symptoms to a variety of abused drugs including morphine, heroin, cocaine, amphetamine, alcohol and nicotine (86). The neurochemical mechanisms explaining the antiaddictive properties of ibogaine are not clearly understood.

Figure 8. Structure of ibogaine

Pharmacokinetics data relative to ibogaine in humans are limited (Figure 8). Following single doses of ibogaine (500 to 800 mg) to individual subjects, maximum ibogaine and noribogaine (its main metabolite) blood concentrations of 30-1250 ng/mL and 700-1200 ng/mL are obtained approximately 2 and 5 hours after drug administration, respectively. Thereafter, ibogaine is cleared rapidly from the blood while noribogaine concentrations remain high. Indeed, concentrations of noribogaine measure at 24 h postdose were in the range of 300-800 ng/mL (87). It is also established that ibogaine is largely deposited in fat and to a lesser extent in brain. Ibogaine and noribogaine are excreted via the renal and gastrointestinal tracts, with a 60 to 70% elimination in urine and feces within 24 hours (85,88).

1.4.7. Lobeline

Lobeline is a lipophilic, non-pyridino, alkaloidal constituent of Lobelia inflate, also known as Rapuntium inflatum, Indian weed, pokeweed, asthma weed, gagroot, vomitwort, bladderpod, eyebright, and Indian tobacco (89) (Figure 9). It has many nicotine-like effects, including tachycardia and hypertension, hyperalgesia (90). Lobeline was previously investigated as a therapeutic agent to treat tobacco dependence and it has been demonstrated to inhibit the effect of amphetamines in behavioral and neurochemical assays (91,92).

Figure 9. Structure of lobeline

To our knowledge, there is still no information about the pharmacokinetics of lobeline in humans. Just one paper from Song et al (93) identified the parent drug and ten metabolites in rat urine 24 h after administration.

1.4.8. LSD

Although drugs producing sensory distortions have been used by man for several millennia, many consider the modern era of psychedelics to have begun when the psychotropic effects of LSD were discovered by Albert Hofmann in 1943. This discovery ushered in an era of intense LSD research, with nearly 1000 articles appearing in the medical literature by 1961 (94). Most of this early research was based upon the drugs' capacity to produce a "model psychosis" (95). By the mid-1960s, LSD and other related drugs had become associated with various counterculture movements, depicted as dangerous, and widely popularized as drugs of abuse. Accordingly, scientific interest in these drugs faded by the late 1960s, but human research with related psychedelics has recently experienced a slight renaissance (84,96-98). What the experience of the 1960s has pointed to many possible therapeutic and non-medical uses. Previous clinical experience, plus more recent informal use, has indicated other potential therapeutic uses for cluster headaches and addictions, among other conditions.

The late Daniel X. Freedman made comments consistent with that assessment, stating, "one basic dimension of behavior…compel-compellingly revealed in LSD states is 'portentousness'-the capacity of the mind to see more than it can explain, to believe in and be impressed with more than it can explicate, to believe in and be impressed with mire than it can rationally justify, to experience boundlessness and 'boundaryless" events, from banal to the profound" (99). Although these descriptions focus on the more spectacular effects that these substances are capable of producing, low doses generally elicit less dramatic results. Typical clinical effects of LSD and related hallucinogens would include the following:

- Somatic symptoms: dizziness, weakness, tremors, nausea, drowsiness, paresthesias, and blurred visions
- Perceptual symptoms: altered shapes and colors, difficulty in focusing on objects, sharpened sense of hearing, and rarely synesthesias.
- Psychic symptoms: alterations in mood (happy, sad, or irritable at varying times), tension, distorted time sense, difficulty in expressing thoughts, depersonalization, dreamlike feelings, and visual hallucinations (100).

LSD can induce disturbances of experience, otherwise observed only in psychoses, such as alteration of cognitive functions, and depersonalization. However, this drug does not appear to produce illness in emotionally healthy persons, but these problems seem to be precipitated in predisposed individuals.

In atypical courses of intoxication, so-called "bad trips", anxiety and excitement predominate. A bad trip is a disturbing experience whose manifestations can range from feelings of vague anxiety and alienation to profoundly disturbing states of unrelieved terror, ultimate entrapment, or cosmic annihilation. The potential causes can be a result of wrong set and settings (101,100).

Figure 10. Structures of LSD and some key metabolites

13-OH-LSD

2-Oxo-3-OH-LSD

LSD

N-Desmethyl-LSD

Desethyl-LSD

LSD acts as an antagonist of peripheral 5-HT receptors, but acts as an agonist at CNS 5-HT receptors. Peak blood concentrations of LSD are typically less than 10 ng/mL. Maximum plasma concentrations following 70 µg dose range up to 2 ng/mL. The maximum blood levels occur around 30-90 min, but psychedelics effects of the drug occur within 5-10 min. The elimination half-life is estimated at 3-6h. Blood concentrations of drug are therefore quite low by 8h post-dose often less than 1 ng/mL. The duration of the action of LSD parallels approximately the blood concentrations, with 'trips' lasting from a few to several hours depending on the dose used (1).

LSD is rapidly metabolized with only about 1-3% of an oral dose excreted in the urine as unchanged LSD. The major metabolites in urine are nor-LSD and 2-oxo-3-OH-LSD (Figure 10). In addition, glucuronide conjugates of 2-oxo-3-OH-LSD are also present. Because of the low blood concentrations, LSD is most often measured in urine. Peak urine concentrations following oral ingestion of a typical street dose are normally less than 10 ng/mL and drop bellow 1 ng/mL within 12-24h. Therefore, extremely sensitive analytical methods are required to detect LSD use for more than 1 day after ingestion of the drug. 2-oxo-3-OH-LSD can be detected in urine up to 96h after administration, whereas LSD can only be detected for 12-24h post-administration (102,103).

1.4.9. Mescaline

Mescaline is one of the classic hallucinogens, which is known as the major alkaloid of the cactus peyote (Figure 11) (33,45).

Peyote grows within a narrow strip of desert along the Texas-Mexico border. When properly cut, the crown ("button") is removed from the plant leaving the root intact. Peyote is not smoked but eaten as peeled fresh buttons, dried whole buttons, dried/ground powder (sometimes also reconstituted in water), or is steeped/reconstituted into a warm tea. Peyote is most commonly consumed as a sacrament in the all-night ceremonies of the Native American Church (104,105). The effects of peyote and mescaline in humans are well studied. Native peyote cults used the cactus because it produces rich visual hallucinations. These psychoactive effects were used in psychiatric studies as a chemically induced model of mental illness (72).

Figure 11. Mescaline

H_3CO NH_2
H_3CO
OCH_3

Typical hallucinogenic doses range from 200 to 500 mg of mescaline with blood concentrations of 3.8 mg/L at 2 h and 1.5 mg/L at 7 h after ingestion (106). Following intake, mescaline is mainly excreted in the urine unchanged from (55-60%) (107).

1.4.10. Psilocin

In recent years, the recreational use of hallucinogenic mushrooms, so-called “magic mushroom”, has become an increasing social problem in several countries. They are not only naturally occurring but also offered as kits for cultivation, and contain the hallucinogenic indole derivatives, psilocin and psilocybin (Figure 12). Differences in the psilocin and psilocybin contents of the fruit bodies depends on the factors such as species, developmental stages, climatic conditions and the availability of soluble nitrogen and phosphorous in the soil (108). Thus, it takes about 30 fruitbodies of Psilocybe semilanceata to produce hallucinatory experience. Other large fungi which also contain psilocybin and psilocin are Psilocybe cubensis Panaeolus spp, Copelandia spp and Gymnopilus spp (109). There are several reports on the contents of psilocin and psilocybin in magic mushrooms (110-112) .

Figure 12. Psilocin and psilocybin

Psilocybin Psilocin

The rapid and extensive cleavage of the phosphoric ester group of psilocybin by alkaline phosphatase and unspecific esterases indicates that psilocybin acts as a prodrug and that its hydroxyl metabolite psilocin represents the true pharmacologically active agent (110,113). Hasler et al (114) compared the pharmacokinetic parameters in plasma and urine after an oral administration of psilocybin to 6 subjects. Within 24h about 0.9% of the applied dose of psilocybin was excreted as free psilocin. However, attention must be paid to the stability of psilocin. According to Tiscione et al (115), urine concentrations of psilocin decrease rapidly even kept in the refrigerator at 4°C. In conclusion, we may find psilocin in urine during 26-48 hours after administration.

1.4.11. Ritalinic acid (methylphenidate)

Methylphenidate (Ritalin) is a phenethylamine derivative used in the treatment of depression, narcolepsy, attention-deficit disorder, and childhood hyperkinesis (116-119). Recently, methylphenidate has become a popular drug of abuse with the usual mode of administration being intravenous injection of dissolved tablets often in combination with the drug pentazocine. Pentazocine (Talwin) is a synthetic benzomorphan derivative that has properties of an analgesic and is about one third to one sixth as potent as morphine. The combination of Ritalin and Talwin is commonly referred to on the street as “poor man’s heroin’. The fact that methylphenidate is commonly prescribed in the treatment of attention-deficit disorder provides a likely source for drugs users.

Figure 13. Ritalinic acid and methylphenidate

H N OCH 3 O

Methylphenidate

H N OH O

Ritalinic acid

Methylphenidate is reported to be absorbed quickly and completely from the gut after oral administration and it is rapidly hydrolyzed in the methyl ester linkage to its metabolite, ritalinic acid (Figure 13). Minor metabolic pathways for both these compounds include parahydroxylation of the aromatic ring, oxidation to 6-oxo-derivates and glucoronide formation. Both methylphenidate and ritalinic acid are usually measured in plasma and urine (120,121). Following oral administration of methylphenidate peak plasma levels at 2 h. Because about 70% of methylphenidate is eliminated in the urine as ritalinic acid, it is obviously a better indicator (more prevalent) that the parent methylphenidate for detecting usage (118,122).

4.12. Scopolamine

The Datura plants are members of the botanical family Solonaceae, which also contains common foods as tomatoes, potatoes, eggplants, peppers, and tobacco. This plant is originated from the tropical areas of Central and South America and it is now a cosmopolitan weed in temperate regions (104). The toxicity of Datura species is well known and has been linked deaths and poisonings for centuries (123). Traditional preparations include adding roots, leaves, or seeds to a fermented drink; drinking an infusion of the leaves or other parts; smoking the leaves; or chewing the fruit. When the plant material is taken orally, the effects last longer than when smoked and will also be more narcotic and hallucinogenic (124,125).

The main toxic components are tropano alkaloids: hyoscyamine, which forms a diastereomeric mixture know as atropine and scopolamine (Figure 14). Scopolamine is an antimuscarinic agent (used as analgesic) and a smooth muscle relaxant. It is also an antispasmodic agent with antinauseant properties, and is extensively used in the treatment of motion sickness and in pre-operative medication (126,127). Weak infusions are used as hypnotics by the elderly and as aphrodisiac by adults. Datura species have also been used in criminal activities (128).

Figure 14. Structure of scopolamine

Up to now, scopolomine metabolism in man has not been verified stringently. An elucidation of the chemical structures of the metabolites extracted from human urine is still lacking. Huaixia et al (129) described the determination of scopolamine and its main metabolites in rat urine after ingesting 55 mg/kg scopolamine. Eighteen metabolites and the parent drug could be detected for up 106 h. In human,

pharmacokinetic parameters are dependent on the dosage form. According to Renner et al (130), scopolamine has a limited bioavailability if orally administered. The maximum drug concentrations occurs approximately 0.5 h after oral administration, and only 2.6% of nonmetabolized scopolamine is excreted in urine.

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II. CONVENTIONAL LC-MS/MS METHODS

2.1. Introduction

The analysis of drugs of abuse, particularly illicit drugs, is one of the most important challenges of forensic and forensic toxicology. As with any other branch of forensic sciences, forensic toxicological analysis must also apply and keep particularly high standards of quality. This is because the analytical result may have a direct and permanent impact on the fate of the person involved. As a consequence, forensic toxicological examinations are subjected to very tight scrutiny. This requirement should concern not only primarily forensic cases, but also extend to all forensic analysis for illicit drugs. It must be kept in mind that each result of drug analysis has potential forensic relevance, irrespective of the primary purpose of examination. It has often been said that the interpretation of results is the most important and difficult part of forensic toxicology. Notwithstanding the value of correct interpretation, it must also be said that the correct result enables any further action. This is particularly true when the analytical result itself may serve as evidence of illegal action.

The analysis of drugs of abuse is not only important in the enforcement of road traffic safety, but also enables the differentiation between the chronic and occasional drug user or makes possible the identification of the source of origin of a particular batch of illegal drugs. Such an analysis would not be possible without the application of chromatography in various forms. Only chromatographic methods successfully combine efficient separation of relevant compound(s) from a biological matrix with specific detection. Among the chromatographic techniques, liquid chromatography-mass spectrometry (LC-MS(/MS)) optimally fulfils the requirements of forensic toxicological analysis due to its high selectivity, the possibility of detection of active metabolites and (emerging in recent years) efficient screening in cases on unclear death. For these reasons, this technique is now finding a more and important place in forensic toxicological laboratories.

Thus, LC-MS(/MS) underwent major evolution in the last decade. An expensive, difficult and not always reliable hyphenated technique turned into a robust analytical tool, applicable in almost all analytical situations.

2.2. History

Beginning in the 1970s, (gas chromatography) GC-MS instrumentation has evolved to an essential tool for both biomedical research and some important routine forensic chemistry Applicability. However, it was the routine introduction on non-disintegrating soft ionization techniques as atmospheric pressure ionization (API) and sophisticated ion analysis methods in the 1990s that finally made the majority of biologically relevant analytes (endogenous metabolites as well as xenobiotics) amenable to highly specific mass spectrometry analysis.

From our current perspective, the hyphenation of LC-MS/MS via an API interface is the only LC-soft ionization-MS technique which has been introduced into routine forensic laboratories so far (1,2). The use of LC-MS/MS systems in bio-analytical research started already in the late 1980s (3,4). Tandem mass spectrometry found its way into forensic laboratories in the earlies 1990s when first flow injection analysis (FIA)-MS/MS methods were introduced for neonatal screening analysis (5). A significant methodological progress was the introduction of LC-MS/MS systems featuring API interfaces, which were broadly acknowledge in bio-analysis in the mid-1990s (6). First implementations in forensic routine laboratories started about a decade ago with realizing the first therapeutic drug monitoring (TDM) assay (7,8). Consequently, this technology allowed a number of important novel assay applicability to be realized in such laboratories, improving their overall quality.

2.3. Analytical background

2.3.1. Ionization

A LC-MS interface has the double task of eliminating the solvents from the LC and producing gas-phase ions from the analyte (9). This can basically be accomplished in two ways:

- the two processes are separated in space, i.e. the analyte is sampled into the vacuum region of the mass spectrometer and then ionized at reduced pressure
- ions are created at atmospheric pressure (hence the name API and then sampled into the vacuum region).

The second strategy has been proved to confer much more robustness and reliability to the ionization process, and, for this reason, API sources have currently almost completely displaced other ion sources that were routinely used previously, e.g. thermospray ionization (TSI).

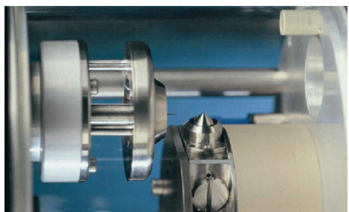
The family of API sources, originally consisting of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), has expanded in recent years to include atmospheric pressure photo-ionisation (APPI), atmospheric pressure laser ionsation (APLI) and sonic spray ionization (SSI). These have all further increased the analytical potential of LC-MS(/MS) in one way or another. Other more obsolete ion sources are TSI, particle beam (PBI), fast atom bombardment (FAB) and matrix-assisted laser desorption ionization (MALDI).

2.3.1.1. Electrospray ionization

ESI was the first to be developed in the family of API sources and, at currently, is the most frequently used ionization mode in the field of analytical toxicology. An ESI source consists of a capillary tube, the ESI probe, to which a voltage (typically 3-5kv) is applied (Figure 15). At flow rates of a few microliters per minute, the difference in potential between the ESI probe and the orifice opening into the vacuum region of the mass spectrometer is sufficient to nebulise the liquid flowing into the capillary. As a result of the limitation in the flow rate applied, this device can be directly coupled to a nano-or micro-LC apparatus, whereas coupling to a normal LC requires splitting of

the eluent. In order to handle higher flow rates, a gas (nitrogen) flowing coaxially to the ESI probe is necessary to maintain a stable spray (pneumatically assisted ESI).

Figure 15: Electrospray



ESI requires the analyte to be ionized in solution. Depending on the voltage polarity, nebulised droplets trapping the ionized analyte will be positively or negatively charged. The reduction in size caused by solvent evaporation accounts for the increase in charge density in the droplet, ultimately leading to its explosion when repulsive forces between charges exceed the cohesive forces of the droplet. This process occurs repeatedly until gas-phase ions are produced by direct emission from the microdroplets (10).

If a compound can be charged at multiple sites in solution, it will carry multiple charges under ESI conditions. This allows the use of mass analysers with limited mass-to-charge (m/z) range (e.g. quadrupoles) to achieve the analysis of high-molecular-weight compounds such as peptides. For example, a compound of molecular weight 12000 carrying 20 charges will appear at m/z 600. The combination of this feature with the ionization mechanism allowing the formation of gas-phase ions from ions in solution makes ESI the best solution for the LC-MS(/MS) analysis of peptides.

Typical ions produced under ESI conditions are:

- in the positive mode: protonated molecular ions $[M+H]^+$, sodium or potassium adducts, or solvent adducts

- in the negative mode: deprotonated molecular ions [M-H]-, or formate or acetate adducts (11)

Clusters ions may sometimes be formed under ESI or other soft ionization API techniques. Adduct formation should be avoided whenever possible, particularly for quantification purposes, as it is often irreproducible. This is not always easy to accomplish. Some quantitative Applicability, however, exploit adduct formation (12-14).

Due to the low amount of energy transferred to the molecule during the ionization process, ESI is a mild ionization technique producing very little fragmentation. As will be discussed later, this has a positive impact on the sensitivity of LC-MS(/MS) analysis as most of the total ion current (TIC) pertains to one ion (the precursor ion in MS/MS mode). The flip side is that ESI-MS does not provide as much structural information. However, fragmentation can be promoted by imparting kinetic energy to the ions of the analyte within the intermediate vacuum region placed between the ion source and the mass analyzer. By applying a difference in potential at the two ends of this region (this parameter is called the orifice, fragmentor, skimmer or capillary voltage, depending on the manufacturer), ions are accelerated and are forced to collide with other molecules species (gas molecules, residual solvent, co-eluting compounds, etc) and the energy gained as a result of these collisions is dissipated by fragmentation (15). The higher the voltage and the length of the path in the intermediate vacuum region, the higher the fragmentation rate. This technique is called in-source collision-induced dissociation (in-source CID) in order to differentiate in from CID obtained under MS/MS conditions (described in the MS/MS section).

In order to correctly select/develop chromatographic separation before ESI-MS, it is important to remember the following fact-signal intensity obtained by ESI depends on the analyte concentration more than on the analyte mass flow (amount per unit time) into the ion source, inferring that better signal-to-noise (S/N) ratios are obtained at lower flow rates. This is said to depend on the solvent evaporation process, which is more efficient at lower flow rates. This improves the transmission of gas-phase ions into the vacuum region.

This explains why a drying gas (nitrogen) and a heating device are also included in currently marketed ESI sources to assist droplet formation and solvent evaporation.

Nevertheless, higher flow rates will certainly result in more frequent servicing of the interface.

In addition to flow rate, the composition of the mobile phase also affects ESI (16,17). As a general rule, because ESI requires the analyte to exist in solution as an ion, the pH of the LC eluent should be selected accordingly. Should this not match with the pH of the mobile phase required for optimal chromatographic separation, it is always possible to modify the latter by post-column addition. However, the rule of thumb 'basic analyte, acidic mobile phase and vice versa' has more than one exception (18)). It should be kept in mind that the chemical environment in the electrospray plume may change dramatically during the spray process (19,20). For example, the production of abundant protonated ions from solutions where their equilibrium concentration is low may be due to a lower than expected pH as a result of solvent oxidation, charge enrichment, uneven droplets subdivision or solvent evaporation.

Buffers are obviously important in order to obtain and control the right pH of the LC eluent. Their selection should be made in the light of two practical considerations:

- non-volatile buffers should be avoided as they deposit into the ion source during the vaporization/ionization process.

- their concentration, as well as the concentration of any other ionized species in the LC eluent, should be kept as low as possible; in fact, an ionized species present at a much higher concentration than the analyte will cover the surface of droplets and prevent the analyte passing into the gas phase, thus reducing its response.

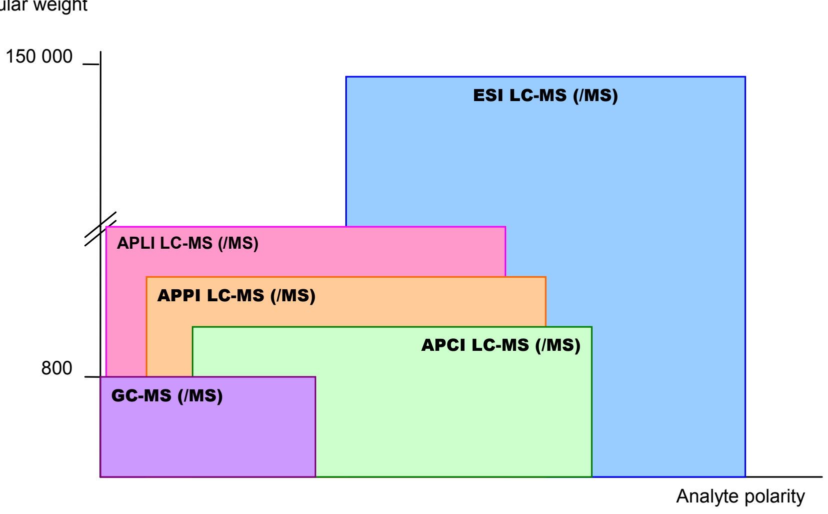
This competition between the analyte and other ions present in the LC eluent is called the matrix effect –a well-known phenomenon that is particularly evident with ESI (21-24). This phenomenon will be described latterly.

Finally, the percentage of organic modifier in the LC eluent should kept as high as possible as it improves the efficiency of solvent evaporation and, as a result, the yield of ions transmitted to the vacuum region. This may be a problem in the case of polar analytes separated under reverse-phase (RP) chromatography. Glucoronide conjugates, for example, require a low percentage of organic modifier to be sufficiently retained in an RP column. Again, post-column addition helps, provided

that the increase in the signal to noise (S/N) ratio due to the increase in the organic modifiers is not counterbalanced by an increase in flow rate (25).

Alternatively, normal-phase chromatography with an aqueous/organic mobile phase enables the retention of polar species with a higher percentage of organic modifier, resulting in higher ionization efficiency when compared with RP separation (26). Figure 16 shows the range of application of LC-ESI-MS(/MS) in terms of analyte polarity and molecular weight compared with GC-MS analysis.

Figure 16. Range of application of different hyphenated chromatographic and MS techniques.



2.3.2. Ion separation

The mass analyzer represents the heart of a mass spectrometer, i.e. the device able to measure the m/z ratios of gas-phase ions. In order to allow a free path of the ions through the analyzer towards the detector, the analyzer must be operated under vacuum. The lower the pressure (typically in the range 10^{-4}-10^{-7} torr), the longer the mean free path of the gas-phase ions. This has an obvious positive effect on sensitivity and mass resolution, although these parameters ultimately also depend on the type of mass analyzer and on the instrumental design adopted by the manufacturer.

Together with sensitivity, other parameters are important in order to decide whether a particular mass analyzer fulfils the requirements of a laboratory. These are mass range, scan speed, mass resolution and mass accuracy.

2.3.2.1. Quadrupole

The quadrupole is the most common mass analyzer for bench-top MS instruments. The most likely reason for this resides in the fact that quadrupoles offer a good compromise of mass range covered, reproducibility of mass spectra, mass resolution and precision for quantification purposes.

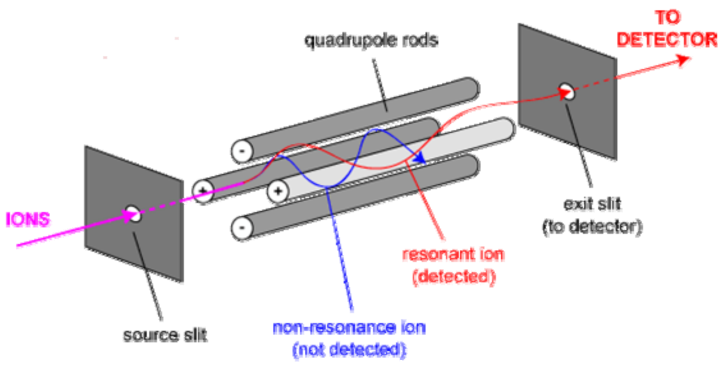
A quadrupole consists of four parallel rods or poles equally spaced around a central axis. An electrical potential is applied to each rod so that each two adjacent poles have opposite polarities. By applying a precisely controlled combination of two electrostatic fields-one direct current (DC) and one at varying radiofrequency (RF)- a resonance frequency for a specific m/z ratio is obtained so that ions at that m/z ratio can reach the detector, whereas ions with lower and higher m/z rations are discarded (Figure 18).

A quadrupole acts as a continuous mass filter, which means that most of the ions that are continuously transferred to the quadrupole from the ion source are lost on their way to the detector and only a few ions (the ions at resonant frequency) are translated into measurable electric signal (Figure 17). By varying the resonant frequency of the quadrupole, a complete set of masses can be scanned at speeds of up to 4000-5000 amu/s, thus obtaining a ‘full-scan’ mass spectrum.

In addition to the above scan mode (typically adopted for the detection and identification of unknown compound), a quadrupole can also be operated in selected-ion monitoring (SIM) mode. In this mode, the quadrupole, is set at one or a few resonant frequencies (depending on the number of ions to be monitored). As a result, both sensitivity and precision in quantification are improved. In fact, since in SIM mode the analyzer spends more time on a specific ion, the amount of signal belonging to this ion reaching the detector is larger than in scan mode. The increase in the S/N ratio is therefore due to a true increase in signal and should not be confused with the increase in the S/N ratio due to the elimination of the huge amount of chemical noise resulting from not monitoring the full mass range.

A typical quadrupole covers a mass range of up to 1000-4000 amu at low resolution (0.7 amu FWHM) and with mass accuracy of 0.1 amu.

Figure 17. A schematic quadrupole analyser



2.3.2.2. Tandem Mass Spectrometry

Traditionally, MS/MS has been implemented in quadrupole instruments where two different quadrupoles, one performing mass selection of precursor (parent) ion(s) and the other mass selection of product (daughter) ion(s), are separated by a collision cell (Figure 18). Here, the precursor ion is accelerated and fragmented into product ions by collision with an inert gas. As the collision cell is also a quadrupole, although simply acting as an ion guide, the two quadrupoles mass anaysers are usually referred to as Q1 and Q3, Q2 being the collision cell, and the instrument is referred to as ‘triple quadrupole’ (QqQ). Fragmentation can be favored by increasing either the kinetic energy (collision energy) of the precursor or the gas pressure in the collision cell.

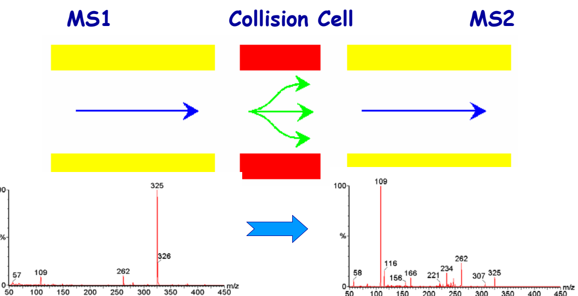
A triple-quadrupole instrument can be operated in a number of different ways, the simpler one being to resemble a single-quadrupole instrument by using either Q1 or Q3 as a pass-all filter. One of the most typical MS/MS scanning modes is the so-called product-ion scan, where one precursor ion is selected by Q1 and the fragment (product) ions obtained after collision are scanned by Q3, thus obtaining a product-ion spectrum. This differs from an in-source CID spectrum in that all fragments detected necessarily originate from the precursor ion.

Other scanning modes, which are very helpful in metabolite profiling studies, are precursor-ion scan and neutral-loss scan (27-29). Both these scan modes are unique to mass spectrometers that provide MS/MS in space. Precursor-ion scan allows one to screen for homologous or similar compounds having a common fragment ion (e.g. different metabolites of a drug): Q1 scans the mass range, whereas Q3 filters a unique product ion and associates it back to the precursor ion that it originated from. The latter is useful for screening compounds with a common neutral fragment loss (e.g. loss of glucoronic acid). In this operating mode, both Q1 and Q3 are synchronously scanned with a fixed mass difference (e.g. 176), and a mass is assigned only to those precursor ions that have lost the predetermined neutral mass.

MS/MS quantification is typically carried out in non-scanning mode by selecting one precursor ion in Q1 and one specific product ion in Q3. This operating mode, a reaction (precursor ion-product ion) instead of an ion is monitored (so-called selected-reaction monitoring [SRM] or, if different reactions are monitored, multiple-

reaction monitoring [MRM] (Figure 19). In this configuration, an MS/MS instrument associates extreme selectivity of detection (the probability that two compounds will share the same precursor AND the same product ion being quite low) with the highest sensitivity. In fact, the addition if a further MS stage implies that a lower number of analyte ions will reach the ion detector. However, as the noise is largely reduced, the resulting S/N ratio will be enhanced.

Figure 18. Multiple Reaction Monitoring



When different reactions are monitored consecutively, as in MRM, care should be taken in order to prevent unwanted contributions to a monitored signal (i.e. the product ion of one reaction) from other reactions monitored in the same acquisition cycle. This phenomenon, called cross-talk, occurs if a precursor ion enters the collision cell when product ions from the previous reaction monitored are still present (30). If the two precursor ions share common fragments, the signal pertaining to one precursor may be overestimated due to the contribution from the other. Different strategies allow one to control this problem, such as:

-adding a pause time between two consecutive transitions (though this will obviously increase the acquisition cycle and will reduce the number of data points per unit time in the chromatogram);

-avoiding the consecutive monitoring of two compounds sharing common product ion (i.e. by monitoring a third reaction in between); or, even better

-optimising LC separation in order to avoid chromatographic overlapping between the cross-talking compounds (31).

Recently designed instruments take care of this problem by speeding up the exit of fragments from the collision cell. Cross-talk should always be considered when developing an analytical method as it is obviously a source of error in quantification.

QTOF (Quadrupole/Time Of Flight), although rather expensive, is also an interesting configuration for MS/MS analysis as it combines the high efficiency of selected ion monitoring in the first mass analyzer (Q) with the high scan rate and high resolving power of the second (TOF).

2.3.3. Ion detection

The detector is the device where ions separated by the mass analyzer are converted into a measurable electric signal (current). The analogue current is the further converted to a digital signal (counts) stored by the data system. The most important characteristics for an ion-detection device are speed, dynamic range and sensitivity (gain).

The most common detection device is the electron multiplier. In an electron multiplier, ions are converted into electrons by means of a dynode electrode-when an ion strikes the dynode surface, electrons are emitted and the introduced current is recorded.

A less common ion-detection system is the photo-multiplier. In a photo-multiplier, ions are initially converted by a dynode into electrons. These electrons are then converted into photons by means of a phosphorous screen. A photomultiplier, operating in a cascading mode, provides signal amplification. The photo-multiplier is sealed and kept under vacuum (photons pass through the glass), and therefore may have a longer lifespan; this is different to the electron multiplier, which is exposed to the internal environment of the mass spectrometer.

TOF mass analyzers have a very high scan speed, and require fast detectors with a large and plane detection are so that many different ions can be detected at the same time. Multi-channel plate detectors providing time responses lower than 1ns and high grain (more than 50 mV per single ion) are typically used for this purpose. Each channel works similarly to a small CEM; because of the easier saturation and also the rapidity of the time-to-digital conversion required, the dynamic range of this type of detector is typically lower than that of the electron and photo-multiplier. Nevertheless, saturation can be observed with any type of ion detector and should be taken into account when a lower than linear response is observed. Apart from affecting quantification, detector saturation may also influence the appearance of the full-scan mass spectrum, thus impairing the performance of library search-based information.

2.4. Characteristics of current LC-MS/MS applications in forensic laboratories

Essential strengths of the LC-MS/MS technology for forensic laboratories include:

- Specificity: the potentially very high analytical specificity of tandem mass spectrometry as LC detector results from using the molecular mass of the analyte and its specific disintegration behavior as detection principle.
- Wide range of applicability with good practicability. In contrast to GC-MS as the "classical" mass spectrometry technique, the application of LC-MS/MS is not limited to volatile molecules (usually with molecular weights below 50 Da). Furthermore, aside from the highly polar analytes (i.e. amino acids), sample preparation is usually simple and does not include derivatization techniques. Mass spectrometry detected LC assays are generally optimized to shorter runtimes. Hence, compared to GC-MS, far higher sample throughput can be realized.
- Flexibility. New assays can typically be developed in house with a high degree of flexibility and within a short run time.
- Information rich detection. A large number of quantitative or qualitative results can be obtained for a single analytical LC-MS/MS run, since due to the fast ion selection electronics, multi-parametric, quasi parallel analysis can be performed with a mass spectrometer.

2.4.1. Handling and robustness

Although routine handling of LC-MS/MS system is easier compared to GC-MS instruments, it is much more complex than operating modern day forensic chemistry analyzers. Incorrect use can cause substantial machine damage and training for several weeks is usually required for technicians to run an instrument. While everyday handling and basic maintenance procedures can doubtlessly be performed by skilled technicians, the main responsibility for LC-MS/MS installations is typically in the hands of an academic. Such an expert is particularly necessary for the development and validation of new LC-MS/MS methods which are –aside some exceptions- individualized ("home brewed") assay

setups tailored to the equipment available. A comprehensive assay validation including a detailed risk assessment has to be carried out. National and international guidelines (e.g. as published by SOFT (32)) are usually the basis of such an undertaking, especially in a forensic environment where especial legal regulations have to be met in some countries.

It is a key feature of LC-MS/MS using API techniques as ESI or APCI, that ideally only a clean beam of ions is transferred into the high vacuum area of an instrument while unionized molecules (LC solvents and sample matrix residuals) do not enter the mass spectrometer. Solid contaminants typically precipitate in the ion source housing around the mass spectrometer's vacuum area entrance orifice. In most cases, contaminated hardware components can be cleaned without venting the mass spectrometer. This is in contrast to GC-MS where essentially the entire effluent of the chromatographic procedure enters the high vacuum area, cleaning of which is very difficult and laborious. Hence, state of the art LC-MS/MS instruments are by far more robust than GC-MS instruments and allow the continuous analysis of large sample batches. Daily measurements series up to 24h duration with short, simple maintenance interventions (e.g. exchange of the LC stationary phases) after several days to weeks can be achieved. Taking into account the high analyte specificity of tandem mass spectrometry, chromatographic analyte separation prior to MS/MS can be minimized, if ion suppression effects are managed. State of the art LC-MS/MS instruments with one- or two-dimensional chromatography setups allow to run up to 20 analyses per hour. Consequently, within 24 h several hundred quantitative analyses can be performed with one LC-MS/MS system in a continuous work mode. In most forensic laboratories far smaller series are run in daily routine, especially if switching from one assay to another requires hardware changes (e.g. of LC columns) causing significant down times.

Typically LC-MS/MS instruments work for months with minimal maintenance but can cause unexpected substantial problems without prior warning. In general, more down-times are related to the LC modules with its large number of mechanical parts compared to the mass spectrometer. Although the chromatographic methods in routine Applicability are kept as simple as possible (no complex gradient, no salt-buffered mobile phases), typically LC problems (e.g. clogging of capillaries, gas bubbles in the fluid system, crystallization of mobile phase additives, microbial growth, abrasion, erosion, and leakage problems) also occur in LC-MS/MS. Mass spectrometry related problems most frequently arise from the API spray capillary

(erosion, blockage) and problems within the ion source housing like matrix accumulation or salt precipitation. More severe problems result from substantial contamination within the vacuum area, problems in the vacuum system or electronic faults. Such events typically require intervention of a service engineer but may be prevented by regular planned maintenance visits.

Troubleshooting, e.g. for decreasing sensitivity or insufficient precision or an individual method, has to include complex considerations. They have to encompass all aspects of the assay as the sample preparation (e.g. injected volume check, early blocking/leakage detection, errors in the composition of mobile phases), the MS/MS-based analyte detection (e.g. hampered by decreasing quality of nitrogen supply causing high background signals, an instable ion spray, source contamination, electrical noise due to a failure of the detector, or decreasing vacuum quality), and used chemicals (e.g. contamination of mobile phases causing ion suppression effects, the instability of internal standards or analytes (33,34).

It might happen that after some maintenance actions or with increased contaminations of some hardware components, the reoptimization of about several MS instruments settings is necessary. Several of these tuning operations have to be performed in a more the less intuitive trial and error manner. Ion spray adjustments (e.g. re-optimization of the capillary position) are typically done manually. Consequently no software read-back is offered, and the contamination of these settings is hardly possible.

Since almost all LC-MS/MS systems are customized by the individual user, not two installations are identical. This makes professional troubleshooting vial “hotline” or service engineers on site additionally difficult; especially if LC and MS are purchased from different manufacturers. With few exceptions, there is no comprehensive support available from the MS industry with covers all components of an installation or specific applicability.

2.4.2. Analytical limitations

Even though the principle of tandem mass spectrometry can allow specific analyte detection, the quality of an individual method has to be critically and carefully assessed. The choice of the optimal internal standard (IS) is one of the most critical steps in designing a LC-MS/MS assay. Whenever a stable isotope labeled derivative of a target analyte is available as IS, very reliable isotope dilution mass spectrometry (IDMS) methods can be developed. If homologue molecules (i.e. a molecule structurally closely related to analyte) have to be used as IS- as in the case for most drugs assays –assay reproducibility and linearity may become a substantial problem. Only IDMS assays can assure co-elution of the targeted analyte with its IS. This is the only possibility to fully compensate sudden signal fluctuations caused by individual matrix induced reduced or increased ion yields (“ion suppression” and “ion enhancement”, e.g. caused by co-administered drugs) and other unpredictable impairments of the ionization efficacy. It should not be overlooked, that a constant drop in ion yields, e.g. due to instrument contamination, will even hamper the performance of an IDMS-based assay due to sensitivity loss .

Additional pitfalls in the accuracy of LC-MS/MS methods can arise from ion-source fragmentation of conjugate derivatives of a target analyte; especially if glycosidic bonds are involved (35) or from isobaric mass transitions of unrelated or related compounds such as metabolites or isomers (36). Interference from isomers of the target analyte can make even IDMS methods invalid.

Manufacturers specify the sensitivity of LC-MS/MS instruments by applying solutions of different reference compounds. Reserpine is widely used for this purpose. It is relatively stable and yields positive and negative ions in both ESI and APCI. However, due to different protocols used (i.e. flow rates, analyte concentration, ion source tuning, ion transmission conditions) direct comparison of the sensitivity of different instruments is hardly possible and rather appropriate; notably, sensitivity of instruments may differ in a compound specific manner. Therefore it is advisable to define analyte related specifications if an instrument is to be implemented for a certain assay. Moreover, it is useful to supply the vendor’s application laboratories with test samples (i.e. extracts from spiked samples) in order to test the feasibility of a specific method intended to be implemented by the costumer). Nominal sensitivity documented on installation of an instrument may differ significantly from the level of sensitivity found in long-term routine application. Long term sensitivity critically

depends on the robustness of a respective instrument system. Furthermore, substantial differences in sensitivity can be observed between identical individual instruments even with similar serial numbers.

2.4.2.1. Matrix effect

LC-MS/MS is a powerful analytical technique for quantitative bioanalysis due to its inherent high sensitivity and selectivity. It is susceptible, however, to matrix effects. The impact of matrix effects on the accuracy, precision and robustness of bioanalytical methods is of growing concern (37-44). Residual matrix effect components, endogenous phospholipids in particular, are a significant source of Imprecision in quantitative analyses commonly conducted by LC-MS/MS. Matrix effects, originally discussed by Kebarle and Tang in the early 1990s, can be described as the difference between the mass spectrometric response for an analyte in standard solution ant the response for the same analyte in a biological matrix, such as plasma (45). Matrix effects result from co-eluting matrix components that affect the ionization of the target analyte, resulting either in ion suppression, or, in some cases, ion enhancement. Matrix effect can be highly variable and can be difficult to control or predict. They are caused by numerous factors, including, but not limited to endogenous phospholipids, dosing media, formulating agents and mobile phase modifiers (46-49). Furthermore, different sources of plasma from the same species can yield different validation results, such as standard curve slope and precision. The severity and nature of suppression or enhancement may be a function of the concentration of the co-eluting metabolites, impurities or degradation products. Furthermore, matrix effects are analyte specific. All of the above factors can cause significant errors in the accuracy and precision of bioanalytical methods. Current international chromatographic method validation guidelines now require that these effects be evaluated as part of quantitative LC-MS/MS method development, validation and routine use (50). Consequently, most current papers describing the quantification of drugs in biofluids discuss matrix effects in some degree.

Several papers describing the evaluation of matrix effects have been published, providing the guidance and techniques necessary for researcher. There are two common methods to assess matrix effects: post-column infusion method (51) and the post-extraction spike method (52). The post-column infusion method provides a qualitative assessment of matrix effects, identifying chromatographic regions most likely to experience matrix effects. This approach is limited in that it does not provide

a quantitative understanding of the level of matrix effect observed by specific analytes, but merely identifies chromatographic regions where an analyte would be most susceptible to suppression or enhancement. However, this process can be very time consuming and require significant optimization, particularly if quantification of multiple analytes in a single run is desired. In contrast, the post-extraction spike method quantitatively assesses matrix effects by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process. In this manner, quantitative effects on ion suppression or enhancement experienced by all analytes in the sample can be measured each time a change is made to the anatycal method.

While more researchers now include an evaluation of matrix effects as part of method development, only some attempt to actually reduce or eliminate matrix effects. Some researchers have focused on optimizing sample preparation to reduce matrix effects (45,53,54), while others have focused on manipulating chromatographic parameters (55,56). Others assess the level of matrix effects and compensate for the alteration of the signal through the use of an IS, often the stable isotope labeled analog of the drug. Still other papers describe the use of flow splitting to reduce matrix effects (57,58). In some cases, researchers opt to use an ionization source, such as APCI, that is less sensitive for their compound, simply because the matrix effects experienced with the more sensitive source are too great. APCI has shown, for certain compounds, less ion suppression and can be a better choice for some assays based on sensitivity and accuracy/precision, but it is not immune to matrix effects (52,59).

In summary, it is evident that LC-MS/MS holds great potential as a complementary technology for laboratory medicine, although at present there are still important limitations of its routine application which have to be deal with. These are in particular issues related to the ease of hardware and software use, the instrument performance and the practicability of LC-MS/MS assays in a routine laboratory setup.

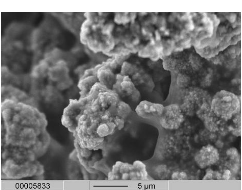
2.5. Sample preparation

It is common knowledge that poor sample preparation, when the most modern and sophisticated techniques are applied, may negatively affect detection and quantification. LC-MS/MS for drugs makes no exception. LC-MS/MS is not a magic tool able to replace optimal sample preparation and separation.

Although less laborious than GC-MS sample work-up, typical LC-MS/MS protocols include several purification steps. Insufficient sample preparation affects LC-MS/MS analysis in two mayor ways. Macromolecular and cellular sample contamination may lead to LC column clogging resulting in instrument down-times. Insufficient removal of substances co-eluting with an analyte into the mass spectrometer can lead to impaired analyte detection, resulting in sensitivity loss or reduced assay specificity and accuracy. Retaining investigated analytes in the chromatographic separation system is a mayor cornerstone in sample preparation. It allows to separate undesirable sample matrix constituents (e.g. salts, phospholipids, etc) and other metabolites from the analyte elution band.

Depending on the analyte concentration range to be addressed, sample preparation or dilution has to be achieved in the course of sample preparation. There are many sample preparation technologies, all of them having certain advantages and disadvantages. The most widely applied simple sample preparation techniques are protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE). Below there is an overview of these sample clean-up techniques.

2.5.1. Protein precipitation (PPT)

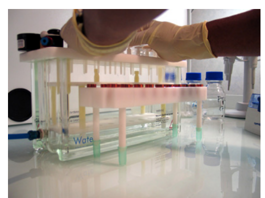


The protein content of human body fluids and tissues is considerable. Numerous techniques have been developed to precipitate protein and remove it before extracting and concentrating drug substances. The solubility of proteins depends on the distribution of hydrophilic and hydrophobic amino residues on the proteins' surface. Proteins that have high hydrophobic amino acid content on the surface have low solubility in aqueous solvent. Charged and polar surface residues interact with ionic groups in the solvent and increase solubility. Knowledge of amino acid composition of a protein will aid in determining and ideal precipitation solvent and method. Protein precipitation reagents include the following: organic solvents (acetone, acetonitrile, methanol), zinc sulfate in methanol, 5-sulfosalicylic acid, perchloric acid, trichloroacetic acid, sodium tungsten and ammonium sulfate. Once proteins have been precipitated, separation of aqueous and solid protein must occur by filtering or centrifugation. Some drugs are occluded (trapped) in the precipitate, but can be at least partially recovered by washing the precipitate with hot water or hot dilute hydrochloric acid. Those drugs readily hydrolyzed by heating under aqueous acidic or basic conditions should be either extracted without protein precipitation or extracted with a mild precipitation reagent such as zinc sulfate-methanol without heating. Esters such as cocaine, benzocaine, meperidine, methylphenidate, and procaine need to be recovered under mild conditions. Alkaloids such as strychnine, nicotine, quinine, various opiates, and some narcotics will not be degraded by moderate heating and acid treatments to precipitate proteins (60-62).

2.5.2. Liquid Liquid Extraction (LLE)

LLE, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase. Once a solvent is chosen, a buffer is usually added to convert drugs to a form that is non-ionic so it will partition readily in organic solvent. The key principle adjusting the pH for extraction is to create non-salt forms of the drugs to be partitioned into the organic solvent. LLE is still frequently used in analytical toxicology, especially for (urgent) screening purposes, when analysis of a wide range of (unknown) compounds rather than target analysis is the objective (63-76). Development of an LLE procedure is, moreover, not time-consuming, although it is difficult to automate, requires high-purity solvents, and can result in the formation of emulsions with incomplete phase separation, the last of which leads to impure extracts. Safe disposal of toxic solvents may also be problematic and expensive.

2.5.3. Solid phase extraction (SPE)



The general definition of SPE could be as follows: Separation of analyte(s) from a mixture of matrix compounds by selective portioning of the compounds between a solid phase (sorbent) and a liquid phase (sample&solvent). SPE is a dynamic process. During extraction, the equilibrium, between analyte concentration in the sample and the analyte concentration on the sorbent, is continuously shifting. Ideally, the analyte 'likes' the sorbent much better than the sample and is completely extracted from the sample. The less sorbent material is required to extract the analyte from the sample, the more selective and thus cleaner the extract will be.

To create a selective method, often simple sample pre-treatment steps are required before the actual extraction. First of all, the sample sometimes contains particulates that have to be filtered out. The SPE cartridge and its frit can filter out particulates, but this may not be sufficient or cause clogging. In this case centrifugation or filtration of the sample is required. Secondly, the sample is often diluted or a buffer is added to neutralize or charge the analyte. By changing the characteristics of the sample or the analyte, the conditions to retain the analyte on the sorbent can be improved. At the same time, to compensate for mistakes occurring from sample pre-treatment all the way to the detection, an internal standard is added to the sample. Sometimes analytes also interact with matrix compounds (protein binding). For the analyte to retain on the cartridge, it preferably must be free in solution. Depending on the type of binding, a solvent can be added to the sample in order to disrupt this drug-protein interaction.

Bellow is described how SPE works for a reversed phase interaction.

- Activation: the function of this step is to wet or activate the functional groups of the sorbent to allow proper interaction with the analyte. If extraction sorbents are hydrophobic they will not be wetted by an aqueous solution. Therefore organic solvents are used. In case the sorbent is wettable, this step may be omitted.

- Equilibration: the function of this step is to create a sorbent chemistry environment similar to that of the sample. If the pH is important for the

extraction chemistry, the sample pH should be adjusted properly in both the sample pre-treatment and the sample conditioning step. It is also important the solvent is fully miscible and compatible with the sample; otherwise precipitation of sample matrix components could potentially cause blockages.

- Sample loading: during sample loading the free analyte will bind the extraction solvent. In this step, the flow rate of the loading step is directly related to the residence time of the analyte on the extraction solvent. A too high flow could result in breakthrough. Some of the matrix compounds will also retain on the sorbent. Usually there is enough sorbent material so this does not negatively impact the capacity. As long as the capacity of the sorbent is sufficient, the amount extracted increases proportional with the sample volume loaded on the cartridge.

- Washing: with a wash step most of the retained matrix compounds are flushed to waste. The wash solvent usually has higher elution strength (% organic solvent) than the solvent used for sample loading. Matrix compounds with a weaker interaction with the sorbent compared to the analyte will go the waste while matrix compounds with stronger interactions will still remain on the cartridge. Use the strongest possible wash solvent in order to get the cleanest extract. However, during the wash, analyte-sorbent interactions can also be partially disrupted causing the analyte to move further in the SPE cartridge. Ideally, the wash is stopped before some of the analyte starts to elute from the cartridge (breakthrough).

- Elution: after the majority of the matrix compounds have been washed to waste, the analyte is eluted from the sorbent. Typically, a strong elution solvent is used to disrupt all the interactions of the analyte with the sorbent. Apart from the analyte, the matrix compounds that have not been washed away are now also eluted with the analyte. These matrix compounds can still cause interferences during detection. The elution step can also be used to even further improve clean-up. This means the analyte elutes completely, but matrix compounds with stronger interactions are retained on the cartridge.

Thus, SPE is currently used as a routine sample clean up method in forensic and toxicology laboratories for a wide range of compounds (62,77-88).

2.5.4. Pros and Cons

The table below shows the advantages and disadvantages it these sample preparation technologies.

Table 2. Overview of sample preparation technologies most important criteria

	Assay Performance			Method development			Workflow	
	Clean-up	Sensitivity	Reproducibility	Time	Simplicity	Validation	Automation	Software Integration
PPT	☹	☹	☹	☺	☺	😐	☺	😐
LLE	☺	😐	☺	😐	😐	☺	☹	☹
SPE	☺	😐	😐	☹	☹	☺	😐	☹

2.6 LC-MS(/MS) methods for the analysis of hallucinogens, cannabinoids, and amphetamines in blood and urine in the literature

A wide variety of body fluid specimens have been utilized for analysis for the presence of drugs of abuse. Blood is widely regarded as the specimen offering the best correlation between drug levels and likely dosing and likely concomitant pharmacological, cognitive, and psychomotor effects. Drug levels found in blood are often quite low (ng/mL) and often short-lived. The analysis of drugs in blood is time-consuming, generally requiring extraction procedures before further analysis can be performed. There have been several publications addressing the application of urine immunoassays to the analysis of blood specimens, after appropriate extractions protocols (89,90,91). Although blood is widely used for drug testing in forensic toxicology settings, the invasiveness of the collection of blood specimens does not lend itself to routine testing in other non forensic context (e.g. workplace, testing environments). Furthermore, there is much greater risk of transmission of infection disease though handling of blood samples than with other alternative matrices not discussed in this thesis (e.g. oral fluid, sweat, hair).

Urine offers the advantages of large specimen volume and relatively high drug concentrations. Urine is 95% water, with sodium chloride and urea in about equal amounts as the main dissolved substances, and with smaller amounts if a wide variety of other constituents. Moreover, urine is relatively easy to collect and analyze. There are a wide variety of immunoassays available for detection of most common drugs of abuse and/or their metabolites in urine. However, one of the most important limitations of urine is the relative difficulty in correlating urine drug/and or metabolite levels with likely dosing and likely impairment.

Accordingly to the scope of this thesis, below it is described the analytical LC-MS(/MS) methods for the determination of hallucinogens, cannabinoids and amphetamines in blood and urine.

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(92)	Cathinone Mescaline	Plasma (1 mL)	SPE (MCX)	Zorbax SCX	Ammonium formate Acetonitrile	ESI+	Ion Trap	Linearity: 10-1000 ng/mL Recovery>72% Matrix effect<7% Inaccuracy<12% Stability
(93)	Chlorpheniramine	Plasma (500 µL)	LLE (Diethyl ether)	Kromasil	Water Acetonitrile Formic acid	ESI+	QqQ	Linearity 0.2-50 ng/mL LOD 0.2 ng/mL Imprecision <10% Inacuracy<8% Stability Recovery>75% Applicability
(94)	Chorpheniramine	Plasma (1 mL)	LLE (Diethyl ether)	Cyclobond I 2000	Diethylamide Methanol Acetonitrile	ESI+	Simple Quad	Linearity 0.13-50 ng/mL Recovery>79% Imprecision<12% Inaccuracy<10% Applicability
(95)	Chlorpheniramine	Plasma (0.5 mL)	LLE (Diethyl ether)	Develosil PhA	Ammonium acetate Acetonitrile Methanol	ESI+	Simple Quad	Linearity 0.52-20.8 ng/mL LOQ 0.52 ng/mL Imprecision<14% Inaccuracy<92% Stability Applicability

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(96)	Chlorpheniramine	Plasma (0.2 mL)	LLE (Methyl butyl ether)	Betasil Diol Zorbax-SB C18	Ammonium formate Methanol	ESI+ APCI+	QqQ	LOQ 0.2 ng/mL Imprecision<38% Matrix effect<30%
(97)	Ibogaine	Urine (0.25 mL)	SPE (HLB)	Zorbax eclipse XDB	Acetonitrile Ammonium formate	ESI+	Simple Quad	Linearity 1.78-358 ng/mL LOQ 1.78 ng/mL Inaccuracy<8% Recovery>70% No matrix effect Stability Applicability
(98)	Ibogaine	Blood (1 mL)	LLE (Methylene chloride: Isopropanol)	ODB Uptisphere C_{18}	Formate buffer Acetonitrile	ESI+	QqQ	Linearity 0.05-5000 ng/mL LOQ 0.05 ng/mL LOD 1 ng/mL Imprecision<15% Inaccuracy<15% Recovery>30% Matrix effect<47% Applicability
(99)	Ibogaine, Psilocin Scopolamine	Urine (50µL)	Hydrolysis	Hypersil Gold	Formic acid Acetonitrile	ESI+	QqQ	Linearity 5-500 ng/mL Imprecision<27% No matrix effect Stability Applicability

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(100)	Kavain	Serum and urine (1 mL)	Hydrolysis LLE (Dichloromethane: Diethylether)	Polar RP HexylPropyl	Ammonium Formate Acetonitrile Water	TIS+	QqQ	No validation Applicability
(101)	Ketamine Norketamine	Urine (1 mL)	Hydrolysis SPE (Isolut HCX)	LiChroCart Purosphere Star RP18e	0.1%Formic acid Acetonitrile	APCI+	Simple Quad	Linearity 2-2000 ng/mL LOQ 2 ng/mL Imprecision<4% Recovery >100% Applicability
(102)	Ketamine Norketamine	Plasma (0.5 mL)	SPE (HLB)	Chiral AGP	Isopropanol Ammonium acetate	ESI+	MSD	Linearity 1-125 ng/mL LOQ 1 ng/mL Imprecision<8% Inaccuracy 0% Recovery >95% Applicability
(103)	Ketamine Norketamine	Urine (1 mL)	LLE (Hexane)	-	0.1% Formic acid Toluene:Acetonitrile	ESI+	MSD	Linearity 0-500 ng/mL LOD 3 ng/mL Imprecision<15% Inaccuracy<15%
(104)	Ketamine Norketamine	Urine (1 mL)	SPE (SPEC Plus)	Brownlee Perkin-Elmer RP-C_{18}	Acetonitrile Acetone Ammonium acetate	TIS+	QqQ	Linearity 1-500 ng/mL LOD 1 ng/mL Recovery>90% Applicability

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(105)	Ketamine	Urine (1.6 mL)	SPE (C18)	Platinum EPS C_{18}	Ammonium Formate Acetonitrile	ESI+	Ion Trap	Linearity 5-1000 ng/mL Imprecision<11% LOD 5 ng/mL No matrix effect Recovery>89% Applicability
(106)	Ketamine	Urine (1.6 mL)	SPE (C18)	Rocket EPS C_{18}	Ammonium Formate Acetonitrile	ESI+	Ion Trap	Linearity 5-160 ng/mL LOQ 5 ng/mL Imprecision<15% Inaccuracy<95% No matrix effects
(107)	Ketamine	Plasma (0.1 mL)	LLE (Dichloromethane: Isopropanol)	ACE 5C_{18}	Formic acid Acetonitirle	ESI+	QqQ	Linearity 1-1000 ng/mL Applicability
(108)	Ketamine Norketamine LSD	Urine (not specified)	Filtered	XTerra RPC_{18}	Ammonium acetate 0.1%Formic acid Methanol	HESI+	QqQ	Linearity 1-1200 ng/mL LOD>0.6 ng/mL LOQ>2.1 ng/mL Imprecision<25% Applicability
(109)	Ketamine Norketamine	Urine (not specified)	Filtered	Supelcosil 18-DB	Ammonium acetate Acetonitrile	ESI+ APCI+	Simple Quad	Linearity 5-250 ng/mL LOD>0.5 ng/mL Imprecision<13% Inaccuracy<10%

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(110)	Ketamine Norketamine	Urine (4 mL)	SPE (MCX and C8)	BEH C_{18}	0.1%Formic acid Acetonitrile	ESI+	QqQ	Linearity 0.1-100 ng/mL LOD> 0.03 ng/mL LOQ 0.1 ng/mL Imprecision<12% Inaccuracy<17% Recovery>61% Dilution integrity<15% No matrix effect Applicability
(111)	Ketamine, norketamine	Blood and urine (50µL)	Hydrolysis PPT (Acetonitrile)	Shiseido Capcell Pak SCX UG 80	Ammonium acetate Acetonitrile	ESI+	QqQ	Linearity 50-5000 ng/mL Imprecision<9.4% Recovery>90% Matrix effect<30% Applicability
(112)	Ketamine Norketamine	Plasma (100 µL)	SPE (MCX)	Nucleodur C_{18}	0.1%Formic acid Acetonitrile	ESI+	Simple Quad	Linearity 5-500 ng/mL LOQ 4 ng/mL Imprecision<2% Recovery>84% No matrix effects Applicability

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(113)	Ketamine Norketamine	Urine (1 mL)	Hydrolysis SPE (World Wide Monitoring)ZSDAU020)	Synergi Hydro RP	Formate buffer Acetonitrile	ESI+	Ion Trap	Linearity 0-1200 ng/mL LOD 0.6 ng/mL LOQ 1.9 ng/mL Imprecision<7% Inaccuracy<6% Matrix effect<9% Recovery>97% Applicability
(114)	LSD	Urine (0.5 mL)	Hydrolysis SPE (HLB)	Atlantis dC_{18}	Ammonium formate Acetonitrile	ESI+	QqQ	Linearity 0.2-100ng/mL) LOD 0.1ng/mL LOQ 0.2 ng/mL Recovery>65% Imprecision<15% Inaccuracy<15% Matrix effect<35% Stability Applicability
(115)	LSD 2-oxo-3-OH-LSD	Blood and Urine (1mL)	LLE (Butyl acetate)	Zorbax SB-C_{18}	0.05% formic acid Acetonitrile	ESI+	QqQ	Linearity 0.01-200 ng/mL LOQ >0.01 ng/mL Imprecision<23% Inaccuracy<12%
(116)	2-Oxo-3-Hydroxy-LSD	Urine (5 mL)	SPE (Anion Exchange)	Eclipse XDB C_{18}	Ammonium acetate Acetonitrile	APCI+	Ion Trap	Matrix effect Stability

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(27)	LSD 2-Oxo-3-OH-LSD	Blood and Urine (1 mL)	LLE (Diethyl ether)	Spherisorb 5 RP 8S	0.1% formic acid Acetonitrile	ESI+	QqQ	Linearity 0.01-4 ng/mL Imprecision<7% LOQ 0.02 ng/mL Inaccuracy<12% Applicability
(117)	2-Oxo-3-OH-LSD	Urine (5 mL)	LLE (Methylene chloride: Isopropanol) SPE (Clean Screen)	Eclipse XDB C_{18}	Ammonium acetate Acetonitriel	APCI+	Ion Trap	Linearity: 1- 8ng/mL (r^2:0.9805) LOQ 0.4 ng/mL
(118)	LSD	Urine (5 mL)	SPE (Bond Elut Certify)	Hypersil C_{18}	Acetate buffer Acetonitrile tryethylamine	ESI+	Simple Quad	Linearity 0.5-10ng/mL LOQ 0.5 ng/mL Imprecision<10%
(119)	LSD	Urine (2 mL)	LLE (Toluene: Diethylether)	Nucleosil C_{18}	Ammonium formate Acetonitrile	ESI+	Simple Quad	Linearity 0.05-20 ng/mL LOQ 0.05 ng/mL Imprecision<14% Recovery>93%
(120)	Mescaline	Urine (1 mL)	SPE (C18)	Zorbax Phenyl	Ammonium acetate Methanol	APCI+	QqQ	Linearity 5-10000ng/mL LOD 3 ng/mL Imprecision<8.5% Recovery>99% No matrix effect Stability Applicability

Table 3: LC-MS(/MS) methods for the analysis of hallucinogens in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(121)	Ritalinic acid Methylphenidate	Urine (0.02 mL)	SPE (HLB)	-	0.1% Formic acid Acetonitrile	ESI+	Simple Quad	Imprecision<11% Inaccuracy 0% LOQ 0.25 ng/mL Recovery>75%
(122)	Ritalinic acid	Plasma and Urine (100µL)	PPT (Acetonitrile)	Hipersil Gold	Ammonium acetate Acetonitrile	ESI+	QqQ	Linearity 0.5-5000 ng/mL LOD>0.16 ng/mL LOQ >0.5 ng/mL Recoveries>77% Imprecision<15% Accuracy<13% No matrix effect Stability Applicability
(123)	Psilocine	Urine (0.1 mL)	Hydrolysis PPT (Methanol)	ODS	Ammonium formate Acetonitrile	ESI+	QqQ	Linearity 500-5000 ng/mL Imprecision<10% LOD 0.5 ng/mL
(124)	Scopolamine	Urine (1 mL)	Hydrolysis SPE (C18)	Zorbax Extend C_{18}	Methanol Ammonium acetate	ESI+	QqQ	No validation Structural information of metabolites

Table 4 : LC-MS(/MS) methods for the analysis of cannabinoids in blood and urine in the literature

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(125)	THC-COOH	Urine (2 mL)	Hydrolysis SPE	Xterra MS C_{18}	Ammonium formate (0.1%FA), Methanol	APCI+	QqQ	Linearity 0-500 ng/mL LOD 0.2 ng/mL, LOQ 5.1 ng/mL, Imprecision <13.4%), Recovery >82%
(126)	THC	Blood (1.2 g)	SPMEM	C_{18}	Methanol Water	APCI+	QqQ	No validation Evaluation of extraction conditions
(127)	THC	Plasma (250µL)	LLE	XTerra MS C_{18}	Ammonium formate Methanol	ESI+	QqQ	Linearity 0.1-10 ng/mL LOQ >0.1 ng/mL, Imprecision <6%, Accuracy <7% , Recovery >85%, No Matrix effect Stability Applicability
(128)	THC, 11-OH-THC, THC-COOH	Blood and urine (1 mL)	Hydrolysis (Urine) SPE (Bond Elut LRC for urine) (Clean Screen ZSTHC020)	Symmetry C_{18}	0.1% Formic acid Acetonitrile	ESI+	QqQ	Linearity 5-2000ng/mL LOD>0.5ng/mL LOQ>2ng/mL, Imprecision <26% Inaccuracy >40%, Recovery >73% No matrix effect

Table 4 : LC-MS(/MS) methods for the analysis of cannabinoids in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(129)	THC, 11-OH-THC, THC-COOH	Plasma (1 mL) Urine (2.5 mL)	Blood: SPE (Bond Elut Certify II) Urine: Hydrolysis LLE (Diethylether: Ethylacetate)	Synergi MAXRP C_{12}	Ammonium formate Acetonitrile	APCI+	QqQ	Linearity 0.2-100 ng/mL LOD 0.1 ng/mL LOQ >0.2 ng/mL, Imprecision <16% , Inaccuracy <26% , Recovery >47%, Stability No matrix effects Applicability
(130)	THC, 11-OH-THC, THC-COOH	1 mL whole blood	PPT (Acetonitrile) SPE (Bond Elut Certify)	BEH C_{18}	0.1% Formic acid Acetonitrile	ESI+	QqQ	Linearity 0.05-50 ng/mL, LOD>0.02ng/mL LOQ> 0.05 ng/mL, Imprecision<10%, Inaccuracy<7%, Recovery>47%, Matrix effect<12% Stability

Table 4 : LC-MS(/MS) methods for the analysis of cannabinoids in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(131)	THC-COOH	Urine (120 µL)	Hydrolysis Dilution	BEH C_{18}	0.1% Formic acid, Acetonitrile	ESI+	QqQ	Linearity 2-1000 ng/mL, LOD 0.2 ng/mL, LOQ 0.7 ng/mL, Imprecision <5%, Inaccuracy <12%, Carryover <0.03%, Hydrolysis efficiency, Matrix effect <40%, Recovery >95% Stability Applicability

Table 5 : LC-MS(/MS) methods for the analysis of amphetamines in blood and urine in the literature

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(132)	MDMA, MDA, MDEA, amphetamine, methamphetamine, ephedrine	Plasma (50 µL)	PPT (Methanol)	Hypersil BDS C_{18}	Ammonium acetate Acetonitrile	ESI+	QqQ	Linearity 0.5-500 ng/mL LOD>0.2 ng/mL LOQ>0.5 ng/mL Imprecision<10% Applicability
(133)	Amphetamine Methamphetamine	Serum (20-150 µL)	SPE (HLB)	Hypersil C_{18}	Ammonium acetate Acetonitrile Methanol	ESI+	QqQ	Linearity 0.3-1000 ng/mL LOQ 0.3 ng/mL Matrix effect<44% Recovery<67% Applicability
(134)	Amphetamine, methamphetamine, MDA, MDMA, MDEA	Urine (5 mL)	On-line SPE (HLB)	-	TFA Methanol	ESI+	QqQ	Linearity 5-500 ng/mL LOD>1 ng/mL Imprecision<16% Inaccuracy<12% Recovery>85% Applicability
(135)	Amphetamine	Plasma (50 µL)	LPME	Chromsep SS	Formic acid Heptanfluorobutiric acid Methanol	ESI+	Ion trap	No validation Evaluation of LPME

Table 5 : LC-MS(/MS) methods for the analysis of amphetamines in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(136)	Amphetamine, methamphetamine, MDA, MDMA, ephedrine	Blood (1 mL)	SPE (MCX)	BEH C_{18}	Pyrrolidine Methanol	ESI+	Single Quad	No validation Evaluation of chromatographic separation
(137)	Amphetamine, methamphetamine, MDA, MDMA, MDEA	Plasma (1 mL)	SPE (HLB)	Atlantis dC_{18}	Ammonium formate Acetonitrile	ESI+	Simple Quad	Linearity 2-250 ng/mL LOD>0.5 ng/mL LOQ 2 ng/mL Recovery>50% No matrix effect Imprecision<15% Inaccuracy<15% No carryover Applicability
(138)	Amphetamine Methamphetamine	Urine (10 mL)	SPE (HLB)°	Symmetry Shield RP_{18}	0.05% Formic acid Acetonitrile	ESI+	Ion Trap	Linearity 5-500 ng/mL LOD 1 ng/mL LOQ 5 ng/mL Imprecision<7% Inaccuracy<6% Recovery>97% No matrix effect Applicability

Table 5 : LC-MS(/MS) methods for the analysis of amphetamines in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(106)	Amphetamine, Methamphetamine, MDA, MDMA	Urine (1.6 mL)	SPE (C18)	Rocket EPS C_{18}	Ammonium Formate Acetonitrile	ESI+	Ion Trap	Linearity 5-160 ng/mL LOQ 5 ng/mL Imprecision<15% Inaccuracy<95% No matrix effects
(114)	Amphetamine, methamphetamine, MDA, MDMA, PMA	Urine (0.5 mL)	LLE (Diethyl ether)	Atlantis dC_{18}	Ammonium formate Acetonitrile	ESI+	QqQ	Linearity (1-1000 ng/mL) Recovery>80% Imprecision<18% Inaccuracy<18% LOD>0.2 ng/mL LOQ>1 ng/mL No matrix effects Applicability
(139)	Amphetamine, Methamphetamine, MDA, MDMA	Urine (0.5 mL)	Hydrolysis SPE (HLB)	Atlantis dC_{18}	Ammonium formate Acetonitrile	ESI+	QqQ	Linearity 1-2000ng/mL) LOD>0.5ng/mL LOQ>1 ng/mL Recovery>80% Imprecision<15% Inaccuracy<15% Matrix effect<30% Stability Applicability

Table 5 : LC-MS(/MS) methods for the analysis of amphetamines in blood and urine in the literature (continued)

Reference	Analyte	Sample	Sample preparation	LC		Interphase	Detector	Validation
				Column	Mobile Phase			
(140)	Amphetamine, methamphetamine, MDA, MDMA, MDEA	Blood and urine (not specified)	Toxitube A	Utisphere ODB C_{18}	Ammonium formate Acetonitrile	ESI+	QqQ	Linearity 0.1-50 ng/mL LOQ 0.1 ng/mL Imprecision<13% Inaccuracy<20% No matriz effects Applicability
(109)	Methamphetamine	Urine (1.6 mL)	SPE (C18)	Platinum EPS C_{18}	Ammonium formate Acetonitrile	ESI+	Ion Trap	Linearity 50-5000ng/mL LOD 10 ng/mL Imprecision<12% Inaccuracy<13% No matrix effects Applicability
(111)	MDMA, MDA, methamphetamine, amphetamine	Blood and urine (50µL)	Hydrolysis PPT (Acetonitrile)	Shiseido Capcell Pak SCX UG 80	Ammonium acetate Acetonitrile	ESI+	QqQ	Linearity 50-5000 ng/mL Imprecision<9.4% Recovery>90% Matrix effect<30% Applicability
(141)	Amphetamine, methamphetamine, MDA, MDMA	Urine (20 µL)	Dilution	Luna C_{18}	0.1%Formic acid Acetonitrile	ESI+	QqQ	Linearity 0-10000ng/mL LOD>2 ng/mL LOQ>7 ng/mL Imprecision<16% No matrix effect Applicability

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III. NEW ADVANCES IN LC-MS/MS METHODS

3.1. Introduction

As described in the previous chapter, samples from biological matrices are usually not directly compatible with LC-MS/MS analyses. Hence, sample preparation has traditionally been done using PPT, LLE, or SPE. Manual operations associated with these processes are very labor intensive and time consuming. Therefore, trends in sample preparation in analytical toxicology are to reduce of solvents, to simplify manipulations, and to reduce the time necessary for sample preparation. Increasing attention is being devoted to the possibility of automating analytical methods. Automation results in greater throughput, improved precision and accuracy, and a minimum of operator intervention, leading to safer sample handling and time-saving procedures. The current trends indicated that automated SPE is now more widely used than in the past. Much of this recent growth stems from increased capabilities of commercially available workstations (1,2). The most attractive feature of on-line SPE is that it almost entirely automates the sample handling process.

In the following, the details of the new trends in on-line SPE techniques will be discussed.

3.2. On-line SPE

A drawback of off-line SPE procedures is that they can be time consuming and cumbersome to perform, often requiring many steps before reaching a concentrated extract suitable for instrumental analysis, of which only a small portion is actually injected onto the chromatographic column.

The increased demand for high-throughput causes a unique situation of balancing cost versus analysis speed as each sample preparation technique offers unique advantages.

As compared to off-line SPE, on-line SPE offers a series of advantages (see Table 6). When working with on-line SPE, off-line SPE elution conditions are no longer applied: Typically, a strong wash solvent is used to elute the analyte from the SPE cartridge. Moreover, the less solvent is required to elute the analyte from the cartridge, the less time is needed for evaporation. With on-line SPE, elution is taking place when the cartridge is switched inline with LC-column and the LC-method is started. As soon as the analyte is eluted from the SPE cartridge, by the mobile phase (isocratic or gradient), the cartridge is switched off-line again. Matrix

compounds with a stronger retention than the analyte, will remain on the cartridge and are not eluted to the LC-column. There is also a difference in analyzed fraction: the reconstitution step is also a dilution step. Usually no the entire reconstituted fraction is injected by the LC. With on-line SPE, all of the analyte loaded on the SPE cartridge, is eluted to the LC column. This means, either less sample is required or higher sensitivity can be reached

Moreover, sample loading is under different conditions: Where with off-line SPE the sample and solvents are sucked through the cartridge using a vacuum manifold, with on-line SPE the sample is applied on the cartridge using a controllable flow rate. During sample loading, the sample is sandwiched between SPE solvents. When sample volumes are small, buffers or additives to break protein binding can be added to the SPE solvents. As long as the mixing between sample and SPE solvents is sufficient, it completely eliminates sample-treatment steps.

Typically, less sample volume is required for on-line SPE, especially when ‘raw’ samples are injected and pre-treatment steps, which are often also a dilution, are not required. However, injection of ‘raw’ samples does not require the use of a suitable autosampler and a disposable extraction column. There is also, a difference in cartridge capacity: When cartridges are constructed for high-pressure (>4000 psi) on-line extraction, a much wider range of sorbent materials and particle size (starting at 8 μm) can be utilized. Chromatographically efficient SPE cartridges combine high extraction capacity with small elution volumes to minimize band broadening and consequently provide increase assay sensitivity.

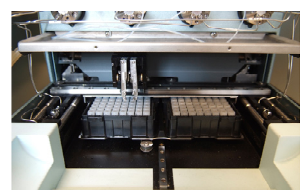
Table 6: Comparative features on on-line and off-line SPE configurations (3)

On-line SPE	Off-line SPE
Automatization and minimal sample handling which translates in better precision and accuracy	Manipulation of the sample, possibility of contamination, and less accuracy and precision
Direct and fast elution of the sample after preconcentration. Minimal degradation	Risk of degradation of compounds (longer overal analysis time)
Minimal consumption of organic solvents (elution with the LC mobile phase)	Consumption of organic solvents for elution
No loss of analytes due to evaporation steps	Possible loss of analytes during evaporation steps
Reduced analysis time and high throughput (simultaneous extraction and analysis of samples in a sequence)	Longer analysis time
Matrix effects; ion suppression or enhancement in MS	Less matrix effects in MS
Reusable cartridges	Disposable cartridges
Limited portability	Availability of portable SPE systems
Expensive equipment	Economical equipment

Thus, the on-line technique can be fully automated. Several generic approaches have recently been developed for on-line sample extraction coupled to LC-MS (4-6). Most on-line SPE approaches use column-switching to couple with the analytical columns. Various column dimensions can be configured for the fast analysis of drug and their metabolites in biological matrix at nanogram per milliliter level or lower.

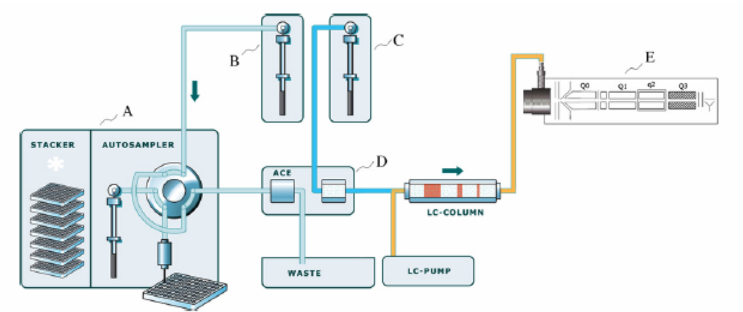
One commercially automated on-line SPE system is the Symbiosis system manufactured by Spark Holland. The system offers large flexibility in processing different types of sample selecting one of the three fully automated operational modes LC-MS(/MS) (direct LC without SPE); XLC-MS(/MS) (one-line SPE coupled to LC-MS(/MS)); AMD (advanced method development).

It includes an autosampler (Reliance), two binary LC pumps, an on-line SPE unit with two high-pressure solvent delivery pumps (HPDs) and a combined valve systems to direct fluid for different steps of SPE (Figure 19). At the beginning of each run, an on-line SPE cartridge is loaded into the unit. After a conditioning step with high organic solvent and an equilibrium step with low organic aqueous solution, a sample is injected onto the cartridge and washed with aqueous solution. Proteins and other matrix materials from the sample are removed during the washing step. Analyte of interest is then eluted onto the analytical column and detected by mass spectrometry. During the sample elution step, a second sample is loaded to a new on-line SPE cartridge for the next analysis.



In this parallel mode, the sample analysis cycle time approximates the LC run time without the time required for the SPE procedures. Since the on-line SPE cartridge is disposable and each sample uses a new cartridge, the carry-over problem from the extraction cartridge is eliminated.

Figure 19. Automated SPE unit coupled to a triple quadrupole mass spectrometer with (A) autosampler with a sample storage compartment, (B and C) high pressure dispensing pumps, (D) automated cartridge exchanger and (E) triple stage quadrupole mass spectrometer.



A generic method for the fast determination of a wide range of drugs in serum or plasma has been presented for the Spark Holland system (7). The method comprises generic solid-phase extraction with HySphere particles, on-line coupled to gradient LC with tandem mass spectrometry detection. The optimized generic SPE-LC-MS/MS protocol was evaluated for 11 drugs with different physicochemical properties. Good quantification for 10 out of 11 of the pharmaceuticals in serum or plasma could be readily achieved. The quantitative assays gave recoveries better than 95%, lower quantification limits of 0.2-2.0 ng/mL, acceptable precision and accuracy and good linearity over 2-4 orders of magnitude. Carry-over was determined to be in the range of 0.02-0.10%, without optimization.

An approach for on-line introduction of IS for quantitative analysis was developed on the Spark Holland system (8). In this approach, analyte and IS were introduced into the sample injection loop using a conventional autosampler (injector) needle pickup from a simple vial. IS was introduced into the sample injection loop on-line from a microresevoir containing the IS solution. As a result, both analyte and IS were contained in the sample loop prior to the injection into the column. The authors demonstrated comparable accuracy and precision to those obtained using off-line IS introduction (i.e. IS and analyte were pre-mixed before injection) while maintaining chromatographic parameters (i.e. analyte and IS elution time and peak width). This new technique was applied for direct analysis of model compounds in rat plasma using on-line SPE LC-MS/MS quantification. On-line IS introduction allows for non-

volumetric sample (plasma) collection and direct analysis without the need of measuring and aliquoting a fixed sample volume prior to the on-line SPE-LC-MS/MS. The method enables direct (plasma) analysis without any sample manipulation and preparation.

Koal et al (9) developed a method for quantification of 7 protease inhibitors in patient plasma samples. Only a sample dilution step was used to dilute samples and add internal standard before the analysis. Run time was only 3.3 min per sample and 6.6. min for the first sample. Alnouti et al (10) reported another study with Symbiosis system connected to a Luna C_{18} analytical column or a Chromolith C18 monolothic column for analysis of two model compounds. Rat plasma spiked with the analytes was diluted with internal standard and injected direcly into the system. Method development including on-line SPE cartridge selection and extraction condition optimization was performed by the Symbiosis system automatically. The total cycle time of 4 min with the Luna C_{18} was reported. The run time was reduced to 2 min per sample for the monolithic column without compromising the quality and validation criteria of the method.

Recently, Jagerdeo et al (11,12) developed a method for the analysis of cocaine and four of its metabolites in whole blood and urine, using Gemini C_6-Phenyl column and the HySphere MM anion cartridge. Very good precision and accuracy for all of the compounds were obtained.

In conclusion, the Symbiosis pharma system offers the entire process of conditioning, sample application, washing and elution taking place at constant flow rates, yielding better precision in comparison with off-line driven extraction procedures. Another important advantage is that no manual transfers are made and that the whole of the eluate is loaded onto the LC column without the need for a pre-concentration step. Therefore, it provides a reliable and simple alternative to improve sensitivity and detection limits for high throughput sample preparation and analysis of compounds in complex matrices (13).

Table7: On-line SPE applications with the Symbiosis system

Reference	Analytes	Sample	SPE		LC		Detection	Validation
			On-line system	Cartridge	Column	Mobile Phase		
(14)	10 drug candidates (names not done)	Plasma (500 µL)	Prospekt	IST Isolute solid-phase CN	Zorbax C_{18} RX	Acetonitrile Water TFA	QqQ	Linearity 2.5-1000 ng/mL LOD: 2.5-5 ng/mL Imprecision <20% Recovery:50-100% Applicability
(15)	Codeine	Plasma (700 µL)	Prospekt	Bond-Elut C_2	Hypersil BDS C_{18},	Phosphate buffer Acetonitrile	UV	Linearity 2-140 ng/mL LOD 0.5ng/mL Imprecision < 5.03% Inaccuracy 1.82% Recovery: 91.9% Applicability
(16)	Piroxicam	Plasma (200 µL)	Prospekt	Bond Elut C_8	Kromasil C_{18},	Acetonitrle Phosphate Buffer.	UV	Imprecision<9.05% Inaccuracy<7.8% Recovery>90% LOQ: 50 ng/mL Applicability

Table7: On-line SPE applications with the Symbiosis system (continued)

Reference	Analytes	Sample	SPE		LC		Detection	Validation
			On-line system	Cartridge	Column	Mobile Phase		
(17)	Camptothecin and metabolites	Plasma (100 µL)	Prospekt	C_{18} analytichem	Symmetry C_{18}	Phosphate buffer Acetonitrile	Fluorescence	Linearity 2.5-25000 ng/mL LOQ: 2.5 and 5 ng/m Imprecision<14.2% Inaccuracy<10.5% Recovery>96% Applicability
(7)	Sulfadiazine, sulfamerazine, taxol, propanolol, carbamazepine, procainamide, caffeine, ranitidine, theophillyne, theobromine, acetaminophen	Serum or plasma (100 µL)	Prospekt	Hysphere Resin GP	Hypersil ODS (C_{18})	Acetonitrile (0.1% Formic acid) Acetate buffer	QqQ	Linearity 2.5-40 ng/mL LOQ: >0.2 ng/mL Imprecision<15% Inaccuracy<15% Recovery>95% Carryover<0.1% Applicability

Table7: On-line SPE applications with the Symbiosis system (continued)

Reference	Analytes	Sample	SPE		LC		Detection	Validation
			On-line system	Cartridge	Column	Mobile Phase		
(10)	Propanolol, ketoconazole	Plasma (5, 50, 100 μL)	Symbiosis	Hysphere C_{18} HD	Luna C_{18}	Acetate buffer Acetonitrile	QqQ	Recovery>90% Linearity 0.1-100 ng/mL Imprecision<10% Inaccuracy<9% Applicability
(10)	Propanolol, ketoconazole, Diclofenac, ibuprofen	Plasma (1 mL)	Symbiosis	Hysphere C_{18} HD	Luna C_{18} Chromolith C_{18}	Acetate buffer Acetonitrile	QqQ	Recoveries:100% Range:1-1000 ng/mL CV%:>90 Bias%<14 Applicability
(18)	Clozapine, desmethylclozapine, clozapine-N-oxide	Serum (50 μL)	Prospekt-2	Hysphere C_{18} HD	Zorbax Eclipse XDB C_{18}	Acetate buffer Methanol	MS	Linearity 10-1000 ng/mL LOQ: 50 ng/mL LOD> 0.15 ng/mL Imprecision<20% Inaccuracy<10% Applicability

Table7: On-line SPE applications with the Symbiosis system (continued)

Reference	Analytes	Sample	SPE		LC		Detection	Validation
			On-line system	Cartridge	Column	Mobile Phase		
(19)	Amitriptyline, nortriptyline, imipramine, desipramine, trazodone, fluoxetine, norfluoxetine, paroxetine, fluvoxamine, sertraline, venlafaxine, norclomipramine, citalopram, clomipramine	Plasma (50 µL)	Symbiosis	Oasis MCX	Gemini C_{18}	Bicarbonate buffer Acetonitrile	QqQ	Recoveries>99% Matrix effect<18% Range: 10-1000 ng/mL CV%<20 Bias%<20 LOQ: 10 ng/mL Stability in the autosampler and freeze/thaw cycles Applicabilty

Table7: On-line SPE applications with the Symbiosis system (continued)

Reference	Analytes	Sample	SPE		LC		Detection	Validation
			On-line system	Cartridge	Column	Mobile Phase		
(20)	Guvacine, kojic acid, theobromide, codeine, emetine, theophylline, hyoscyamine, quinine, catechin, chlorogenic acid, dihydrorobinetin, harmine, caffeic acid, khellol glucoside, berberine, coumaric acid, ellagic acid, etc	Stock solutions (10 µL)	Symbiosis	HysPhere Resin GP	Luna C_{18}	0.1% Formic acid Acetonitrile	DAD	Evaluation of trapping efficiency Influence of flow rate of loading solvent Influence of acetonitrile content in loading solvent Influence of analyte content Capacity of GP cartridges
(21)	8 drug compounds (names not done)	Blood (5-10 µL)	Symbiosis	HysPhere C_{18} HD	X-Bridge C_{18}	0.1% Formic acid Methanol	QqQ	Recovery>50% Stability Applicability

Table7: On-line SPE applications with the Symbiosis system (continued)

Reference	Analytes	Sample	SPE		LC		Detection	Validation
			On-line system	Cartridge	Column	Mobile Phase		
(11)	Cocaine, ecgonine, ecgonine methyl ester, benzoylecgonine, cocaethylene	Whole blood (500 µL)	Symbiosis	Hysphere MM Anion Exchange	Gemini C_6-Phenyl	0.1%Formic acid Acetonitrile	QqQ	Linearity: 4-500 ng/mL LOQ : 8-47 ng/mL LOD : 3-16 ng/mL Imprecision<9% Inaccuracy<7% Applicability
(12)	Bennzoylecgonine, ecgonine methyl ester, ecgonine, cocaethylene	Urine (1 mL)	Symbiosis	Hysphere MM Anion Exchange	Gemini C_6-Phenyl	0.1% Formic acid Acetonitrile	QqQ	Linearity 7-1000 ng/mL Imprecision<9% Inaccuracy<5% LOD: 3-23 ng/mL LOQ : 7-69 ng/mL Applicability

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IV. STUDY OBJECTIVES

The aim of this work was to optimize LC-MS/MS methods in this laboratory for characterization and quantification of popular drugs in biological matrices, and additionally develop a new emerged technology: the on-line SPE-LC-MS/MS (Symbiosis system). Applications were chosen because of the specificity of the method and/or the frequent need of analysis of a large number of samples.

At the moment of the development of a method there were several significant aspects to take into account a) the amount of sample required can be an analytical limitation, b) forensic toxicologists may be required to analyze drugs in blood to evaluate an impairment status, c) the analysis of urine is often demanded as the time window for the detection of drugs is wider than for blood and d) complete validation is required to guarantee the robustness of the method.

4.1. Hallucinogens in urine (LC-MS/MS)

Until recently in Europe, LSD was the substance that dominated the field of hallucinogenic drug use. The emergence of hallucinogenic plants and mushrooms as a potentially widespread drug trend laid relatively dormant in Europe until the late 1990s when they began to be marketed alongside other 'natural' products by smart shops in the Netherlands. It seems that interest in natural hallucinogens appear to be related to a 'return to nature' trend and has been facilitated by the rapid expansion on internet sales and information. The screening of hallucinogens is quite complex because of the wide structural variety of compounds in these group. In the toxicology section of the NICC there was no method available to quantify the hallucinogens, while their analysis is often demanded. Due to the fact that concentrations of hallucinogens in blood are quite low, urine was considered as the best choice due to the larger sample volume, higher drug concentrations, and wider time window of detection of drugs.

4.1.1. We aimed at developing an LC-MS/MS method for the analysis of a broad collection of hallucinogens and related compounds as chlorpheniramine, ketamine, ritalinic acid (including metabolites) in urine using a simple off-line SPE and LC-MS/MS with the highest sensitivity.

4.1.2. This method was to be applied for the routine analysis of urine samples in the laboratory.

4.2. Cannabis and Amphetamines in blood and urine

Since the 1990s, cannabis use has risen markedly among general and school populations in many EU countries. While consumption patterns remain largely occasional, there also signs of more intensive use which could cause health or social problems and in time lead to dependence.

Globally, after cannabis, amphetamines are among the most commonly consumed illicit drugs. In Europe today, in terms of the absolute numbers, cocaine use may be higher, but the geographic concentration of cocaine in a few countries means that for most of the European Union, some form of synthetically produced drug remains the second most commonly used illicit substance.

During 2008, the section of toxicology of the NICC was requested to analyze 1939 blood samples, collected in a context of drugged driving, and 675 urine samples, collected in different forensic settings for the analysis of drugs of abuse.

70.1% of these blood samples were positive for cannabis and 24.4 % for amphetamines.

In the case of the urines, 55.9% were positive for cannabis and 6.7% for amphetamines.

Therefore, the need of rapid, sensitive and robust methods using LC-MS/MS for the analysis of cannabis and amphetamines in blood and urine was a clear next objective of the Institute.

4.2.1. Analysis of THC and metabolites in blood by LC-MS/MS:

Up to now, in the section of toxicology of the NICC the analysis of THC and metabolites was carried out in 1 mL blood with GC-MS (LLE + derivatization). It was a very long sample preparation process and in many cases the sample volume was insufficient to perform the analysis (as usually the analysis of other drugs is demanded too).

4.2.1.1. Our aim was to develop a simple, fast and sensitive LC-MS/MS method for the confirmation of THC, 11-OH-THC and THC-COOH in blood.

4.2.1.2. The method needed to be validated requiring a small sample volume.

4.2.1.3. The method was to be applied to the analysis of authentic blood samples for routine analysis.

4.2.2. Analysis of THC-COOH in urine by on-line SPE-LC-MS/MS:

4.2.2.1. The aim of this study was to develop and completely validate a simple, rugged and high-throughput on-line SPE-LC-MS/MS method for rapid analysis of THC-COOH in urine.

4.2.2.2. The method would require a minimum sample handling.

4.2.2.3. In addition, the aim was to determine the presence or absence of THC-COOH in urine with respect to the internationally accepted cut-off of 15 µg/L.

4.2.3. Analysis of amphetamines in blood and urine by on-line SPE-LC-MS/MS

4.2.3.1. The aim of this study was to develop and completely validate a simple, rugged on-line SPE-LC-MS/MS method for rapid and simultaneous bio-analysis of 7 amphetamines in blood and urine.

4.2.3.2. The method would have enough sensitivity to reduce the sample volume (50-100 µL).

4.2.3.3. Moreover, the total process from the sample preparation to the analysis needed to be completely automated.

4.2.3.4. The validated method was to be applied to the routine analysis of authentic blood and urine samples.

V. APPLICATIONS

5.1. LC-MS/MS METHOD

FOR THE SIMULTANEOUS ANALISIS OF MULTIPLE HALLUCINOGENS, CHLORPHENIRAMINE, KETAMINE, RITALINIC ACID, AND METABOLITES IN URINE.

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Liquid Chromatography–Tandem Mass Spectrometry Method for the Simultaneous Analysis of Multiple Hallucinogens, Chlorpheniramine, Ketamine, Ritalinic Acid, and Metabolites, in Urine

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Abstract

A validated method for the simultaneous analysis of multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid, and several metabolites is presented. The procedure comprises a sample clean-up step, using mixed-mode solid-phase extraction followed by liquid chromatography (LC)–tandem mass spectrometry analysis. Chromatographic separation was achieved using a Sunfire C_8 column eluted with a mixture of formate buffer, methanol, and acetonitrile. The applied LC gradient ensured the elution of all the drugs examined within 14 min and produced chromatographic peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time and two precursor-product ion transitions for the non-deuterated analogues. Validation of the method was performed using 500 µL of urine. The limits of quantification (LOQ) for LSD and 2-oxo-3-hydroxy-LSD were 0.05 and 1 ng/mL, respectively, and ranged, for the other hallucinogens, from 0.5 to 10 ng/mL. Linear and quadratic regression was observed from the LOQ of each compound to 12.5 ng/mL for LSD, 50 ng/mL for 2-oxo-3-hydroxy-LSD and 500 ng/mL for the others ($r^2 > 0.99$). Precision for the QC samples, spiked at a minimum of two concentrations, was calculated [%CV and %bias < 20% for most of the compounds, except for bufotenine and cathinone (%bias < 24%), and ibogaine (%bias < 30%)]. Extraction was found to be both reproducible and efficient with recoveries > 87% for all the analytes. Furthermore, the processed samples were demonstrated to be stable in the autosampler for at least 24 h. Finally, the validated method was applied to the determination of chlorpheniramine, ketamine, LSD, and psilocin in authentic urine samples.

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Introduction

Hallucinogens are mostly of vegetal origin (e.g., mushrooms, cacti, and other plants), and their effects were already known by the ancestral cultures (1–7). The most powerful among them and semisynthetic in nature, lysergic acid diethylamide (LSD), was discovered about 60 years ago (8,9). Recently, the illicit use of hallucinogens has re-emerged, especially among teenagers and well-educated adults (10,11). Typical clinical effects of hallucinogens include dizziness, weakness, tremors, nausea, drowsiness, paraesthesia, blurred vision, altered shapes and colors, difficulty in focusing on objects, sharpened sense of hearing, alteration in mood, tension, distorted time sense, difficulty in expressing thoughts, depersonalization, dreamlike feelings, and visual hallucinations (12,13). In addition, one of the well-known side effects of LSD is the occurrence of flashbacks. These are repeated hallucinatory and other perceptual experiences occurring after the initial LSD experience has subsided (14). Over the last few years, human hallucinogenic drug research has regained attention. The main areas of current interest are the pharmacology of these drugs. However, information about how they affect the brain is scarce (15–18). No dose-response relationship has been reported for these compounds. Despite their high degree of physiological safety and lack of dependence liability, hallucinogens have been branded by law enforcement officials as among the most dangerous drugs that exist (1,12,19). Indeed, although there are no reports that confirm that LSD or other classical hallucinogens have directly caused overdose death, fatal accidents may occur particularly when these drugs are used recreationally in unsupervised settings. During the last decade, the use of prescription drugs for non-medical purposes (e.g., for recreation and for psychic effects—to get high,

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to have fun, to get a lift, or to calm down) has increased (20). Ketamine is catalogued as a synthetic anesthetic for human and veterinary surgery; it induces sedation, immobility, and amnesia. It has also gradually become popular on the European party scene for its hallucinogenic effects and, when used in combination with ecstasy ("candy-flipping"), as a stimulant (21,22). Methylphenidate (with ritalinic acid as its main metabolite) is a phenethylamine derivative used in the treatment of depression, narcolepsy, attention-deficit disorder, and childhood hyperkinesis. Recently, it has become a popular drug of abuse because of its properties similar to morphine when combined with pentazocine (a synthetic derivative that has properties of an analgesic) (23). Chlorpheniramine is used to relieve or prevent the symptoms of hay fever and other allergies and to prevent motion sickness, nausea, vomiting, and dizziness. Human poisoning caused by accidental or intentional ingestion has been reported (24–26).

Consequently, methods for the extraction and determination of all of these compounds from human samples are required for both diagnosis and effective treatment of the intoxication and for forensic purposes. Several techniques have been used for the toxicological analysis of hallucinogens and the licit drugs in different biological matrices, including gas chromatography–mass spectrometry (GC–MS) (21,24,27–32) and liquid chromatography–mass spectrometry (LC–MS) (8,16,22,23,33–40). However, most of the reported methods cover only a single substance or mixtures of a few hallucinogens and/or other drugs or they need time-consuming derivatization or several cycles.

LC–MS–MS detection is now considered the method of choice for the quantitative determination of thermolabiles and polar drugs in biological matrices because of simple pretreatment, high sensitivity, and relatively short analysis time. Until now, no report dealing with the simultaneous analysis of a large series of hallucinogens in urine has been published.

In the present study, a convenient and sensitive LC–electrospray ionization (ESI)-MS–MS method has been developed and validated for the determination of multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid, and metabolites in urine. In addition, this method was applied to the determination of chlorpheniramine, ketamine, LSD, and psilocin in authentic urine samples.

Experimental

Chemicals

Ammonium formate, formic acid, and sodium hydroxide (0.1N) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonia solution (32%, extra pure) and glacial acetic acid were from Merck (Darmstadt, Germany). Acetonitrile and methanol (LC–MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands). Solid-phase extraction (SPE) cartridges Oasis® MCX (30 mg, 1 mL) were from Waters (Milford, MA).

Individual certified stock solutions of bufotenine, cathinone, chlorpheniramine, ibogaine, ketamine, mescaline, and norketamine (at a concentration of 1 mg/mL in methanol); psilocin (as a solid); LSD (at a concentration of 25 µg/mL in methanol); 2-oxo-3-hydroxy-LSD (0.1 mg/mL in methanol); and the deuterated analogues psilocin-d_{10} (as a solid), ketamine-d_4, LSD-d_3, mescaline-d_9, and norketamine-d_4 (at a concentration of 0.1 mg/mL in methanol) were from LGC Promochem (Molsheim, France). Standard reference material of scopolamine, kavain, lobeline, and ritalinic acid (all of them as a solid) and ritalinic acid-d_5 (0.1 mg/mL in methanol) were purchased from Sigma-Aldrich. Liquichek™ external quality controls (QC) were purchased from Bio-Rad Laboratories (Irvine, CA) (expressed in the text as C1 and C3). Participation in proficiency tests ["UKNEQAS for Drugs of Abuse in urine" (Cardiff, U.K.)] were also used in the evaluation of the accuracy of the method.

Specimens

Urine used for the preparation of blanks, calibrators, and QC samples was obtained from healthy drug-free volunteers. A batch of authentic human urine was also obtained from two subjects who admitted having used "magic mushrooms"; urine samples were collected 1, 4, 8, and 24 h after ingestion. The samples were analyzed anonymously. In addition, a human sample was collected from one subject who ingested one tablet containing 3 mg of chlorpheniramine in the morning followed by another tablet of the same drug in the evening. A urine sample was collected one day later. Finally, a dog urine sample was obtained from a veterinary clinic. The dog had received a dose of a mixture of compounds (containing ketamine) prior to a surgical operation.

Preparation of standard solutions

An internal standard (I.S.) working solution of each of the deuterated analytes was prepared (1 µg/mL in methanol, except for LSD-d_3 which was at 10 ng/mL).

A working solution containing all of the non-deuterated analytes was prepared (10 µg/mL in methanol for all the compounds except for LSD, which was 250 ng/mL, and for 2-oxo-3-hydroxy-LSD, which was 1 µg/mL).

Working solutions for the calibrators were prepared monthly and stored at 4°C. The QCs were prepared in one single batch, aliquoted at the beginning of the validation of the study, and stored at –20°C until analysis.

Sample preparation and extraction

Six-hundred microliters of 0.1M sodium acetate buffer (pH 4) and 50 µL of I.S. working solution were added to 500 µL of urine. After conditioning with 1mL of methanol, 1 mL of water, and 1 mL of sodium acetate buffer (0.1mM, pH 4), the diluted urine samples were applied onto the SPE columns. Sample clean-up was optimized using a variety of washing protocols: 1. successive 1-mL washes of sodium acetate buffer (0.1M, pH 4) and methanol; 2. 1-mL washes of the same buffer and a mixture of methanol and water (80:20, v/v); and 3. 1-mL washes of buffer and a mixture of methanol and water (50:50, v/v). After washing, cartridges were dried by applying full vacuum for 5 min before elution with 0.5 mL of 5% ammonia in methanol. Following the extraction, the elution solution was

completely evaporated to dryness under a gentle stream of nitrogen. The dry residue was then reconstituted in 600 µL of the aqueous mobile phase (A) used in the LC gradient. Twenty microliters was injected into the LC–MS–MS.

LC

LC was performed using a Waters Alliance 2695 separation module. All aspects of system operation and data acquisition were controlled using MassLynx NT 4.1 software (Waters). Analytes were separated on a Sunfire® C_8 column (3.5 µm, 2.1 mm × 100 mm) (Waters) using a gradient elution with ammonium formate buffer 10mM pH 3.5 (A) and acetonitrile/methanol (2:1, v/v), at a flow rate of 0.3 mL/min. A gradient was carried out starting from 2% B at 3.5 min, B was then increased to 20% over the next 0.5 min and maintained over the next 4 min. From 8 min to 11 min, B was linearly increased to 98% and maintained for 4 min before returning to its initial conditions within 0.5 min and equilibrating for 6.5 min, which resulted in a total run time of 22 min.

MS

A Quattro Ultima tandem MS (Waters) fitted with a Z-Spray ion interface was used for all analyses. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis: capillary voltage, 1.0 kV; source block temperature, 120°C; a desolvation gas (nitrogen) heated to 250°C and delivered at a flow of 800 L/h. The appropriate multiple reaction monitoring (MRM) conditions for the individual analytes and their respective deuterated analogues were determined by direct infusion into the MS. The cone voltage (CV) was adjusted to maximize the intensity of the protonated molecular species $[M + H]^+$ and collision-induced dissociation of each protonated molecule was performed. Collision gas (argon) pressure was maintained at 2.7×10^{-3} mbar and the collision energy (eV) adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis.

Method validation

Linearity, within-run precision, and between-run precision. Quantification was performed by integrating of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. Thus, the deuterated I.S. were used to correct for variability in the extraction procedure. Where it was not possible to obtain a commercially available deuterated analogue for any compound, the deuterated analogue of another close compound was selected. The different I.S. were assigned to the different analytes in the following combinations: LSD-d_3 was used for the calculations of

Table I. MRM Transitions and Conditions for All Compounds and their Deuterated Analogues

Compound	Rt (min)	Precursor Ion (*m/z*)	Product Ions* (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Bufotenine	3.2	205.20	160.10, 58.10	20	18, 15
Cathinone	5.4	150.10	132.20, 117.10	25	12, 20
Psilocin	5.7	205.20	160.10, 58.10	20	15, 18
Mescaline	8.0	212.20	195.20, 180.20	35	10, 20
Scopolamine	8.0	304.10	156.20, 138.00	20	15, 20
2-Oxo-3-Hydroxy-LSD	8.7	356.20	237.20, 222.20	40	25, 35
Norketamine	8.9	224.10	207.10, 125.00	30	10, 25
Ketamine	9.2	238.20	125.10, 207.10	20	30, 15
Ritalinic acid	9.2	220.20	84.20, 56.30	40	20, 45
Ibogaine	12.0	311.30	174.20, 122.10	20	35, 35
LSD	12.0	324.30	223.20, 208.20	25	25, 30
Chlorpheniramine	12.2	275.20	230.10, 167.10	20	20, 40
Lobeline	12.5	338.40	216.30, 96.10	40	20, 25
Kavain	13.4	231.10	185.10, 115.10	40	12, 12
Psilocin-d_{10}	5.7	215.20	66.10	10	18
Mescaline-d_9	8.0	221.30	204.30	20	10
Norketamine-d_4	10.0	228.20	211.20	30	10
Ketamine-d_4	8.2	242.20	129.10	35	30
Ritalinic acid-d_5	9.2	225.10	83.90	20	20
LSD-d_3	12.0	327.30	226.60	20	20

* Underlined transitions were used for quantification.

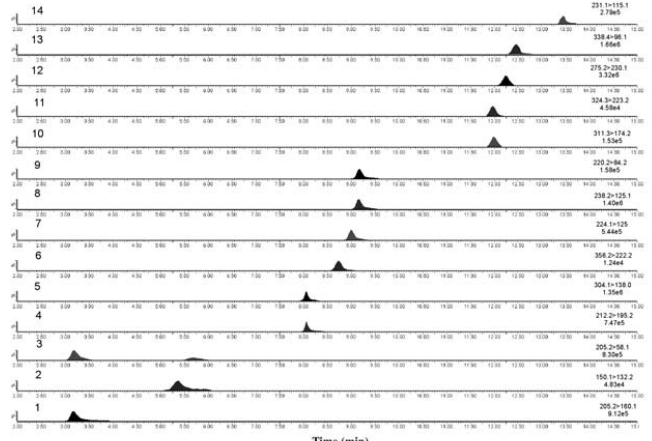

Figure 1. MRM chromatograms obtained with a single injection of a 500 µL extracted urine sample enriched with 10 ng/mL (at 0.25 ng/mL and 1 ng/mL for LSD and 2-oxo-3-hydroxy-LSD, respectively) of bufotenine (1) (peak at 3.2 min), cathinone (2), psilocin (3) (peak at 5.7 min), mescaline (4), scopolamine (5), 2-oxo-3-hydroxy-LSD (6), norketamine (7), ketamine (8), ritalinic acid (9), ibogaine (10), LSD (11), chlorpheniramine (12), lobeline (13), and kavain (14).

peak-area ratios and concentrations of LSD; ketamine-d_4 for ketamine and lobeline; mescaline-d_9 for bufotenine, mescaline, and scopolamine; norketamine-d_4 for norketamine; psilocin-d_{10} for cathinone and psilocin; and ritalinic acid-d_5 for chlorpheniramine, ibogaine, kavain, 2-oxo-3-hydroxy-LSD, and ritalinic acid.

Assay linearity was investigated by constructing calibration curves (n = 8) which ranged from 0.05 to 12.5 ng/mL (0.05, 0.25, 0.625, 1.25, 3.125, 6.25, and 12.5 ng/mL) for LSD; from 1 to 50 ng/mL (1, 2.5, 5, 12.5, 25, and 50 ng/mL) for 2-oxo-3-hydroxy-LSD; and from 10 to 500 ng/mL (10, 25, 50, 125, 250, and 500 ng/mL) for the other compounds.

QCs were analyzed for every run in blank urine at least at two concentration levels: at 4, 40, and 400 ng/mL for ketamine, lobeline, norketamine, and ritalinic acid; 40 and 400 ng/mL for psilocin, bufotenine, cathinone, chlorpheniramine, scopolamine, mescaline, and ibogaine; 4 and 40 ng/mL for 2-oxo-3-hydroxy-LSD; and 0.1, 1, and 10 ng/mL for LSD. Within-run precision and between-run precision were estimated by replicate (n = 2) analysis of the QC samples performed on six different days, according to Peters et al. (41). The precision was expressed as the %CV (variation coefficient). A comparison of the calculated concentrations of the QC samples to their respective nominal values was used to assess the accuracy (bias) of the method.

The limit of quantification (LOQ) was defined in this study as the concentration of the lowest calibrator that was calculated to be within ± 20% of the nominal value and with a %CV less than 20%.

The limit of detection (LOD) was estimated from blanc urine samples, spiked with decreasing concentrations of the analytes, where the response of the qualitative ion could reliably differentiate from background noise and with signal-to-noise ratio (S/N) of the qualifier equal to or greater than 3.

Selectivity, stability, recovery, and assessment of matrix effects. Selectivity was evaluated by analyzing urine samples from eight healthy volunteers who did not take any of the targeted compounds for several days before urine sampling and checked for the absence of the compounds of interest by analyzing the samples with the present technique before using as blanks.

The stability of the processed sample, when placed in the autosampler (maintained at 6 ± 2°C) was checked by repeated injections of two extracted QC samples (n = 5, each) at 40 and 400 ng/mL, except for LSD (1 and 10 ng/mL) and 2-oxo-3-hydroxy-LSD (4 and 40 ng/mL). Five extracted samples at each concentration were spiked with the I.S. at time zero and after 24 h, and the stabilities were estimated by comparing the peak response ratios at each concentration. Stability was tested against a lower percentage limit corresponding to 90% of the mean value of control samples by one-sided t-test ($P < 0.05$).

Extraction recoveries were estimated by comparing the ratio of the peak areas of the non deuterated compounds to the peak areas of the I.S. (i.e., responses) of a 40 ng/mL QC (n = 3) (except LSD at 1 ng/mL and 2-oxo-hydroxy-LSD at 4 ng/mL) when the non-deuterated compounds were added before the extraction step with those obtained when the non deuterated analytes were added after sample extraction. In both cases, the deuterated analogues were added after the extraction.

Matrix effect was investigated by adding the compounds at 40 ng/mL concentrations (except LSD at 1 ng/mL and 2-oxo-hydroxy-LSD at 4 ng/mL) to six different extracted blank urines just before injection, and the peak I.S. areas were compared to those obtained from the QCs at the same concentration and diluted in the same volume (aqueous mobile phase A) (n = 6).

Results and Discussion

Method validation

The applied gradient ensured the elution of all the drugs examined within 14 min and produced chromatographic peaks of acceptable symmetry. Selectivity of the method was achieved by

Table II. Dynamic Range, LOD, LOQ, and Equation of a Typical Calibration Curve with the Corresponding Coefficient of Determination (r^2)

Compound	LOD (ng/mL)	LOQ (ng/mL)	Dynamic Range (ng/mL)	Equation	r^2
Bufotenine	0.0125	10	10–500	$y = 0.000058159x^2 + 2.2625x – 0.074542$	0.999783
Cathinone	1.25	10	10–500	$y = 0.689329x – 1.03924$	0.996005
Psilocin	0.5	10	10–500	$y = 0.000199849x^2 + 2.27876x – 2.61572$	0.999747
Mescaline	0.5	10	10–500	$y = 1.44018x + 4.1591$	0.996820
Scopolamine	0.025	10	10–500	$y = –0.000304532x^2 + 1.63741x + 2.60315$	0.999785
2-Oxo-3-Hydroxy-LSD	0.0125	1	1–50	$y = 0.855494x – 0.535242$	0.992852
Norketamine	0.5	2	2–500	$y = 0.000250638x^2 + 1.64552x – 0.152192$	0.999092
Ketamine	0.125	0.5	0.5–500	$y = 0.000332073x^2 + 1.13911x – 0.145666$	0.999446
Ritalinic acid	2.5	2	2–500	$y = 0.000319152x^2 + 0.754816x + 0.141469$	0.999222
Ibogaine	0.5	10	10–500	$y = 0.000141267x^2 + 0.612077x – 0.888373$	0.999783
LSD	0.0003	0.05	0.05–12.5	$y = 8.56868x – 0.118143$	0.999201
Chlorpheniramine	0.0125	10	10–500	$y = 0.0044298x^2 + 17.04x – 6.09868$	0.998370
Lobeline	0.05	2	2–500	$y = 0.00144394x^2 + 1.3557x – 1.11657$	0.999948
Kavain	0.25	10	10–500	$y = 1.26145x – 3.39317$	0.996979

a combination of retention time, precursor, and product ions and determined acceptable in terms of absence of interference in the blank urine samples analyzed. With the exception of 2-oxo-3-hydroxy-LSD and ibogaine, the most prominent precursor-product transition was used for quantification and the next most abundant used as qualifiers (Table I). For 2-oxo-3-hydroxy-LSD and ibogaine, an elevated background response was noted when using the most prominent precursor product transition. Improved sensitivity (based on the signal-to-noise ratio) was achieved when using the next most abundant for quantification.

For the corresponding deuterated analogues, only one transition was monitored. Injection of single solutions did not produce interference in the other MRM channels, except for psilocin and bufotenine. As these compounds show the same transitions, many efforts were made to improve the chromatographic separation. Using the optimized chromatographic conditions, these two compounds were separated with retention times (Rt) of 3.2 and 5.7 min for bufotenine and psilocin, respectively. Figure 1 shows the MRM chromatograms of an extracted urine blank sample spiked at 10 ng/mL (at 0.25 ng/mL and 1 ng/mL for LSD and 2-oxo-3-hydroxy-LSD, respectively).

The linearity of the method was verified over the concentration range. After evaluating different types of regression, the calibration curves were constructed using a 1/*x* weighted quadratic regression for all of the compounds except for cathinone, mescaline, 2-oxo-3-hydroxy-LSD and kavain, to where linear regression was observed to be more suitable. The study was based on the standard error of the fit and minimization of bias of the calibrators. In all cases a $r^2 > 0.99$ was obtained. A typical example of a calibration curve, dynamic range, LOD, and LOQ for each compound is shown in Table II.

Within-run precision (%CV) was lower than 11% for all the analytes at the QC sample concentrations. The between-run precision (%CV) was also < 20%. A bias < 16% was observed for all the compounds except for bufotenine, cathinone, and ibogaine, which reached 23.6%, 23.8%, and 29.4%, respectively. Although for these compounds it could be considered out of

Table III. Within-Run Precision (%CV), Between-Run Precision (%CV), and Bias (expressed as percent of deviation) of the QC Samples

	Concentration (ng/mL)	Within-Run Precision (%CV)	Between-Run Precision (%CV)	Bias (%)
Bufotenine	400	5.1	8.9	13.0
	40	5.8	18.6	23.6
Cathinone	400	5.1	17.4	18.3
	40	3.2	19.0	23.8
Psilocin	400	3.7	2.6	5.5
	40	2.1	11.1	10.7
Mescaline	400	3.8	9.7	7.5
	40	2.6	4.3	9.8
Scopolamine	400	7.2	5.1	5.4
	40	5.1	9.8	12.9
2-Oxo-3-hydroxy-LSD	40	5.4	8.3	5.4
	4	7.3	9.3	4.0
Norketamine	400	1.8	4.7	1.5
	40	2.1	5.1	10.8
	4	5.4	5.8	13.3
Ketamine	400	4.6	4.6	1.7
	40	2.3	6.7	9.1
	4	2.5	5.8	13.8
Ritalinic acid	400	4.5	8.5	1.2
	40	2.0	7.4	4.9
	4	5.6	7.6	9.3
Ibogaine	400	7.9	13.7	15.6
	40	8.3	14.5	29.4
LSD	10	8.1	7.9	9.2
	1	9.2	11.8	4.0
	0.1	3.4	9.6	10.0
Chlorpheniramine	400	7.1	9.4	6.3
	40	6.0	9.6	9.3
Lobeline	400	4.8	7.6	2.2
	40	2.5	8.8	8.4
	4	4.8	4.2	15.0
Kavain	400	5.2	8.5	4.6
	40	4.8	9.1	3.3

Table IV. Extraction Recovery and Matrix Suppression*

	Recovery	Matrix Effect	
	(*n* = 3)	(*n* = 6)	(%CV)
Bufotenine	106.9	63.8	6.8
Cathinone	99.5	69.9	9.9
Psilocin	87.6	85.0	5.6
Mescaline	103.7	78.9	9.6
Scopolamine	106.7	68.8	9.0
2-Oxo-3-Hydroxy-LSD	107.4	101.5	7.5
Ketamine	105.3	78.2	6.2
Ritalinic acid	107.9	134.0	9.5
Norketamine	106.0	87.5	8.7
LSD	107.1	75.0	12.1
Chlorpheniramine	104.7	67.5	8.8
Ibogaine	109.5	68.9	12.0
Lobeline	109.4	53.9	5.3
Kavain	108.6	92.1	9.8

* Data represent the mean of several experiments with a 40 ng/mL calibrator.

Table V. Summary of Psilocin Urine Concentrations Observed in Authentic Samples from Two "Magic Mushroom" Users

	Time (h)			
ID	0	4	8	24
1	407.9 ng/mL	1358.7 ng/mL	757.0 ng/mL	16.6 ng/mL
2	113.7 ng/mL	624.5 ng/mL	N.A.*	N.A.

* Not available.

the validation requirements, in the literature similar results has been reported and considered 'sufficient for screening purposes' (42). The results are summarized in Table III.

The stability in the autosampler of the spiked samples at two concentrations was monitored at 6 ± 2°C for 24 h. No statistical significant differences could be observed for the two concentrations for all compounds ($P < 0.05$) (data not shown).

One of the limiting factors of LC–MS(–MS) applications is the potential presence of matrix effects leading to suppression or enhancement of the analyte response. It may be eliminated or minimized by 1. changing and improving sample preparation procedures, 2. performing the assay under more efficient chromatographic conditions to separate analytes of interest from undetected endogenous compounds that may effect the efficiency of ionization of analytes, and 3. by evaluating and changing the LC–MS interface and the mechanism of ionization of analytes (43,44). We evaluated several washing procedures for SPE of the hallucinogens during method development. Looking for a compromise between sample clean-up with a minimum loss of analyte, the best results were obtained by washing the cartridges with 1 mL washes of sodium acetate (0.1M, pH 4) and a mixture of methanol and water (50:50, v/v). Very high and reproducible recoveries were obtained with this SPE procedure for all analytes. On the other hand, some analytes presented a significant matrix effect (> 20%), which could be also the cause of the analytical variation (higher %bias) for some of them. Nevertheless, it has been stated that the use of deuterated I.S. would partially compensate for ion suppression effects (42). Indeed, taking into account that this is a screening method for urine analysis and, in this case, the sensitivity is not an issue (so false results are unlikely), we can conclude that the matrix effect found in some of the compounds are still acceptable. The results of the extraction recovery and matrix effect study are presented in Table IV.

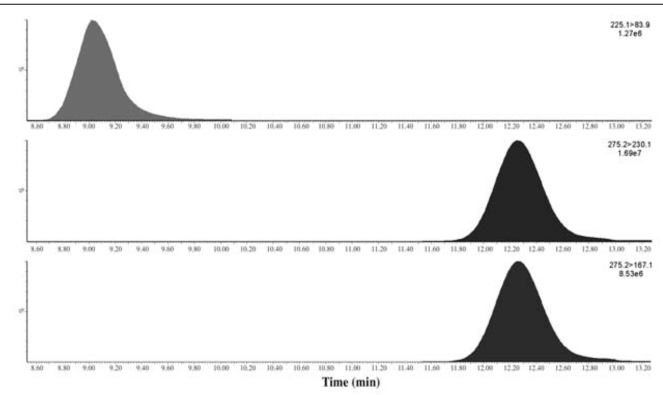

Figure 2. Typical MRM chromatograms obtained following the analysis of an authentic urine specimen from a chlorpheniramine user (225.6 ng/mL). The figure shows the response for the I.S., ritalinic acid-d_5 (top trace), and for the two transitions of chlorpheniramine (quantifier and qualifier; middle and bottom traces respectively). Peak intensity is shown in the top right-hand corner of each trace.

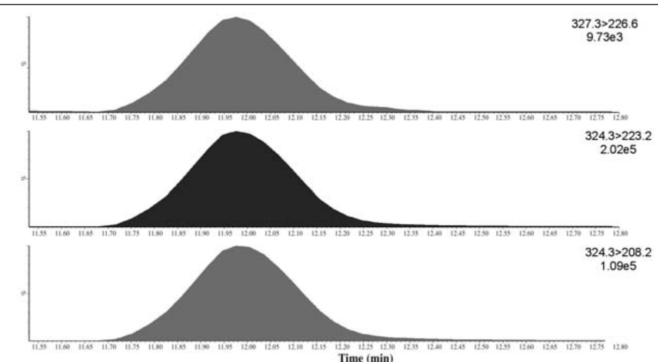

Figure 3. MRM chromatograms obtained following the analysis of an external QC (Cardiff, U.K.) containing 2 ng/mL of LSD. The figure shows the response for the two LSD transitions (qualifier and quantifier, middle and bottom traces respectively) and the I.S., LSD-d_3 (top trace). Peak intensity is shown on the right-hand corner of each trace.

Samples

The validated LC–MS–MS method was applied to the analysis of authentic urine samples. A dilution 1 in 10 was performed to those samples with concentrations of drugs out of the calibration range. In the first case, two young men had admitted to ingestion of some "magic mushrooms" to have fun. Urine samples were collected after 1, 4, 8, and 24 h. All samples were positives for psilocin (Table V). The highest concentrations were observed after 4 h after ingestion, which corresponds to an earlier report (45). One case of chlorpheniramine was obtained from a man who ingested two doses of a medicament (containing 3 mg of chlorpheniramine), one in the morning and the second one at night. The sample was collected one day after. Chlorpheniramine was detected at 225.6 ng/mL (Figure 2). Finally, we received a dog urine sample from a veterinary clinic. This sample came from a German dog that had a dose of ketamine prior to a surgical operation. A urine sample was collected 1 h later. High concentrations of ketamine (> 500 ng/mL) and much lower concentrations of norketamine (14.7 ng/mL) were observed in the dog urine sample.

This method has been also successfully applied to the analysis of external quality control samples ("ring test" organized by the "UKNEQAS for Drugs of Abuse in Urine") and C1 and C3 (Bio-Rad Laboratories). Figure 3 shows the typical chromatograms obtained following the analysis of one of the external QC samples. The results obtained for the LSD samples are shown in Table VI.

Table VI. Summary of Urinary LSD Concentrations in the External QC Urine Samples ("ring test" organized by the "UKNEQAS for Drugs of Abuse in Urine") and C1 and C3 (Bio-Rad Laboratories)

Sample ID	Concentration (ng/mL)	Bias (%)	Adulterated With
1	9.4	6.0	Dodecyl benzene sulfate
2	2.0	0.0	
3	6.4	14.7	
4	2.3	–15.0	
5	7.6	0.0	Sulfuric acid
6	14.5	N.A.*	
C1	0.2	5.6	
C3	0.7	6.2	

* Not available.

Conclusions

In this report, a validated and highly sensitive LC–ESI-MS–MS method is described for the simultaneous quantification of multiple hallucinogens, ketamine, chlorpheniramine, ritalinic acid, and their metabolites. The method combined SPE with LC–MS–MS and provided thorough clean-up of the matrix to avoid ion suppression or enhancement, in combination with high recovery, excellent precision, and bias in the linear range investigated, using 500 µL of sample. The method was successfully applied to authentic urine samples.

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V. APPLICATIONS

5.2. SIMULTANEOUS ANALYSIS OF THC AND ITS METABOLITES IN BLOOD USING LC-MS/MS

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Simultaneous analysis of THC and its metabolites in blood using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Cannabis is considered to be the most widely abused illicit drug in Europe. Consequently, sensitive and specific analytical methods are needed for forensic purposes and for cannabinoid pharmacokinetic and pharmacodynamic studies. A simple, rapid and highly sensitive and specific method for the extraction and quantification of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy- Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (THC-COOH) in blood is presented. The method was fully validated according to international guidelines and comprises simultaneous liquid–liquid extraction (LLE) of the three analytes with hexane:ethyl acetate (90:10, v/v) into a single eluant followed by separation and quantification using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Chromatographic separation was achieved using a XBridge C_{18} column eluted isocratically with methanol:0.1% formic acid (80:20, v/v). Selectivity of the method was achieved by a combination of retention time, and two precursor–product ion transitions. The use of the LLE was demonstrated to be highly effective and led to significant decreases in the interferences present in the matrix. Validation of the method was performed using 250 μL of blood. The method was linear over the range investigated (0.5–40 μg/L for THC, 1–40 μg/L for 11-OH-THC, and 2–160 μg/L for THC-COOH) with excellent intra-assay and inter-assay precision; relative standard deviations (RSDs) were <12% for THC and 11-OH-THC and <8% for THC-COOH for certified quality control samples. The lower limit of quantification was fixed at the lowest calibrator in the linearity experiments. No instability was observed after repeated freezing and thawing or in processed samples. The method was subsequently applied to 63 authentic blood samples obtained from toxicology cases. The validation and actual sample analysis results show that this method is rugged, precise, accurate, and well suited for routine analysis.

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1. Introduction

Cannabis is considered to be the most widely abused illicit drug in Europe. Indeed, statistical information shows that 30% of the under-forties age group have already consumed this drug [1,2].

Δ^9-Tetrahydrocannabinol (THC) is the main psychoactive constituent. During marijuana smoking, THC is rapidly absorbed in larger amounts than when taken orally and, due to its strong lypophilic nature, it spreads rapidly throughout the body. It is mainly metabolized to 11-hydroxy- Δ^9-tetrahydrocannabinol (11-OH-THC) by the human body. This metabolite is still psychoactive and is further oxidized to 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (THC-COOH). In humans and animals more than 100 metabolites could be identified but 11-OH-THC and THC-COOH are the most predominant. Metabolism mainly occurs in the liver by cytochrome P450 enzymes CYP2C9, CYP2C19 and CYP3A4 [3].

Urine drug concentration data do not provide adequate answers to demanding clinical and forensic questions. These are more readily answered with quantitative blood data which provides more information related to the current state of impairment. However, the analysis of blood can be more challenging due to the presence of lipophilic and proteinaceous compounds not usually found in urine, the need for substantially lower sensitivity limits and the lower sample volume available.

Due to the high specificity and the increased signal-to-noise in combination with short chromatographic run times, liquid

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chromatography–tandem mass spectrometry (LC–MS/MS) allows for specific, selective and sensitive analysis of compounds with a wide polarity range in samples of various nature. It offers the possibility to simplify sample preparation, although this approach should be treated with caution due to the possibility of ion suppression or enhancement as a result of the matrix. Consequently, attention must be paid to the choice of the sampling method and the influence of the collected matrix on the LC–MS/MS analysis.

Several methods have been described for the quantification of THC and its metabolites in blood. Immunochemical methods, mainly ELISA, are generally used as screening tools for cannabis use [4]. However, for workplace drug testing, driving under the influence of drugs and for forensic cases, the confirmation of positive immunoassay test results is necessary. It is usually performed by gas chromatography–mass spectrometry (GC–MS) methodologies [5–7]. However, GC requires time-consuming sample preparation and the need to use various derivatization techniques. In contrast to GC, no decomposition of the cannabinoids occurs during liquid chromatography and hence the cannabinoid acid forms may be analyzed directly. Several papers report the application of LC–MS(/MS) [8–14]. Most of them require high sample volume (1 mL) to achieve high sensitivity, they are focused on just one compound and/or the method is not fully validated (following all criteria for chromatographic assays). All of these aspects are significant at the moment of development of a method since (a) the amount of sample required is an analytical limitation, (b) forensic toxicologist may be required to analyze THC and other hydroxylated metabolites in blood to evaluate an impairment status and (c) complete validation is required to guarantee the robustness of the method.

Our aim was to develop and fully validate a simple, fast and sensitive LC–MS/MS method for the confirmation of THC, 11-OH-THC and THC-COOH in blood which required only a minimal volume of sample and with an efficient sample clean-up procedure.

2. Experimental

2.1. Chemicals

Individual stock solutions of THC, 11-OH-THC and THC-COOH (all certified at a concentration of 1 mg/mL in methanol), and the internal standards (I.S.) $[^2H_3]$THC (THC-d_3), $[^2H_3]$11-OH-THC (11-OH-THC-d_3) and $[^2H_9]$THC-COOH (THC-COOH-d_9) (certified concentration of 0.1 mg/mL in methanol) were obtained from LGC Promochem (Molsheim, France).

Methanol (LC–MS grade), 0.1% formic acid in water (UPLC grade) and water (HPLC grade) were purchased from Biosolve (Valkenswaard, The Netherlands). N-hexane (for chromatography), ethylacetate (for chromatography) and acetic acid (glacial) were obtained from Merck (Darmstadt, Germany).

External quality control (QC) samples were obtained from Medichem World (Steinenbronn, Germany).

2.2. Specimens

Pooled blank blood samples were used for development and validation of the procedure and were obtained from a local blood bank. Authentic samples were obtained from toxicology cases.

2.3. Preparation of standard solutions and sample extraction

Separate working solutions of the drugs, for tuning and selectivity experiments, were prepared in the laboratory at a concentration of 4 mg/L in methanol. A mixed working solution of non-deuterated compounds at 4 mg/L for THC and 11-OH-THC and of 16 mg/L for THC-COOH, in methanol was used for the preparation of calibrators. A mixed I.S. working solution of 1 mg/L for THC and 11-OH-THC and of 4 mg/L for THC-COOH, was prepared in methanol. Working solutions were stored at 4 °C, and were prepared monthly.

To obtain the lower concentrations needed for internal standardization and validation of each experiment, further dilutions in methanol were prepared the same day.

The extraction procedure was carried out in 10 mL disposable screw top vials of high quality glassware (Chromacol, Herts, UK) using 250 μL of blood. Fifty microliters of the I.S. working solution, 750 μL of deionised water and 200 μL of 10% acetic acid (glacial) were added. After adding 4 mL of hexane:ethyl acetate (90:10, v/v) mechanical shaking was carried out for 30 min. Then, the samples were centrifuged (10 min at 3000 × *g*), the organic phase was transferred to a 5 mL disposable screw top vial (Chromacol) and then evaporated to dryness with a vacuum centrifuge (Jouan, Saint Herblain, France). The extract was reconstituted in 120 μL of mobile phase and 30 μL was injected into the LC–MS/MS system.

2.4. LC–MS/MS

2.4.1. Chromatography

LC was performed using a Waters Alliance 2695 separation module (Waters, Milford, MA, US). Analytes were separated on a XBridge C_{18} column (150 mm × 2.1 mm, 3.5 μm) (Waters), eluted isocratically with methanol:0.1% formic acid (80:20, v/v), delivered at a flow rate of 0.3 mL/min. The total run time of the method was 13 min.

2.4.2. Mass spectrometry

A Quattro Ultima tandem MS (Waters) fitted with a Z-Spray ion interface was used for all analyses. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis: capillary voltage, 1.0 kV; source block temperature, 120 °C, desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 800 L/h. The appropriate multiple reaction monitoring (MRM) conditions for the individual analytes and their respective deuterated analogues were determined by direct infusion into the MS/MS. The cone voltage (CV) was adjusted to maximize the intensity of the protonated molecular species $[M+H]^+$ and collision-induced dissociation of each protonated molecule was performed. Collision gas (argon) pressure was maintained at 2.7×10^{-3} mbar and the collision energy (eV) adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis.

All aspects of system operation and data acquisition were controlled using MassLynx NT 4.2 software with automated data processing using the TargetLynx™ software (Waters). The statistical treatment of data was carried out using Excel 2000 (Microsoft).

2.5. LC–MS/MS assay validation

The analytical validation was performed according to the recommendations of Peters and Maurer [15,16], Shah et al. [17] and the SOFT/AAFFS Laboratory Guidelines [18].

2.5.1. Linearity, limit of quantification (LOQ), limit of detection (LOD), precision and accuracy

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. Freshly prepared working solutions of 200, 50, 12.5 and 2.5 μg/L for THC and 11-OH-THC, and of 800, 200, 50 and 10 μg/L for THC-COOH in methanol were used to prepare blood calibrators at a concentration of 40, 20, 15, 10, 5, 2, 1

and 0.5 μg/L for THC and 11-OH-THC, and of 160, 80, 60, 40, 20, 8, 4, and 2 μg/L for THC-COOH. Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression, with a 1/*x*-weighing factor for all compounds.

The limit of quantification (LOQ) was estimated by replicate analysis ($n=2$) over eight different days and it was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) less than 20%.

The limit of detection (LOD) was estimated from blank blood samples, spiked with decreasing concentrations of the analytes. It was defined as the concentration where the response of the qualitative ion could reliably be differentiated from background noise, i.e. signal-to-noise ratio (S/N) equal to or greater than 3:1, and with acceptance criteria for ion ratios equal to or lower than 30% and retention time deviations lower than 3.5% relative to that of the corresponding control or calibrator [18].

Three blood QCs for THC and 11-OH-THC and four blood QCs for THC-COOH at three and four different concentrations, respectively, were included and analyzed in every batch: (a) external QC1 (Medichem): concentration THC (2.0 μg/L), 11-OH-THC (2.7 μg/L), THC-COOH (26.1 μg/L), (b) external QC2 (Medichem): concentration THC (2.9 μg/L), OH-THC (2.2 μg/L), THC-COOH (53.1 μg/L), (c) "in house" QC 'low' at 3 μg/L for each compound and (d) "in house" QC 'high' at 120 μg/L for THC-COOH. The "in house" QCs were prepared by different operators from different working solutions of the calibrators and it was stored at −20 °C until use.

Intra-assay and inter-assay precision was evaluated by replicate ($n=2$) analysis of the QC samples performed over eight different days. Precision (expressed as $\%RSD_r$ for intra-assay precision and $\%RSD_t$ for inter-assay precision) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level—α of 0.05). Comparing the mean of calculated concentrations of QC samples to their respective nominal values, provided data on the accuracy of the method.

2.5.2. Selectivity and specificity

The selectivity of the method against endogenous interferences was verified by examination of the chromatograms obtained after the extraction of eight different blank blood samples from different origin. Moreover, specificity was also assessed by the analysis of blood samples spiked at 200 μg/L with other drugs of abuse and their metabolites (amphetamine, methamphetamine, MDA, MDMA, MDEA, ephedrine, PMA, benzoylecgonine, cocaine, morphine, codeine, 6-MAM, hydromorphone, hydrocodone, dihydrocodeine, oxycodone, oxymorphone, ethylmorphine, norcodeine, buprenorphine, methadone, EDDP, meperidine, fentanyl, pholcodine and tramadol) usually found in Belgium.

2.5.3. Stability

The stability of the drugs in blood samples that had been processed and then stored in the autosampler awaiting LC–MS/MS analysis was monitored; 250 μL blank blood spiked at the initial concentration of 34 μg/L ($n=12$) and extracted. The I.S. was added to control samples ($n=6$) and the concentrations were determined immediately. Another pool of samples was kept in the autosampler at 6 ± 2 °C and analyzed, previously spiked with the I.S., after 48 h ($n=6$).

For an evaluation of freeze/thaw stability, the control samples at 34 and 2.5 μg/L ($n=6$, at each concentration) were spiked with the I.S. and analyzed immediately. The stability samples, spiked at 34 and 2.5 μg/L ($n=6$, at each concentration), were subjected to three freeze/thaw cycles. For each freeze/thaw cycle, the samples were frozen at −20 °C for at least 24 h, thawed, and then maintained at ambient temperature for 4 h. After the three cycles, the samples were spiked with the I.S. and analyzed. Stability was tested against a lower percentage limit corresponding to 90–110% of the ratio (mean value of stability samples/mean value control samples) with a 90% of the confidence interval of the stability samples between 80% and 120% of the mean of the control samples.

2.5.4. Assessment of matrix effects

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different analyses were carried out. The first one involved a post-column infusion experiment. The study was based on a continuous post-column infusion of a mixture of the analytes and their internal standards (10 μg/L at a flow rate of 10 μL/min) to produce a constant elevated response in the MRM channels. The interference of this constant response was monitored in the whole run following the injection of extracted blood samples from different origin ($n=6$) and compared to the response following the injection of mobile phase only. A second type experiment consisted of a comparison of the peak responses of the analysis of a blank sample spiked at 37.5 and 2.5 μg/L ($n=6$, for each concentration) with those obtained from mobile phase spiked at the same concentration levels.

2.5.5. Recovery

Extraction recoveries were estimated by comparing the ratio of the peak areas of the non-deuterated compounds to the peak areas of the I.S. (i.e. responses) of blood samples spiked at 37.5 and 2.5 μg/L ($n=6$, for each concentration) when the non-deuterated compounds were added before the extraction step with those obtained when the non-deuterated analytes were added after sample extraction. In both cases, the deuterated analogues were added after the extraction.

3. Results and discussion

The method was validated for specificity, linearity, LOQ, LOD, precision, accuracy, recovery and matrix effect by the analysis of spiked blood samples. In each case, a weighted (1/*x*) linear regression line was applied. Other weighting factors were tested before validation of the method but they did not give significantly better results. Correlation coefficients higher than 0.998 were achieved in the range investigated, i.e. from 0.5 to 40 μg/L for THC, from 1 to 40 μg/L for 11-OH-THC and from 2 to 160 μg/L for THC-COOH. Fig. 1 shows the MRM chromatograms obtained following the analysis of the lowest calibrator (0.5 μg/L for THC, 1 μg/L for 11-OH-THC and 2 μg/L for THC-COOH). At this concentration a S/N ratio >10:1 was observed for the qualifier and the criteria for LOQ were satisfied.

The applied isocratic mobile phase (methanol:0.1% formic acid, 80:20, v/v) ensured the elution of all the drugs examined within 11 min and produced chromatographic peaks of acceptable symmetry. In the early stages of method development an isocratic gradient of 90:10 methanol: 0.1% formic acid (v/v) was tested: although the compounds eluted earlier, giving a shorter run time, many interferences were observed at the retention time (2.5 min) of 11-OH-THC and THC-COOH when analysing blank blood samples.

Selectivity of the method was achieved by a combination of retention time, precursor and two product ions. The most prominent precursor–product transitions were used for quantification of THC and THC-d_3 and the next most abundant, used as qualifier. For the other compounds, an elevated background was noted when using the MRM transition based on the most prominent product. Improved sensitivity (based on S/N) was achieved when the MRM transition was based on an alternative product ion (Table 1). The

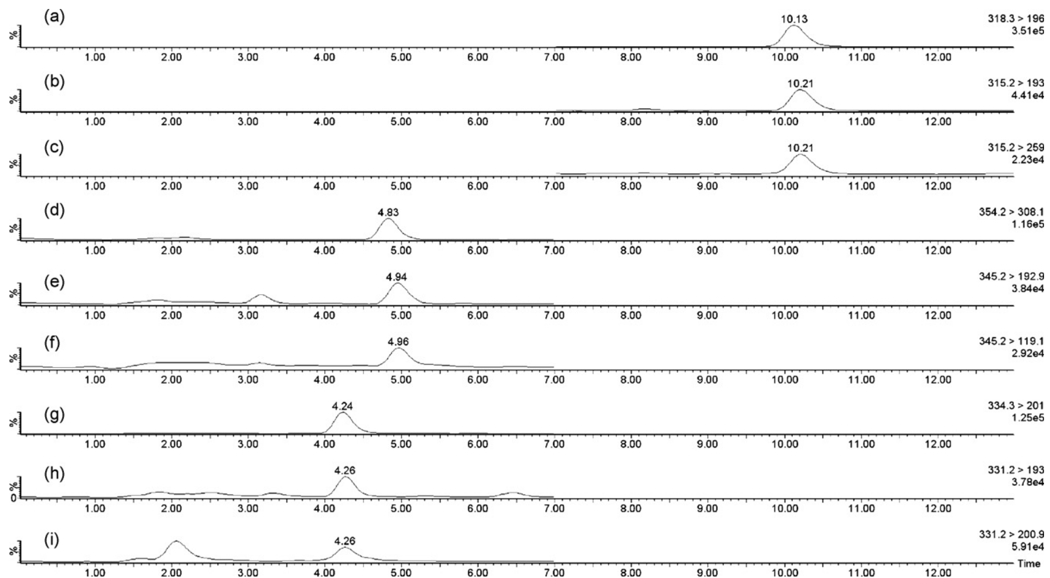

Fig. 1. MRM chromatograms obtained following the analysis of a spiked blood sample at the LOQ for (a) THC-d_3, (b) THC (quantifier), (c) THC (qualifier), (d) THC-COOH-d_9, (e) THC-COOH (quantifier), (f) THC-COOH (qualifier), (g) 11-OH-THC-d_3, (h) 11-OH-THC (quantifier), and (i) 11-OH-THC (qualifier). Peak intensity is shown in the top right-hand corner of each trace.

Table 1
MRM transitions and conditions for all compounds and their deuterated analogues. Underlined transitions were used for quantification.

Compound	Precursor ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energy (eV)
THC	315.2	259.0 193.0	30	20 20
11-OH-THC	331.2	200.9 193.0	30	20 20
THC-COOH	345.2	192.9 119.1	30	30 30
THC-d_3	318.3	196.0	30	20
11-OH-THC-d_3	334.3	201.0	30	20
THC-COOH-d_9	354.2	308.1	30	20

ion ratios (quantifier/qualifier) were 2.1, 0.8 and 1.5 for THC, 11-OH-THC and THC-COOH, respectively. For the corresponding deuterated analogues, only one transition was monitored. Injection of single analyte solutions did not produce interference in the other MRM channels. Fig. 2 shows the fragmentation structures of THC (a), 11-OH-THC (b), THC-COOH (c) to the quantifier ion.

Table 2
Dynamic range, LOD, LOQ, relative standard deviation (RSD, %) at the LOQ and equation of a typical calibration curve with the corresponding coefficient of determination (r^2). The limit of quantification (LOQ) was estimated by replicate analysis ($n = 2$) over eight different days.

	THC	11-OH-THC	THC-COOH
Dynamic range (μg/L)	0.5–40	1–40	2–160
LOD (μg/L)	0.5	1	2
LOQ (μg/L)	0.5	1	2
RSD (%) at LOQ	6.8	9.7	6.4
r^2	0.999465	0.999695	0.998958

Linearity data, LOQ and LOD are shown in Table 2. No interference peaks were observed in the cannabinoids MRM channels when blank blood samples spiked with other drugs were analysed. The LOD and the LOQ were identical for all compounds, since lower concentrations did not meet the acceptance criteria for ion ratios. In Belgium, the concentration of THC in a blood sample from a driver

m/z 315 → A → m/z 193

m/z 331 → B → m/z 193

m/z 345 → C → m/z 193

Fig. 2. Fragmentation of THC (a), 11-OH-THC (b), THC-COOH (c) to the quantifier ion.

Table 3
Intra-assay (expressed as RSD_r, %) and inter-assay precision (expressed as RSD_t, %) and accuracy of the QC samples. Intra-assay and inter-assay precision was evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days and it was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level—α of 0.05).

	THC			11-OH-THC			THC-COOH		
	RSD_r (%)	RSD_t (%)	Accuracy (%)	RSD_r (%)	RSD_t (%)	Accuracy (%)	RSD_r (%)	RSD_t (%)	Accuracy (%)
External control 1	3.7	11.5	91.3	4.0	5.2	94.7	3.8	7.8	95.4
External control 2	2.3	7.2	99.4	2.8	5.7	0.0	2.3	4.4	100.8
'In house' QC 'low'	1.4	7.1	96.0	3.9	4.9	105.8	6.4	6.2	99.8
'In house' QC 'high'	–	–	–	–	–	–	3.3	4.6	97.9

Table 4
Matrix effect, matrix effect RSD (%) and extraction recovery (%) evaluated at two different concentrations ($n = 6$, for each concentration).

	THC		11-OH-THC		THC-COOH	
	37.5 μg/L	2.5 μg/L	37.5 μg/L	2.5 μg/L	37.5 μg/L	2.5 μg/L
Mean matrix effect (%)	−8.5	0.4	23.6	26.5	−6.6	0.8
Matrix effect RSD (%)	8.1	8.5	2.6	8.4	6.7	5.4
Recovery (%)	109.4	108.1	110.8	111.5	105.0	104.8

that constitutes an offence is 2 μg/L. For other European counties like France and Germany (for plasma samples) it is fixed at 1 μg/L [19].

The intra- and inter-assay precisions were satisfactory, with all RSDs lower than 12% (Table 3). Results indicated that the accuracy of the assay was >90%.

The stability of processed samples (spiked at 34 μg/L and then stored in the autosampler at 6 ± 2 °C) was monitored after 48 h. No statistical significant difference could be observed for any of the compounds. Furthermore, all compounds, when spiked in blood at two concentrations, i.e. 34 and 2.5 μg/L, were demonstrated to be stable after three freeze/thaw cycles.

The matrix effect, defined as the effect of co-eluting residual matrix components on the ionization of the target analyte, typically results in either signal suppression or enhancement. Moreover, interfering matrix components can affect the reproducibility and accuracy of the developed procedure, leading to compromising or erroneous results. Consequently, in the development of any LC–MS(/MS) method, an efficient sample clean-up and use of appropriate internal standardization is necessary and the potential for any such ion suppression and enhancement should be assessed. Post-column infusion experiments (based on the method described by Bonfiglio et al. [20]) were performed to provide information of the effect of the matrix throughout the course of the elution time for the analytes. Fig. 3(a)–(c) shows the responses obtained following an injection of an extracted blank blood sample and a mobile phase only control (top and bottom chromatograms for each compound, respectively). A second experiment was carried out and we compared peak responses obtained when the cannabinoids were spiked to a blank blood sample with the response obtained when the

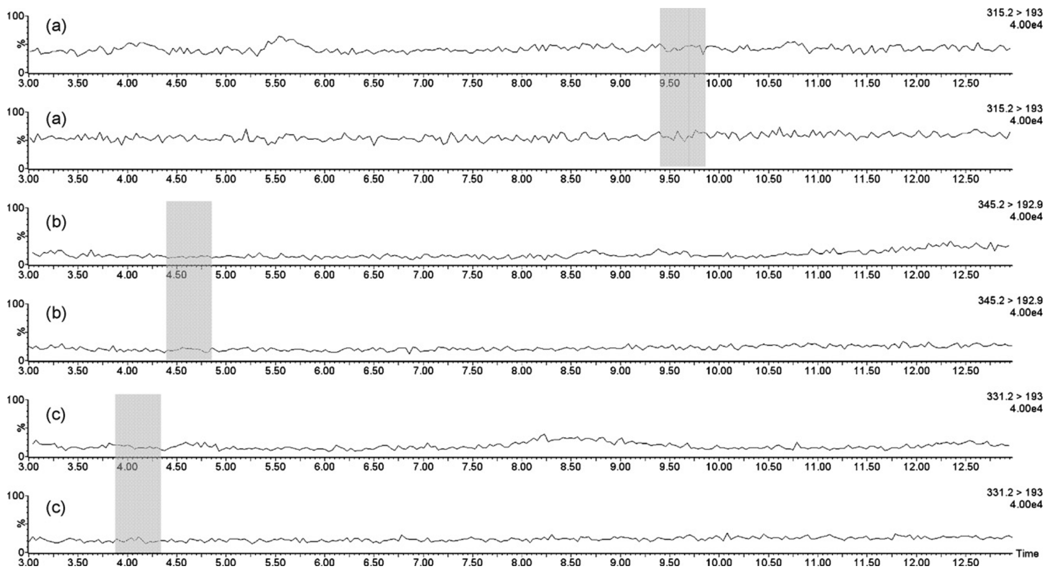

Fig. 3. Evaluation of the effect of the matrix on (a) THC, (b) THC-COOH and (c) 11-OH-THC by post-column infusion following an injection of extracted blank blood (top trace for each compound) and mobile phase only control (bottom trace). The dotted areas indicate the elution-position of each drug. MRM transition and peak intensity are shown on the right-hand corner of each trace.

cannabinoids were added to a mobile phase only at the same concentration. For 11-OH-THC some ion enhancement was observed, nevertheless it has been stated that the use of deuterated I.S. would partially compensante for matrix effects [21,22]. The results of the matrix effects at the two different concentrations are presented in Table 4.

The results of the extraction recovery study are also presented in Table 4. Very high and reproducible recoveries were obtained with this LLE procedure for all analytes. It must be pointed out the importance of adding the solution of 10% acetic acid to achieve a pH lower than 4.5 due to the pK_a of THC-COOH.

The validated LC–MS/MS method was applied to the analysis of 63 authentic samples from toxicology cases. Concentrations of THC-COOH were quite variable and generally high. The corresponding 11-OH-THC concentrations remained quite low. Samples with a concentration above the linear range of the calibration curve were diluted 1:2 with blank blood and re-analyzed. The median and minimum–maximum range (in μg/L), respectively, were as follows: THC (7.45 [1.3–34.1]), 11-OH-THC (2.7 [1.0–13.4]) and THC-COOH (44.8 [7.9–224.3]).

4. Conclusions

Reliable analytical data are a prerequisite for correct interpretation of toxicological findings in the evaluation of scientific studies, as well as in daily routine work. Unreliable analytical data might not only be contested in court, but could also lead to unjustified legal consequences for a defendant. Therefore, new analytical methods to be used in forensic and/or clinical toxicology require careful method development and thorough validation of the final method.

In this report, a fully validated and highly sensitive LC–MS/MS method is described for the simultaneous analysis of THC, 11-OH-THC and THC-COOH in blood. The method combined LLE with LC–MS/MS and provided a thorough clean-up of the matrix to minimize ion suppression and enhancement, in combination with high recovery, excellent precision and accuracy in the linear range investigated, using 250 μL of sample. The method was successfully applied to authentic samples.

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V. APPLICATIONS

5.3. ON-LINE SPE-LC-MS/MS

FOR HIGH THROUGHPUT ANALYSIS OF THC-COOH

IN URINE

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Short communication

On-line solid-phase extraction combined with liquid chromatography–tandem mass spectrometry for high throughput analysis of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in urine

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ABSTRACT

A simple, rapid and highly sensitive method for the analysis of THC-COOH in urine, using automated on-line solid-phase extraction (SPE) combined with liquid chromatography (LC)–mass spectrometry (MS/MS), is developed and fully validated according to international guidelines. Chromatographic separation was achieved on an Atlantis dC_{18} column with an isocratical gradient, ensuring the elution of THC-COOH within 4.1 min. The total process time was 6 min and 500 μL of sample was required. SPE using C_8 cartridges was highly effective, reproducible and led to significant decreases in the interferences present in the matrix. The method showed an excellent intra- and inter-assay precision (relative standard deviation (RSD) <7% and bias <13%) for four external quality control (QC) samples and three 'in house' QCs. Responses were linear over the investigated range ($r^2 > 0.99$, 5–200 μg/L). Limits of quantification (LOQ) and detection (LOD) were determined to be 5 μg/L and 0.25 μg/L, respectively. Furthermore, the analyte and the processed samples were demonstrated to be stable. Moreover, no carryover was observed after the analysis of high concentrated urine samples (5000 μg/L THC-COOH)). The method was subsequently applied to authentic samples previously screened by a routine immunoassay method.

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1. Introduction

High-throughput analysis is becoming increasingly important in all areas of science; the forensic sciences being no exception. Moreover, due to the development of more potent drugs, drug concentrations in biological samples are often present at much lower levels than before. Therefore, fast analytical techniques with higher sensitivity and selectivity are needed. One of the bottlenecks in bio analysis is sample preparation, especially if the method requires manual extraction techniques. An elegant system for the rapid analysis of complex samples can be obtained by on-line coupling of SPE to LC–MS detection. With this procedure, the sample is injected directly into the SPE-MS system and the rate-limiting off-line extraction step is eliminated. As a result, automation leads to higher sample throughput and increased sensitivity as the whole sample extract is analyzed and not a fraction. Other advantages are safer sample handling and improved precision as operator intervention is minimized [1].

Cannabis is one of the most widely used illicit drug in the world, being the most frequently detected drugs in cases of driving under the influence of drugs (DUID) in several countries [2]. Cannabis use is detected by identifying the presence of the major psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (THC) or its metabolites in biological fluids. The major metabolite found in urine is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), which exists in both the free and glucuronide form [3–5]. Analytical procedures are well-documented for determining the presence of THC-COOH in urine using gas chromatography–mass spectrometry (GC–MS(/MS)) [6–10], liquid chromatography–mass spectrometry (LC–MS(/MS)) [1,6,11–13] and immunoassays [14–17]. However, to date, no report has been published dealing with the analysis of THC-COOH in urine by any type of on-line SPE-LC–MS/MS.

Therefore, the aim of this study was to develop and validate a simple, rugged and high-throughput on-line SPE-LC–MS/MS method for quantification of THC-COOH in urine.

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2. Materials and methods

2.1. Reagents

Individual stock solutions of THC-COOH (1 mg/mL in methanol), and the internal standard (IS) [$^9H_3$] THC-COOH (THC-COOH-d_9) (0.1 mg/mL in methanol) were from LGC Promochem (Molsheim, France).

Water (HPLC grade), methanol (LC–MS grade), 0.1% formic acid in water (UPLC/MS grade) and acetonitrile (LC–MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Potassium hydroxide (powder), triethylamine, (puriss.p.a.) and trifluoroacetic acid (puriss.p.a.) were purchased from Sigma–Aldrich (Steinheim, Germany). HySphere C_8 cartridges were from Cofraz (Elsene, Belgium). Glacial acetic acid was from VWR (Leuven, Belgium).

External quality control (QC) urine samples (Medidrug U-screen cut-off −25% and +25%) were obtained from Medichem World (Steinenbronn, Germany). Liquicheck external quality controls C1 and C3 for urine were purchased from Bio-Rad Laboratories (Irvine, CA).

2.2. Specimens

Blank urine samples were obtained from drug-free volunteers. Authentic urine samples were obtained from forensic toxicology cases.

2.3. Preparation of standard solutions for calibrators and QC samples

Two different working solutions of the non-deuterated compound at 10 mg/L in methanol were prepared. The first was used for preparation of the calibrators and the second for the 'in house' QC samples. The internal standard (IS) working solution of 1 mg/L was prepared in methanol. Working solutions were prepared monthly and stored at 4 °C. The 'in house' QCs were stored at −20 °C until use.

The external QCs were prepared following the indications of the manufacturer. Each vial of the Medidrug U-screen controls was reconstituted exactly with 5.0 mL of bidistilled water, swirled gently and allowed to equilibrate for 20 min at room temperature (RT). Before sampling, the vial was gently homogenized for 5 min using a rotation mixer. After reconstitution, the QCs were stable for 7 days (at 2–8 °C in the dark). The Liquicheck controls were equilibrated to RT and swirled gently to ensure homogeneity before sampling. Once the control was opened, it was stable for 30 days when stored tightly capped at 2–8 °C.

2.4. SPE-LC–MS/MS

2.4.1. Sample preparation: hydrolysis

Fifty microliters of potassium hydroxide 10 M and 50 μL of the IS working solution (0.25 mg/L) were added to 500 μL of urine and the samples were incubated at 60 °C for 15 min. Similar hydrolysis procedures have been described by other authors [1,18]. Samples were cooled to RT before the addition of 300 μL distilled water. Before injection onto the on-line SPE system, the samples were acidified by adding 600 μL of glacial acetic acid.

2.4.2. XLC (on-line SPE)

Sample extraction was performed using the on-line SPE Symbiosis™ Pharma System (Spark Holland™, Emmen, The Netherlands). The entire system was operated by SparkLink for Masslynx™ software (version 4.1, Waters).

The following XLC program was used: after conditioning with 2 mL of methanol and 1 mL of water, 200 μL of the diluted and acidified urine sample was applied to the SPE cartridge using 2 mL of water as transport solvent. Clean-up was accomplished through successive 2 mL washes of 0.1% formic acid, and methanol:0.1% formic acid (50:50, v/v) in order to remove salts and endogenous interferences present in the biological samples. Elution of the analytes from the cartridge was achieved by application of the LC mobile phases (0.1% formic acid (A) and acetonitrile (B)) (standard (gradient pump) elution mode) during the chromatographic run. Whilst the elution step was being performed, a new cartridge was conditioned, loaded and washed in the left clamp. Following the elution step, several automated clamp and valve washes with water, 0.2% triethylamine and 0.1% trifluoroacetic acid were performed to avoid carryover between samples.

2.4.3. Chromatographic conditions

Analytes were separated using an Atlantis dC_{18} column, 3 μm, 3 mm × 100 mm (Waters). Separation was carried out in isocratic mode (0.1% formic acid:acetonitrile, 20:80, v/v). The complete run time was 6 min.

2.4.4. Tandem mass spectrometry

A Quattro Premier tandem mass spectrometer (Waters) was used. Ionization was achieved using electrospray in positive ionization mode (ESI+). Nitrogen was applied as nebulisation and desolvation gas at a flow rate of 600 L/h and heated to 350 °C. Capillary voltage and source block temperature were 3 kV and 120 °C, respectively.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards (200 μg/L, in 0.1% formic acid–acetonitrile (20:80, v/v)) were infused into the mass spectrometer and the cone voltage (CV) was optimised to maximise the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mbar) and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis (Table 1).

2.5. On-line SPE-LC–MS/MS assay validation

Validation was performed based on the FDA guidelines and recent publications concerning validation of bio analytical methods [19,20].

Table 1
MRM transitions and conditions for THC-COOH and its deuterated analogue. Underlined transition was used for quantification.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
THC-COOH	345.2	<u>299.3</u> 193.1	30	20 30
THC-COOH-d_9	354.2	308.4	30	20

2.5.1. Linearity, limit of quantification (LOQ), limit of detection (LOD), precision and bias

It is known that the concentration of drugs in urine can vary considerably (e.g. from some μg/L to few thousands of μg/L of THC-COOH) depending on the individual and the time of collection, In routine urine toxicological analysis, it is necessary to fix the quantification range depending on the cut-off established in the laboratory. The aim in our toxicological laboratory is to determine the presence of THC-COOH in urine with respect to the Belgian legal cut-off of 15 μg/L. Thus, the established quantification range has been determined to guarantee a good quantification around this concentration.

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogue. Freshly prepared working solutions of 1000 μg/L and 100 μg/L in water were used to prepare urine calibrators at a concentration of 200, 100, 50, 20, 10 and 5 μg/L using HPLC-grade water. Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression, with a $1/x$-weighting factor.

The limit of quantification (LOQ) was estimated by replicate analysis ($n = 2$) over 8 different days and was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) less than 20%.

The limit of detection (LOD) was estimated from blank urine samples, spiked with decreasing concentrations of the analyte. It was defined as the concentration for which the response of the qualitative ion could reliably be differentiated from background noise, i.e. signal-to-noise ratio (S/N) equal to or greater than 3:1. The acceptance criteria for ion ratios equal to or lower than 20% and retention time deviations lower than 3.5% relative to that of the corresponding control or calibrator.

Seven QCs were analyzed, four external QCs (two each from Medichem and Bio-Rad Laboratories) and three 'in house' QCs.

Intra- and inter-assay imprecision was evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days. Imprecision (expressed as $\%RSD_r$ for intra-assay imprecision and $\%RSD_t$ for inter-assay imprecision) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level (α) of 0.05). Bias of the method was determined by comparison of the mean of calculated concentrations of QC samples to their respective nominal values.

2.5.2. Selectivity and specificity

The selectivity and specificity of the method against endogenous interferences was verified by examination of the chromatograms obtained after the extraction of eight different blank urine samples from healthy volunteers, and after the analysis of authentic urine samples from cocaine and amphetamine users. Moreover, blank urine samples ($n = 3$) spiked with amphetamine, methamphetamine, MDA, MDMA, ephedrine, PMA, mCPP, morphine, codeine, benzoylecgonine, codeine, 6-MAM, fentanyl, pholcodine, hydromorphone, hydrocodone, norcodeine, dihydrocodeine, oxycodone, oxymorphone, cocaine, methadone, EDDP, 27 benzodiazepines, zolpidem, zopiclone, zaleplon, THC and 11-OH-THC were also analyzed to check for interferences.

2.5.3. Stability

The autosampler stability of processed samples at concentrations of 160 μg/L and 15 μg/L ($n = 6$ at each concentration) was monitored as follows; one pool of samples were determined immediately, while another pool of samples was analyzed after remaining in the autosampler at 6 ± 2 °C for 24 h and at RT for 72 h (a weekend). All samples were spiked with the IS just before analysis.

Stability of THC-COOH in the matrix was determined through spiked blank urine samples with concentrations of 160 μg/L and 15 μg/L ($n = 6$ at each concentration). Stability was checked after storage at 2–6 °C for 72 h (weekend) and after three freeze/thaw cycles.

All the stability experiments were tested against a lower percentage limit corresponding to 90–110% of the ratio (mean value of stability samples/mean value control samples) with a 90% of the confidence interval of the stability samples between 80 and 120% of the mean of the control samples.

2.5.4. Assessment of matrix effects

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different experiments were carried out.

The first one involved a post-column infusion experiment [21]. This experiment was based on a continuous post-column infusion of THC-COOH and its internal standard (10 μg/L at a flow rate of 10 μL/min) to produce a constant response in the MRM channels. This constant response was monitored throughout the whole run following the injection of urine samples from different origin ($n = 6$) and compared to the response following the injection of mobile phase only.

The second experiment consisted of a comparison between the peak responses of THC-COOH spiked to a blank urine sample at concentrations of 160 μg/L and 15 μg/L ($n = 6$, for each concentration) with those obtained after being spiked in the mobile phase at the same concentration levels [22].

2.5.5. Recovery

Extraction recoveries were estimated by performing the following experiments: blank urine samples spiked at 160 μg/L and 15 μg/L ($n = 6$, for each concentration) were loaded and washed in a first SPE cartridge while a second cartridge was placed in series to determine the breakthrough of the first one. Both cartridges were subsequently eluted independently. Recovery was considered to be the ratio between the response obtained after elution of the first cartridge and the total response (sum of both, the first and the second SPE cartridge).

2.5.6. Carryover

Carryover was evaluated by the analysis of blank urine samples spiked with the IS after the analysis of the upper calibrator (200 μg/L, $n = 8$), after the analysis of authentic urine samples from cannabis users ($n = 8$) and after the analysis of a highly concentrated sample (5000 μg/L).

2.5.7. Dilution integrity

Spiked blank urine samples at 3200 μg/L and 600 μg/L were rediluted 1:20 (v/v) with blank urine ($n = 6$) and analyzed to evaluate the dilution integrity.

3. Results and discussion

The method was validated for selectivity, linearity, LOQ, LOD, imprecision, bias, analyte and processed sample stability, matrix effect, recovery, carryover and dilution integrity.

The applied chromatographic method ensured the elution of THC-COOH within 4.1 min and produced peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, precursor and two product ions. The most prominent precursor-product transition was used for quantification and the next most abundant as qualifier (Table 1). For the corresponding deuterated analogue, only one transition was monitored. Fig. 1 shows the MRM chromatograms obtained following the analysis of the urine

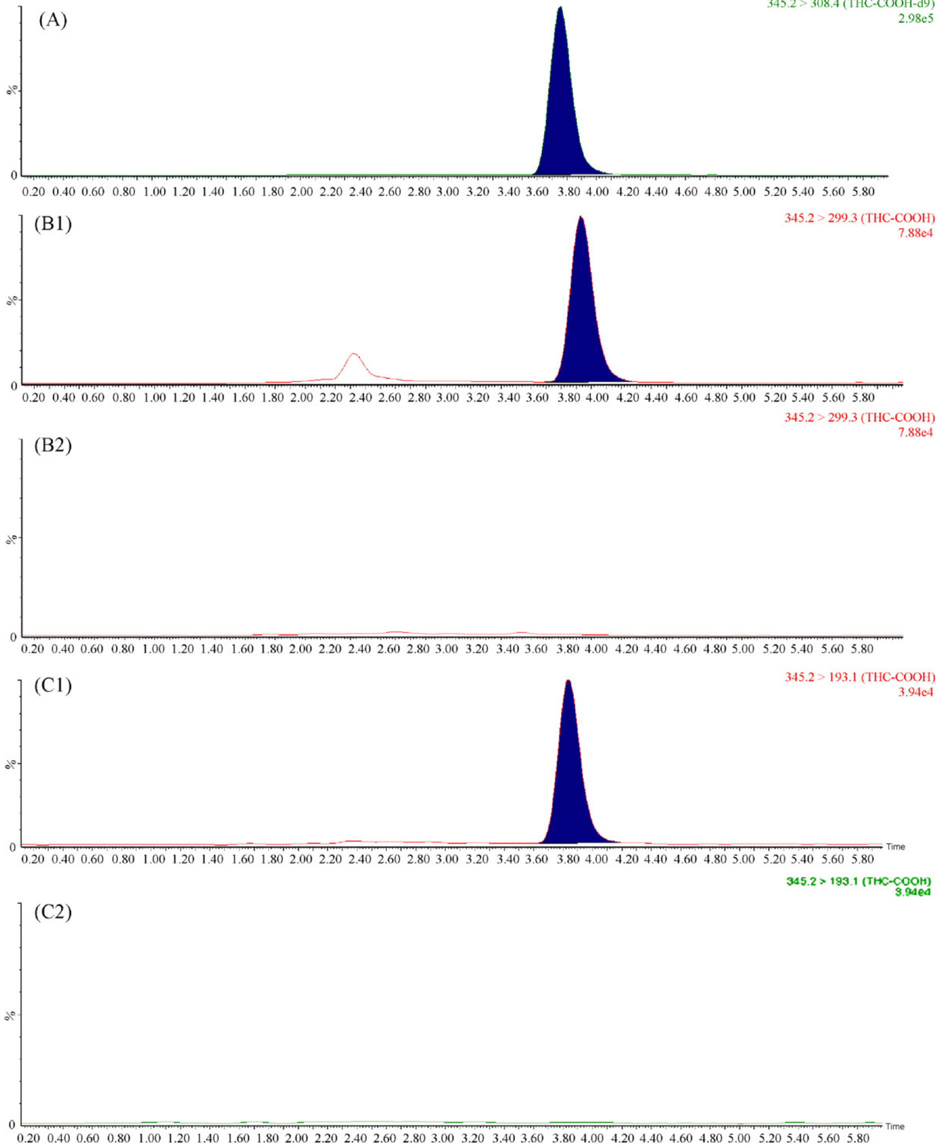

Fig. 1. MRM chromatograms obtained following the analysis of a spiked urine sample with 5 μg/L (LOQ) and a blank urine injected after the highest calibrator: (A) IS, (B1) quantifier of THC at LOQ, (B2) quantifier of THC in the blank urine, (C1) qualifier of the LOQ and (C2) qualifier of the blank urine. Peak intensity is shown in the top right-hand corner of each trace. Blank urine samples are displayed at the same peak intensity of the LOQ.

lowest calibrator (5 μg/L) and a blank injected after a high concentrated sample. No interferences were observed after the analysis of blank urine samples spiked with over—the counter-drugs and cannabinoids, ensuring the selectivity of the method.

During pre-validation experiments, the linearity was tested up to 5000 μg/L ($r > 0.99$). However, due to the legal cut-off in Belgium for THC-COOH, which is 15 μg/L, the quantification range applied in the laboratory was 5–200 μg/L. Correlation coefficients of the weighed ($1/x$) linear regressions for this range were higher than $r^2 = 0.99$.

The LOQ was determined at 5 μg/L as a S/N > 10:1 was observed for the qualifier and the criteria for LOQ were satisfied. LOD was 0.25 μg/L.

The intra- and inter-assay imprecision were satisfactory, with all RSDs lower than 7% (Table 2). The results indicated that the bias of the assay was <13%.

Stability of the processed samples in the autosampler was monitored after 24 (at 2–6 °C) and 72 h (RT). No instability was observed during this period of time. Moreover, THC-COOH spiked to blank urine samples was also stable after the three freeze/thaw cycles and after 72 h in the fridge.

Table 2
Intra-assay (expressed as RSD_r (%)) and inter-assay precision (expressed as RSD_t (%)) and bias of the LOQ and QC urine samples. Intra-assay, inter-assay precision and bias were evaluated by replicate (n = 2) analysis of the QC samples performed over eight different days.

	Nominal value (μg/L)	Average (μg/L) (n = 16)	RSD_r (%) (n = 16)	RSD_t (%) (n = 16)	Bias (%) (n = 16)
C1	6.0	6.4	2.9	4.1	7.2
C3	18.5	20.1	1.9	2.8	8.8
Medichem cutoff −25%	37.5	34.3	5.2	5.8	−8.5
Medichem cutoff +25%	62.5	55.1	1.9	5.1	−11.8
'In house' QC low	15.0	13.1	6.2	6.8	−12.3
'In house' QC medium	80.0	79.8	2.0	4.4	−0.3
'In house' QC high	160.0	156.8	2.9	5.4	−2.0

Post-column infusion experiments were performed to provide information of the matrix effect throughout the course of the elution time for the analyte and its IS. No significant changes in response were observed. The second experiment performed to assess matrix effects compared the peak area responses, obtained when the compound was spiked into blank urine samples, with the responses obtained when the compound was added to mobile phase at the same concentration. No significant matrix effect (mean 3.6%) was observed with this on-line SPE procedure.

Moreover, for the recovery studies, no breakthrough was observed in the second cartridges placed in series, so recovery was 100%.

No carryover was observed in the analysis of a blank urine sample injected after the analysis of the upper calibrator (200 μg/L), neither after the analysis of authentic urine samples or after a highly concentrated sample (5000 μg/L).

The dilution integrity test demonstrated a bias <2% and an RSD (%) of 13.4% and 11.6% for the diluted blank urine samples at 3200 μg/L and 600 μg/L, respectively.

4. Samples

Thirty-four urine samples from cannabis users were analyzed in one run with the present method. Concentrations varied considerably. Those samples with concentrations above the upper calibrator were diluted 1/20 with blank urine and reanalyzed. The median was 652 μg/L with minimum–maximum range of 12.1–3681.

5. Conclusions

In this report a fully validated and highly sensitive automated LC–MS/MS method is described for the analysis of THC-COOH in urine. The method combined on-line SPE with LC–MS/MS and provided a thorough clean-up of the matrix in combination with high recovery, excellent precision and bias within the investigated linear range. The method was successfully applied to authentic samples from cannabis users.

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V. APPLICATIONS

5.4. HIGH THROUGHPUT ANALYSIS OF AMPHETAMINES IN BLOOD AND URINE WITH ON-LINE SPE-LC-MS/MS

High-Throughput Analysis of Amphetamines in Blood and Urine with Online Solid-Phase Extraction-Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

An automated online solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS–MS) method for the analysis of amphetamines in blood and urine was developed and validated. Chromatographic separation was achieved on a Nucleodur® Sphinx RP column with an LC gradient (a mixture of 10 mM ammonium formate buffer and acetonitrile), ensuring the elution of amphetamine, methamphetamine, MDMA, MDA, MDEA, PMA, and ephedrine within 11 min. The method was fully validated, according to international guidelines, using only 100 and 50 µL of blood and urine, respectively. The method showed an excellent intra- and interassay precision [relative standard deviation (RSD) < 11.2% and bias < 13%] for two external quality control samples (QC) for both matrices and three and two 'in house' QCs for blood and urine, respectively. Responses were linear over the investigated range ($r^2 > 0.99$, 2.5–400 µg/L for blood and 25–1000 µg/L for urine). Limits of quantification were determined to be 2.5 and 25 µg/L for blood and urine, respectively. Limits of detection ranged from 0.05 to 0.5 µg/L for blood and 0.25 to 2.5 µg/L for urine, depending on the compound. Furthermore, the analytes and the processed samples were demonstrated to be stable (in the autosampler for at least 72 h and after three freeze/thaw cycles), and no disturbing matrix effects were observed for all compounds. Moreover, no carryover was observed after the analysis of high concentration samples (15,000 µg/L). The method was subsequently applied to authentic blood and urine samples obtained from forensic cases, which covered a broad range of concentrations. The validation results and actual sample analyses demonstrated that this method is rugged, precise, accurate, and well-suited for routine analysis as more than 72 samples are analyzed non-stop in 24 h with minimum sample handling. The combination of the high-throughput online SPE and the well-known sensitivity and selectivity assured by MS–MS resulted in the elimination of the bottleneck associated with the sample preparation requirements and provided increased sensitivity, accuracy, and precision.

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Introduction

Amphetamine is a psychostimulant drug known to produce wakefulness and increase focus and appetite. The main effects reported after use of amphetamines are euphoria, increased energy, muscle tremor, hypertension, increased blood temperature, and dry mouth (1). Designer amphetamines chemically related to amphetamine include methamphetamine, methylenedioxyamphetamine (MDA), methylendioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), and 4-methoxyamphetamine (PMA). They also present similarities to some naturally occurring weak stimulants such as ephedrine.

Globally, after cannabis, amphetamines are among the most commonly consumed illicit drugs. According to the annual report of the European Monitoring Center for Drugs and Drug Addiction (EMCDDA), surveys conducted in the European countries in recent years showed an increase in amphetamine and ecstasy consumption (2). Methamphetamine use was not observed in Belgium but was reported in other countries (e.g., U.S., Lithuania, Slovenia, Russia, and Poland).

Several techniques have been used for the toxicological analysis of amphetamines in blood and/or urine including gas chromatography with mass spectrometry (GC–MS) (3,4) and liquid chromatography with tandem mass spectrometry (LC–MS–MS) (5–10). With the advent of increased selectivity when using LC–MS–MS, the possibility of omitting the sample preparation step arose (11–13) because sample preparation is considered the main bottleneck as it is often time consuming. Therefore, several clinical and forensic laboratories simply use direct injection of the biological sample. However, sample preparation is often required to reduce matrix effects. The increased demand for high-throughput causes a unique situation of balancing cost versus analysis speed. An elegant system for the rapid analysis of complex samples can be obtained by online coupling of solid-phase extraction (SPE) to LC–MS detection. With this procedure, the sample is injected directly into the SPE-MS system, and the rate-limiting off-line extrac-

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tion step is eliminated. As a result, automation leads to higher sample throughput, and increased sensitivity as the whole sample extract is analyzed and not a fraction. Other advantages are safer sample handling and improved precision as operator intervention is minimized (14,15). Several generic approaches have recently been developed for online sample extraction coupled to LC–MS (15–21).

Wu et al. (22) developed a method for the direct analysis of amphetamines for urine by column-switching connecting a SPE column before the analytical column. However, the method was not completely validated because no studies of matrix effects and carryover were carried out. According to the literature, when extractions are performed using automated liquid handlers, the potential of carryover and cross-contamination increases (23). Thus, it must be included in method validation.

The aim of this study was to develop and completely validate a new simple, rugged, and high-throughput online SPE-LC–MS–MS method for rapid and simultaneous analysis of amphetamine, methamphetamine, MDMA, MDA, MDEA, PMA, and ephedrine in blood and urine. This online SPE-LC–MS method offers the entire process of conditioning, sample application, washing, and elution taking place at constant flow rates. Another important advantage is that no manual transfers are made and that the whole extracted sample is loaded onto the LC column without the need for a preconcentration step. Disposable cartridges were applied for each injection minimizing the carryover. The method was fully validated with respect to precision, accuracy, quantification and detection limit, stability, sample throughput, and carryover.

Materials and methods

Reagents

Individual stock solutions of amphetamine, methamphetamine, MDA, MDMA, and PMA (all certified at a concentration of 1 mg/mL in methanol), and the internal standards (IS) [$^3H_{11}$] amphetamine (amphetamine-d_{11}), [$^3H_5$] methamphetamine (methamphetamine-d_5), [$^3H_5$] MDA (MDA-d_5), [$^3H_5$] MDMA (MDMA-d_5), [$^3H_6$] MDEA (MDEA-d_6), and [$^3H_3$] ephedrine (ephedrine-d_3) (certified concentration at 0.1 mg/mL in methanol) were from LGC Promochem (Molsheim, France). HPLC-grade water, LC–MS-grade methanol, UPLC–MS-grade 0.1% formic acid in water, and LC–MS-grade acetonitrile were purchased from Biosolve (Valskenswaard, The Netherlands). Ammonia solution (32%, extra pure) was from Merck (Darmstadt, Germany). Ammonium formate (99.995+% powder) and triethylamine (puriss.p.a.) were purchased from Sigma Aldrich (Steinheim, Germany). Oasis HLB Prospekt cartridges were from Waters (Milford, MA). External quality control (QC) blood samples were obtained from Medichem World (Steinenbronn, Germany). Liquichek external quality controls C1 and C3 for urine were purchased from Bio-Rad Laboratories (Irvine, CA). The concentrations of the amphetamines in these external QCs are displayed in Table I (for blood) and Table II (for urine) as 'nominal value'.

Specimens

Pooled blank blood samples were used for development and validation of the procedure and were obtained from a local blood bank whereas drug-free volunteers provided blank urine.

Authentic blood and urine samples were obtained from forensic cases or from 28 volunteers who received either placebo or a dose of MDMA (75 mg). Blood samples were freshly collected 1 h and 15 min after ingestion, and the venotubes (without anticoagulant) were directly centrifuged for 10 min at 3000 rpm. The serum obtained was stored at –20°C prior to analysis. The study protocol was approved by the ethics committee of the University Hospital of Maastricht in the Netherlands.

Preparation of standard solutions

Two different working solutions of the non-deuterated compounds at 4 mg/L in methanol were prepared. The first was used for preparation of the calibrators and the second for the 'in house' QC samples. The internal standard (IS) working solution of 1 mg/L was prepared in methanol. Working solutions were prepared monthly and stored at 4°C. The 'in house' QCs were stored at –20°C until use. The external QCs were prepared following the indications of the manufacturer.

To obtain the lower concentrations needed for internal standardization and validation of each experiment, further dilutions in water were prepared the same day.

SPE-LC–MS–MS

Sample preparation: XLC (online SPE)

Sample extraction was performed using the online SPE Symbiosis™ Pharma System (Spark Holland™, Emmen, the Netherlands). It comprises two integrated units: the Reliance™ autosampler with two integrated binary LC pumps and the online SPE unit Prospekt-2 system, which includes the automated cartridge exchange (ACE) unit and two high-pressure dispensers. The entire system was operated by SparkLink for Masslynx™ software version 4.1 (Waters, Milford, MA). The extraction procedure was carried out in glass screw-neck vials of high-quality Waters glassware.

Initially, 10 SPE cartridges were evaluated to determine the optimal SPE sorbent for the extraction of all analytes. The evaluated cartridges were HySphere CN, C2, C8, C8EC, C18, Resin SH, and Resin GP (SparkHolland) and Oasis HLB, MCX, MAX cartridges (Waters). The solvents used for the SPE procedure were thoroughly investigated to determine the optimal combination to maximize recovery of the analytes of interest while eliminating carryover and elution of any endogenous components causing matrix effects.

Nine hundred and fifty microliters of ammonium formate buffer (10 mM) and 50 µL of the IS working solution (0.1 mg/L) were added to 100 µL of blood and 50 µL of urine, respectively. The following XLC program was subsequently used: after conditioning with 2 mL of methanol, 1 mL of water and 1 mL of water (5% NH_4OH), 150 µL of the diluted blood or urine sample was applied onto the SPE cartridge using 1.5 mL of water (5% NH_4OH) as transport solvent. Clean-up was accomplished with 1 mL washes of 5% NH_4OH and water in order to remove salts and endogenous interferences present in

the biological samples. The cartridge was then physically moved with a robotic arm to the elution (right) clamp in line with the LC pumps, thus leaving the extraction (left) clamp ready to start with a new sample. While the elution step was being performed, a new cartridge was conditioned, loaded, and washed in the left clamp. The elution was performed with the LC gradient of the mobile phase [ammonium formate buffer 10 mM (A) and acetonitrile (B)] [standard (gradient pump) elution mode] during the whole chromatographic run. Following the elution step, several automated clamp and valve washes with water, acetonitrile, and 0.2% triethylamine were carried out to avoid contamination between samples.

Chromatographic conditions. Several C_{18} analytical columns (XBridge, Waters), SunFire (Waters), Gemini (Phenomenex, Torrance, CA), Chromolith (Merck, Whitehouse Station, NJ) and Nucleodur Sphinx (Macherey-Nagel, Dueren, Germany) were initially evaluated in terms of chromatographic retention, peak shape, and carryover. The Nucleodur Sphinx RP column (3 µm, 120 × 2 mm) (FilterSer-vice, Eupen, Belgium) was finally chosen. A gradient was performed starting from 30% B and a flow rate of 0.25 mL/min for 3 min. From 3 to 8 min, B was subsequently increased to 35%. Then, from 8 to 9 min, B was linearly increased to 95% and kept for 1.5 min before returning to the initial conditions. Before the next injection, the column was equilibrated for 9.5 min to assure the appropriate initial conditions of the gradient for the next injection.

Tandem MS. A Quattro Premier tandem MS (Waters) was applied. Ionization was achieved using electrospray in positive ionization mode (ESI^+). Nitrogen was used as nebulization and desolvation gas at a flow rate of 600 L/h and heated to 350°C. Capillary voltage and source block temperature were 1 kV and 120°C, respectively.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards [200 µg/L, in 10 mM ammonium formate/acetonitrile (50:50, v/v)] were infused into the MS, and the cone voltage (CV) was optimized to maximize the intensity of the protonated molecular species $[M + H]^+$. Collision-induced dissociation (CID) of each proto-

Table I. Intraassay*, Interassay Precision†, and Bias of the LOQ and QC Blood Samples

		Amphetamine	Methamphetamine	MDA	MDMA	MDEA	PMA	Ephedrine
LOQ	Nominal value (µg/L)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
	Average (µg/L)	2.2	2.5	2.4	2.6	2.4	2.7	2.5
	RSDr (%)	2.2	1.5	4.5	11.1	2.5	3.5	5.5
	RSDt (%)	9.2	7.2	7.2	10.4	3.0	4.3	7.0
	Bias	–12.7	–0.2	–3.5	2.5	–3.3	8.1	–1.3
External QC 1	Nominal value (µg/L)	94.4	85.3	58	102.8	69.8	N/A	N/A
	Average (µg/L)	97.6	83.9	59	101.9	67.5	N/A	N/A
	RSDr (%)	4.4	3.7	4.3	3.9	4.7	N/A	N/A
	RSDt (%)	5.7	4.1	3.8	3.9	4.4	N/A	N/A
	Bias	3.4	–1.7	1.8	–0.9	–3.2	N/A	N/A
External QC 2	Nominal value (µg/L)	51.1	128.8	99.3	76.9	57.6	N/A	N/A
	Average (µg/L)	52.8	126.7	106.2	75	56.4	N/A	N/A
	RSDr (%)	2.7	3.8	2.6	3.3	2.8	N/A	N/A
	RSDt (%)	6.1	4.4	3.2	4.9	4.8	N/A	N/A
	Bias	3.4	–1.7	6.9	–2.4	–2.1	N/A	N/A
In house QC Low	Nominal value (µg/L)	3.5	3.5	3.5	3.5	3.5	3.5	3.5
	Average (µg/L)	3.2	3.6	3.5	3.4	3.5	3.7	3.5
	RSDr (%)	4	2.2	3.9	8.7	3.5	2.5	2.5
	RSDt (%)	6.2	4.9	4.5	9.2	4.3	3.5	3.4
	Bias	7.3	2.7	–0.7	–1.7	–0.3	5.9	–0.8
In house QC Medium	Nominal value (µg/L)	50	50	50	50	50	50	50
	Average (µg/L)	53.1	51.3	52.7	51.2	50.7	N/A	51.5
	RSDr (%)	3.5	1.8	1.6	2.5	1.9	N/A	2.7
	RSDt (%)	3.8	4.3	2.7	2.6	3.1	N/A	2.7
	Bias	6.1	2.5	5.5	2.4	1.3	N/A	3
In house QC High	Nominal value (µg/L)	160	160	160	160	160	160	160
	Average (µg/L)	163.1	159.3	160.8	160.2	159.7	158.2	161.7
	RSDr (%)	2.5	3.2	1.4	2	1.3	1.7	2.9
	RSDt (%)	2	4.5	2.7	2.2	2.7	2.5	2.9
	Bias	1.9	–0.4	0.5	0.1	–0.2	–1.2	1.1

* Expressed as RSDr (%).
† Expressed as RSDt (%).

nated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mBar) and the collision energy (eV) adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis.

Online SPE-LC–MS–MS assay validation

Validation was performed based on the FDA guidelines and recent publications concerning validation of bioanalytical methods (24,25).

Linearity, limit of quantification (LOQ), limit of detection (LOD), precision, and accuracy (bias)

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. Freshly prepared working solutions at 4000 µg/L were used to prepare blood calibrators at concentrations of 400, 200, 100, 50, 25, 12.5, 5, and 2.5 µg/L for blood and at 1000, 800, 500, 250, 200, 125, 100, 50, and 25 µg/L for urine. Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression with a 1/x-weighing factor for all compounds.

The LOQ was estimated by replicate analysis (n = 2) over eight different days and was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) of less than 20%. The LOQ was calculated performing the analysis of variance: a ‘single factor’ ANOVA test [significance level (α) of 0.05].

The LOD was estimated from blank blood and urine samples, spiked with decreasing concentrations of the analytes. It was defined as the concentration where the response of the qualitative ion could reliably be differentiated from background noise [i.e., signal-to-noise ratio (S/N) equal to or greater than 3:1]. The acceptance criteria for ion ratios should be equal to or lower than 20% and retention time deviations lower than 3.5% relative to that of the corresponding control or calibrator.

Five QCs were analyzed for blood: two external QCs (Medichem) and three in house QCs. For urine, four QCs were monitored: two external from Bio-rad and two in house. The in house QCs were prepared by different operators from different working solutions different of those for the calibrators and stored at –20°C until use.

Table II. Intraassay*, Interassay Precision†, and Bias of the LOQ and QC Urine Samples

		Amphetamine	Methamphetamine	MDA	MDMA	MDEA	PMA	Ephedrine
LOQ	Nominal value (µg/L)	25.0	25.0	25.0	25.0	25.0	25.0	25.0
	Average (µg/L)	22.1	24.8	25.0	23.8	24.2	27.2	24.7
	RSDr (%)	7.8	5.3	4.2	6.4	5.9	3.5	5.6
	RSDt (%)	7.0	5.9	4.5	6.1	5.3	3.5	5.8
	Bias	–11.6	–0.8	0.1	–4.9	–3.4	8.9	–1.2
External C 1	Nominal value (µg/L)	100.0	100.0	100.0	100.0	100.0	N/A‡	N/A
	Average (µg/L)	105.3	101.8	96.5	99.6	99.5	N/A	N/A
	RSDr (%)	5.0	4.0	5.0	4.5	4.7	N/A	N/A
	RSDt (%)	4.5	9.3	4.5	5.2	4.8	N/A	N/A
	Bias	5.3	1.8	–3.5	–0.4	–0.5	N/A	N/A
External C 3	Nominal value (µg/L)	625.0	625.0	312.0	312.0	312.0	N/A	N/A
	Average (µg/L)	635.2	620.5	305.9	299.5	317.5	N/A	N/A
	RSDr (%)	2.2	3.4	3.0	3.2	2.3	N/A	N/A
	RSDt (%)	3.2	6.5	3.4	4.4	3.1	N/A	N/A
	Bias	1.6	–0.7	–1.9	–4.0	1.8	N/A	N/A
In house QC Low	Nominal value (µg/L)	80.0	80.0	80.0	80.0	80.0	80.0	80.0
	Average (µg/L)	80.8	80.0	81.5	81.0	81.0	80.1	79.6
	RSDr (%)	4.9	3.6	3.4	2.6	2.3	4.2	3.2
	RSDt (%)	4.4	2.9	4.1	2.6	2.3	3.9	3.3
	Bias	1.0	0.0	1.9	1.3	1.2	0.1	–0.5
In house QC High	Nominal value (µg/L)	400.0	400.0	400.0	400.0	400.0	400.0	400.0
	Average (µg/L)	419.9	405.0	407.5	406.8	399.9	407.9	402.9
	RSDr (%)	3.4	3.3	4.3	1.9	3.6	3.1	2.6
	RSDt (%)	3.3	3.1	3.9	3.0	3.4	3.1	2.6
	Bias	5.0	1.3	1.9	1.7	0.0	2.0	0.7

* Expressed as RSDr (%).
† Expressed as RSDt (%).
‡ N/A (not available).

Intraassay and interassay precision was evaluated by replicate (n = 2) analysis of the QC samples performed over eight different days. Precision (expressed as $\%RSD_r$ for intraassay precision and $\%RSD_t$ for interassay precision) was determined by performing the analysis of variance: a single factor ANOVA test [significance level (α) of 0.05]. Comparing the mean of calculated concentrations of QC samples to their respective nominal values, provided data on the accuracy of the method.

Selectivity

The selectivity of the method was verified by examination of the chromatograms obtained after the extraction of eight different blank blood and urine samples from different origins.

Moreover, a blank urine and blood sample spiked with morphine, codeine, benzoylecgonine, codeine, 6-MAM, fentanyl, pholcodine, hydromorphone, hydrocodone, norcodeine, dihydrocodeine, oxycodone, oxymorphone, cocaine, methadone, EDDP, 27 benzodiazepines, zolpidem, zopiclone, zaleplon, THC, 11-OH-THC, and THC-COOH were also analyzed to check for interferences.

Stability in the autosampler and after three freeze/thaw cycles

The autosampler stability of diluted samples at concentrations of 160 and 3.5 µg/L for blood and 400 or 80 µg/L in urine (n = 6 at each concentration) were monitored as follows: one pool of samples were determined immediately and another pool of samples were analyzed after remaining in the autosampler at 6 ± 2°C for 24 and 72 h (a weekend). All samples were spiked with the IS just before analysis.

Stability studies of amphetamines after three freeze/thaw cycles in both matrices were determined through spiked blank urine and blood samples with concentrations of 160 and 3.5 µg/L for blood and 400 or 80 µg/L in urine (n = 6 at each concentration).

All the stability experiments were tested against a lower percentage limit corresponding to 90–110% of the ratio (mean value of stability samples/mean value control samples) with a 90% confidence interval of the stability samples between 80 and 120% of the mean of the control samples.

Assessment of matrix effects

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different analyses were carried out. The first one involved a post-column infusion experiment (26). This experiment is based on a continuous post-column infusion of a mixture of the analytes and their internal standards (10 µg/L at a flow rate of 10 µL/min) to produce a constant elevated response in the MRM channels. This constant response was monitored throughout the whole run following the injection of urine and blood samples from different origin (n = 6) and compared to the response following the injection of mobile phase only.

The second experiment consisted of a comparison between the peak responses of the analytes of interest spiked into blank blood (160 and 3.5 µg/L) or urine (400 and 800 µL) (n = 3 for each concentration) and those obtained after being spiked in the mobile phase at the same concentration levels (27).

Recovery

Extraction recoveries were estimated by performing the following experiments: a blank sample spiked at 160 and 3.5 µg/L (and the IS to each concentration) for blood and 400 and 80 µg/L (and the IS to each concentration) for urine (n = 3 for each concentration) were loaded and washed in a first SPE cartridge while a second cartridge was placed in series to determine the breakthrough of the first one (breakthrough is the maximum volume from which 100% recovery can be achieved). Both cartridges where subsequently eluted independently. Recovery was considered to be the ratio between the response obtained after elution of the first cartridge and the

Table III. MRM Transitions and Conditions For All Compounds and Their Deuterated Analogues

	Precusor Ion (m/z)	Product Ions* (m/z)	Cone Voltage (V)	Collision Energy (eV)
Amphetamine	136.1	<u>91.1</u> 118.9	15	15 10
Methamphetamine	150.0	<u>91.1</u> 119.1	20	15 10
MDA	180.1	105.0 <u>134.9</u>	15	20 15
MDMA	194.2	105.0 <u>163.1</u>	20	25 15
MDEA	208.2	105.0 <u>163.1</u>	20	15 15
PMA	166.1	121.1 <u>149.0</u>	15	18 10
Ephedrine	166.1	<u>133.0</u> 148.3	15	20 10
Amphetamine-d_{11}	147.1	98.0	15	15
Methamphetamine-d_5	155.0	91.9	20	20
MDA-d_5	185.0	167.9	18	10
MDMA-d_5	199.2	165.1	15	15
MDEA-d_6	214.2	165.9	20	12
Ephedrine-d_3	169.1	151.1	20	15

* Underlined transitions were used for quantification.

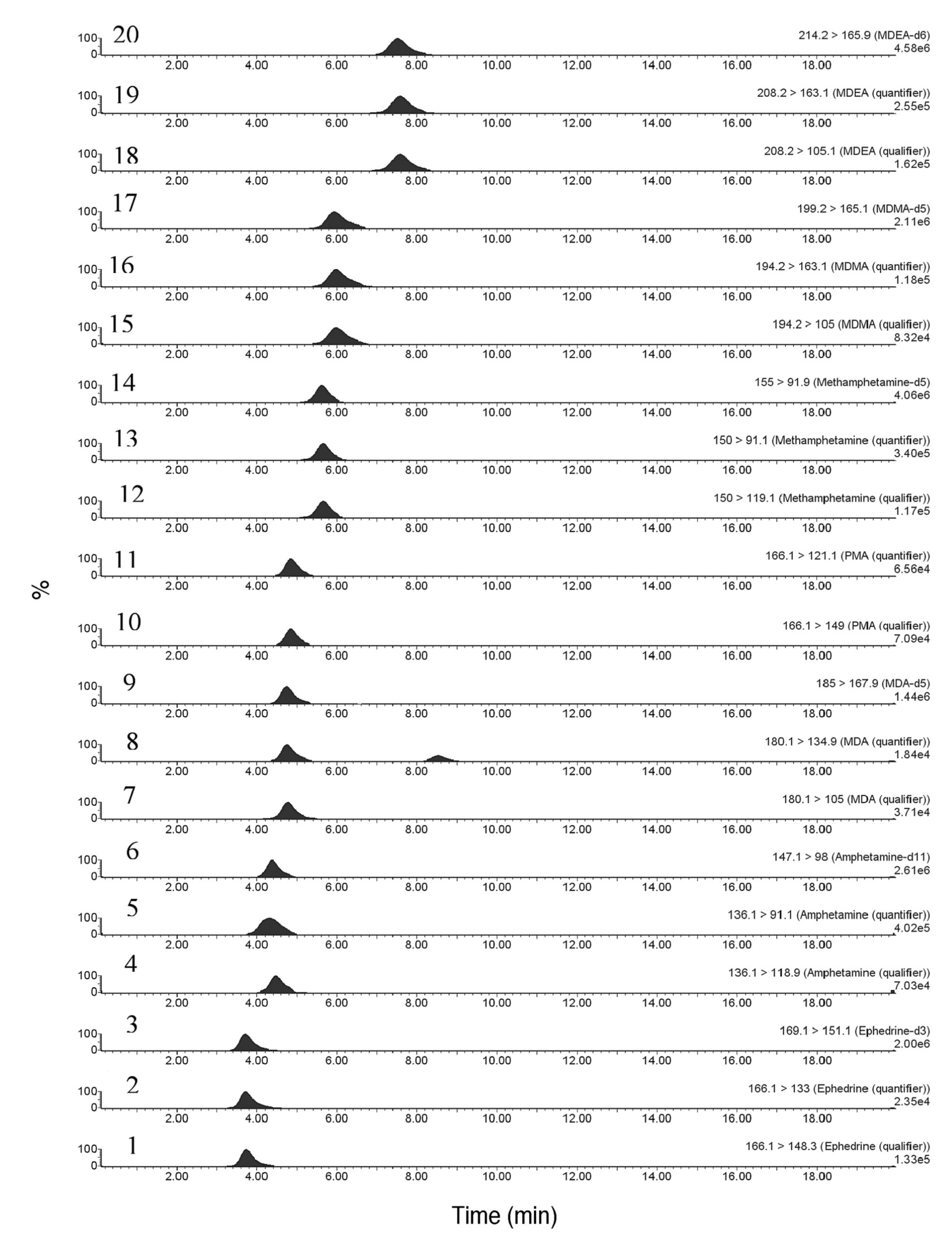

Figure 1. MRM chromatograms obtained following the analysis of a spiked blood sample with 2.5 µg/L. Chromatogram identification: 1, ephedrine (qualifier); 2, ephedrine (quantifier); 3, ephedrine-d_3; 4, amphetamine (qualifier); 5, amphetamine (quantifier); 6, amphetamine-d_{11}; 7, MDA (qualifier); 8, MDA (quantifier); 9, MDA-d_5; 10, PMA (qualifier); 11, PMA (quantifier); 12, methamphetamine (qualifier); 13, methamphetamine (quantifier); 14, methamphetamine-d_5; 15, MDMA (qualifier); 16, MDMA (quantifier); 17, MDMA-d_5; 18, MDEA (qualifier); 19, MDEA (quantifier); and 20, MDEA-d_6. Peak intensity is shown in the top right-hand corner of each trace.

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total response (sum of both the first and second SPE cartridge).

Carryover

Carryover was evaluated by the analysis of blank blood and urine samples spiked with the IS after the analysis of the upper calibrator (n = 8) for both matrices and after the analysis of a very concentrated urine sample (n = 3) (15,000 µg/L).

Results and Discussion

During optimization of the SPE, the Oasis MCX, C_{18}, and HLB cartridges demonstrated excellent recovery for all analytes of interest. HLB cartridges were the best choice because of optimal analyte elution from the MCX, an extremely high pH was required, which was not compatible with the chosen analytical column. The HLB cartridge is a macroporous polimer made of a balanced ratio of two monomers, the lipophilic divinylbenzene and the hydrophilic *N*-vinylpyrrolidone.

The applied gradient ensured the elution of all the drugs examined within 11 min and produced chromatographic peaks of acceptable symmetry.

The method was validated for selectivity, linearity, LOQ, LOD, precision, accuracy, matrix effect, recovery, stability, and carryover by the analysis of spiked blood and urine samples.

Selectivity of the method was achieved by a combination of retention time, precursor, and two product ions. The most prominent precursor-product transitions were used for quantification and the next most abundant used as qualifier (Table III). For the corresponding deuterated analogues, only one transition was monitored. Injection of single analyte solutions did not produce interference in the other MRM channels. No interferences were observed after the analysis of blank urine samples spiked with several over-the-counter drugs, ensuring the selectivity of the method. The ion ratios quantifier versus qualifier were as follows: amphetamine 2.5, methamphetamine 2.9, MDA 2.2, MDMA 1.4, MDEA 1.5, PMA 1.0, and ephedrine 5.6.

The quantification range applied was 2.5–400 µg/L for blood and 25–1000 µg/L for urine. Correlation coefficients of the weighed (1/x) linear regressions for this range were higher than r^2 = 0.99.

Figure 1 shows the MRM chromatograms obtained following the analysis of the blood lowest calibrator (2.5 µg/L). At this concentration a S/N >10:1 was observed for the qualifier, and the criteria for LOQ were satisfied. LOD ranged from 0.05 µg/L to 0.5 µg/L for blood and 0.25 µg/L to 2.5 µg/L for urine, depending on the compound. The intra- and interassay precision

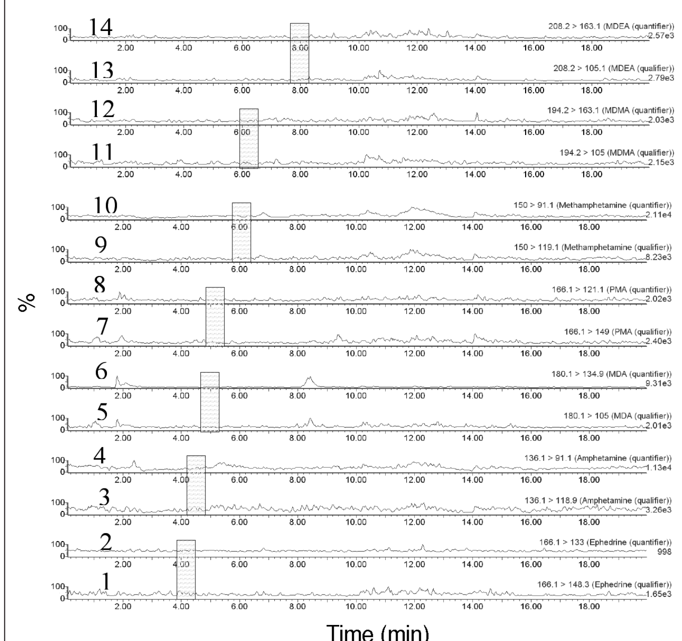

Figure 2. MRM chromatograms obtained following the analysis of blank urine analyzed after a high concentrated sample (15,000 µg/L). Chromatogram identification: 1, ephedrine (qualifier); 2, ephedrine (quantifier); 3, amphetamine (qualifier); 4, amphetamine (quantifier); 5, MDA (qualifier); 6, MDA (quantifier); 7, PMA (qualifier); 8, PMA (quantifier); 9, methamphetamine (qualifier); 10, methamphetamine (quantifier); 11, MDMA (qualifier); 12, MDMA (quantifier); 13, MDEA (qualifier); and 14, MDEA (quantifier). Dotted area represents the retention time of each amphetamine. Peak intensity is shown in the top right-hand corner of each trace.

Table IV. Extraction Recovery and Matrix Effects for Blood and Urine*

	Urine		Blood	
	Recovery (%)	Matrix effect (%)	Recovery (%)	Matrix effect (%)
Amphetamine	99.8	–17.8	99.9	–3.6
Methamphetamine	99.1	–3.7	100	–14.3
MDA	100.0	–12.0	99.8	–11.0
MDMA	100.0	–4.7	100	–13.9
MDEA	99.6	7.7	99.9	–31.3
PMA	100.0	–9.8	100	–12.0
Ephedrine	99.7	–14.3	97.6	–6.4

* Data represent the mean of six experiments at two concentration levels (n = 3) for both matrices; blood (n = 3) at 160 and 3.5 µg/L; urine (n = 3) at 400 and 80 µg/L.

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were satisfactory with all RSDs lower than 12% (Table I and II for blood and urine, respectively). Results indicated that the bias of the assay was < 13%. Post-column infusion experiments were performed to provide information of the matrix effect throughout the course of the elution time for the analytes. A second experiment was carried out where peak responses obtained when the compounds were spiked to a blank blood and urine samples were compared with the response obtained when the compounds were added to a mobile phase only at the same concentration. In both experiments, no matrix effects were observed except for MDEA. For MDEA some ion suppression was observed for blood (–31.3%); nevertheless, the use of deuterated IS partially compensates for the observed matrix effect, and thus, it did not compromise the quantification as demonstrated with the results obtained for the intra- and interassay precision (28). The results of the matrix effects and recovery at the two different concentrations are presented in Table IV. Very high and reproducible recoveries were obtained with this online SPE procedure for all analytes. The stability of samples in the autosampler after 24 h and 72 h and after three freeze/thaw cycles were monitored. No compound presented instability during this period of time.

After optimization of the method, no carryover was observed in the analysis of a blank sample injected after the analysis of the upper calibrator (neither with blood nor urine). No significant carryover was observed after the analysis of a blank urine sample analyzed after the injection a very high concentrated authentic urine sample (15,000 µg/L) (Figure 2). In addition, during analyses of authentic urine samples (n = 50) from forensic and toxicology cases, a blank was injected after each sample to check for carryover.

Samples

Blood samples were collected from 28 healthy volunteers 1 h and 15 min after the ingestion of a single dose of MDMA (75 mg) or a placebo. The blood samples were centrifuged for 10 min at 3000 rpm, and the serum obtained was stored at –20°C prior to analysis. The results are presented in Table V. Concentrations of MDMA were generally low. The median and minimum-maximum range (in µg/L) for MDMA were 48.3 (919.7–77.00). The concentrations of the authentic urine samples varied considerably. Those urine samples with concentrations higher than the upper calibrator (1000 µg/L) were diluted 1:10 and reanalyzed. Figure 3 shows the MRM chromatogram of the analysis of a urine sample from an amphetamine user.

Table V. Results Obtained From the MDMA Study*

ID	MDMA	MDA
P2101A	45.8	< LOQ
P2101B	–	–
P2102A	50.0	< LOQ
P2102B	–	–
P2103A	–	–
P2103B	46.0	< LOQ
P2104A	50.6	< LOQ
P2104B	–	–
P2105A	–	–
P2105B	50.4	< LOQ
P2106A	–	–
P2106B	22.9	0
P2107A	61.5	3.6
P2107B	–	–
P2108A	–	–
P2108B	24.3	< LOQ
P2109A	–	–
P2109B	19.7	< LOQ
P2110A	77.0	< LOQ
P2110B	–	–
P2111A	–	–
P2111B	66.0	< LOQ
P2112A	64.9	< LOQ
P2112B	–	–
P2113A	46.5	< LOQ
P2113B	–	–
P2114A	40.4	< LOQ
P2114B	–	–

* Concentrations are given in µg/L.

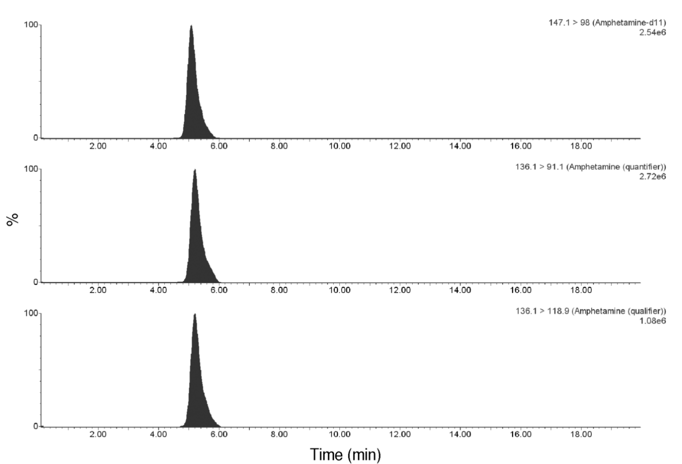

Figure 3. Typical MRM chromatograms obtained following the analysis of an authentic urine sample with a concentration of 103 µg/L for amphetamine. The figure shows the response for the two transitions (quantifier and qualifier). Peak intensity is shown in the top right-hand corner of each trace.

Conclusions

LC–MS–MS offers high specificity, good precision and accuracy, a wide dy-

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namic range, and high sensitivity. Due to its mass selectivity, it was expected that method development time and sample turnover time would be reduced significantly. However, ion-suppresion requires that the majority of biological matrix constituents are removed prior to LC–MS–MS analysis making sample preparation a time consuming element in the development of LC–MS–MS bioassays. During the process of method development the demands on sensitivity, precision and accuracy become more stringent, which resulted in increasing assay development time (ranging from day 1 for 'quick and dirty' work up to several weeks for a fully validated assay applicable for pre-clinical study samples). Especially, for clinical studies, high demands on specificity, accuracy, and precision must be complied. Apart from full automation, state-of-the-art online SPE provides high precision and sensitivity and a higher sample throughput as compared to liquid liquid extraction (LLE) or off-line SPE (21).

In this article, a fully validated and highly sensitive LC–MS–MS method is described for the simultaneous analysis of the main amphetamines in blood and urine. The method combined online SPE with LC–MS–MS and provided a thorough clean-up of the matrix in combination with high recovery, excellent precision, and accuracy in the linear range investigated using just 100 µL and 50 µL of blood and urine samples. The method was successfully applied to authentic samples from drug users and sensitive enough to detect a single use of MDMA in healthy volunteers. This method is certainly of interest on the field of forensic toxicology.

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VI. CONCLUSIONS

Conclusions

Conclusiones

Conclusions

This thesis intended to demonstrate the usefulness of present day LC-MS/MS applications used in forensic routine laboratories, and instrument improvement, supporting the successful spreading of LC-MS/MS.

6.1. Analysis of multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid and metabolites:

-A validated method for the simultaneous analysis of multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid and several metabolites with LC-MS/MS was developed and validated.

-Sample preparation consisted of a SPE using mixed mode cation exchange cartridges because most of the selected hallucinogens have a pKa higher than 7. After evaporation of the elution SPE solvent, samples were reconstituted in mobile phase for their analysis by LC-MS/MS. The method required 500 µL of urine achieving a LOQ for LSD and 2-Oxo-3-Hydroxy-LSD of 0.05 and 1 ng/mL, respectively and ranged, for the other hallucinogens, from 0.5 to 10 ng/mL. Linear and quadratic regression was observed from the LOQ of each compound to 12.5 ng/mL for LSD, 50 ng/mL for 2-oxo-3-OH-LSD, and 500 ng/mL for the others. The precision and bias were < 20% for most of the compounds, except for bufotenine and cathinone (%bias <24%), and ibogaine (%bias <30%)). External QCs containing LSD were analysed within each batch. Excellent recoveries for all the analytes were obtained (>87%). No instability was observed when the processed samples were kept in the autosampler for at least 24h.

-The method was applied to the analysis of urine samples from two young men who declare to have ingested magic mushrooms. Urine samples were collected after 1, 4, 8 and 24 h. Maximum concentrations of psilocin were found at 4 hours after ingestion, and it was still present after 24 hours. Authentic samples containing ketamine and chlorpheniramine were also analysed with the present method.

6.2. Analysis of cannabis in blood:

-An LC-MS/MS method was developed for the analysis of THC, and its main metabolites, 11-OH-THC and THC-COOH, in 250 µL of blood. Sample clean-up consisted of a LLE with hexane: ethyl acetate (90:10, v/v). No significant matrix effect was observed and excellent recoveries were obtained. The linearity range was 0.5-40 µg/L for THC, 1-40 µg/L for 11-OH-THC, and 2-160 µg/L for THC-COOH, and excellent intra-assay and inter-assay precision; RSDs were < 12% for THC and 11-OH-THC and < 8% for THC-COOH for certified QC samples were obtained. The compounds demonstrated to be stable in the processed samples and after three freeze/thaw cycles.

-Finally, 63 authentic blood samples from cannabis users were analyzed which demonstrated the usefulness of the method. The median and minimum maximum range (in ng/mL), respectively, were as follows: THC (7.45, [1.3-34.1]), 11-OH-THC (2.7, [1.0-13.4]) and THC-COOH (44.8, [7.9-224.3]).

6.3. Analysis of cannabis in urine:

-On-line SPE-LC-MS/MS was applied to the analysis of THC-COOH in urine (500 µL). Samples were hydrolyzed at strongly basic conditions (KOH) and then acidified before injection. C8 cartridges demonstrated to be highly effective to reduce matrix interferences and give a high recovery. The total run time was 6 min working at isocratic conditions. The method showed an excellent intra- and inter-assay precision (RSD <7% and bias <13%) for four external QC samples and three in house' QCs. The dynamic range was from 5 to 200 µg/L (r^2>0.99). LOQ and LOD were determined to be 5 µg/L and 0.25 µg/L, respectively. Furthermore, the analyte and the processed samples did not present instability and no carryover was observed after the analysis of high concentrated urine samples.

-Then, the method was consequently applied to 34 authentic samples analysed in one batch. The median was 652 ug/L with a minimum-maximum range of [12.1-3681]

6.4. Amphetamines in blood and urine:

-An on-line SPE-LC-MS/MS method was developed for the analysis of amphetamine, methamphetamine, MDMA, MDA, MDEA, PMA and ephedrine in blood and urine. The method was fully validated, following international guidelines, using only 100 µL and 50 µL of blood and urine, respectively. Very good intra- and inter-assay precision were obtained for all the compounds (RSD <11.2% and bias <13%) for two external QC for both matrices, and three and two 'in house' QCs for blood and urine, respectively. Linearity was demonstrated in the range of 2.5 ng/mL to 400 µg/L for blood and from 25 to 1000 µg/L for urine. LOQ was 2.5 µg/L and 25 µg/L for blood and urine, respectively. The analytes as well as the processed samples were stable (in the autosampler at least during 72 h and after three freeze/thaw cycles) and no matrix effects and carryover were observed.

-Finally, the method was applied to 28 volunteers who ingested consumed either a placebo or a dose of MDMA. The median and minimum-maximum range (in ng/mL) for MDMA were 48.3, [19.7-77.0], respectively. The method was also applied to authentic blood and urine from forensic cases.

General conclusion

Toxicology in all its application areas is heavily dependent on analytical methods. In the wake of analytical developments in other scientific fields, we have witnessed the introduction of a variety of new instrumental analytical approaches, especially in the field of forensic toxicology. The essence of the discipline entails that one is continuously confronted with new and unexpected cases in which very often one is asked to find the well-known needle in a haystack, actually often without knowing that the needle looks like! In scientific terms, toxicologist most of the time has very little foreknowledge and is forced by these circumstances to make use of all his intellectual creativity and analytical skills. As a result, new trends are continuously appearing in the analytical tool chest of the toxicologist. Some of these are short lived, but others grow to become new standard tools.

LC-MS/MS offers high specificity, good precision and accuracy, a wide dynamic range and high sensitivity. Due to its mass selectivity, it was expected that method development time and sample turnover time would be reduced significantly.

However, ion-suppression requires that the majority of biological matrix constituents are removed prior to LC-MS/MS analysis making sample preparation a time consuming element in the development of LC-MS/MS bioassays. During the process of method development the demands on sensitivity, precision and accuracy become more stringent, resulting in increasing assay development time (ranging from 1 day for 'quick and dirty' work up to several weeks for a fully validated assay applicable for (pre)-clinical study samples). Especially, for forensic studies, high demands on specificity, accuracy and precision must be complied. Apart from full automation, on-line SPE provides high precision and sensitivity and a higher sample throughput as compared to LLE or off-line SPE.

The LC-MS/MS methods (using off-line and on-line sample clean up) were applied to authentic blood and urine samples obtained from forensic cases which covered a broad range of concentrations. A reduction of sample volume was obtained compare to current GC-MS analysis used in the laboratory, and currently, these methods are working for routine forensic cases in the NICC for a number of years. They have demonstrated to be robust, accurate, while using external QCs within each batch.

In this thesis on-line SPE-LC-MS/MS has been evaluated in terms of practical gain in speed and efficiency that can be achieved compared to current LC-MS/MS systems.

The combination of the high throughput on-line SPE with the well-known sensitivity and selectivity assured by MS/MS resulted in the elimination of the bottleneck associated with the sample preparation requirements and provided increased sensitivity, accuracy and precision. Table 8 summarizes the approximate time employed in each step for the applications presented in this work (considering a batch of 15 samples).

Table 8. Time required in each application included in this thesis

Sample prep	Application	Number of compounds	Samples (n)	Hydrolysis (min)	Sample preparation SPE or LLE (min)	Evaporation (min)	Reconstitution (min)	Run time (min)
Off-line	Hallucinogens in urine	14	15	-	20	20	5	20
	Cannabis in blood	3	15	-	30	20	5	13
On-line	Cannabis in urine	1	15	15	-	-	-	6
	Amphetamines in blood	7	15	-	-	-	-	20
	Amphetamines in urine	7	15	-	-	-	-	20

*Shaded area corresponds to the sample preparation time.

As it can be observed from this table, the use of on-line SPE-LC-MS/MS technique has made possible the development of faster methods by reducing the sample preparation time and increasing the sample throughput. Conditioning, washing and elution steps can be performed automatically and to extract one sample while another is being analyzed by LC-MS/MS. Other important advantages of the on-line coupling are decreased risk of contamination of the sample, elimination of analyte losses by evaporation or by degradation during sample preconcentration, and improved precision and accuracy. Higher sensitivity is also achieved due to the transfer and analysis of the totality of the extracted species to the analytical system, in contrast to off-line clean-up procedures where only an aliquot of the extract is injected into the column. In addition, the on-line SPE has low solvent consumption requirements thereby decreasing the costs of organic solvents waste disposal.

Moreover, the on-line system avoids mistakes due to elimination of sample preparation steps. As everything is automated, a higher number of samples may be analysed in a raw, giving the possibility of including more QC samples within each batch, being of significant importance in terms of laboratory accreditation.

However, there are several limitations when working with on-line SPE-LC-MS/MS. In our experience, when automating a SPE method one must keep in mind that a

manual procedure cannot be transferred without minor modifications. Pressures, flow-rates, and injection solvent composition which must be suitable to the type of SPE (stability!), must be optimized again. Thus, the compounds of interest when developing a method must present similar physic-chemical properties (less flexibility than the off-line SPE procedures). Carryover is also an important parameter to evaluate when working with the Symbiosis system. Although disposable cartridges are used within each injection, precipitation and/or adsorption of the analytes to the tubing may occur. Carryover is a matter of proper combination of hardware and chemistry. In addition, the compatibility of the SPE elution solvent with the pH range of the analytical column is another consideration to take into account (e.g. when working with cation exchange cartridges the elution solvent is at highly organic pH, while common analytical columns usually can work up to a pH of 8). Strong changes in pH should be avoided.

Conclusions

Cette thèse a eu pour objet de démontrer l'utilité des applications actuelles avec LC-MS/MS employées dans les laboratoires médico-légaux et du développent instrumental, grâce au grand développement de la technique LC-MS/MS.

6.1. Analyse de multiples hallucinogènes, chloropheniramine, ketamine, acide ritalinique et plusieurs métabolites

- Une méthode utilisant le LC-MS/MS a été développée et validé pour l'analyse simultanée de multiples hallucinogènes, chloropheniramine, ketamine, acide ritalinique et plusieurs métabolites dans l'urine.

-La préparation de l'échantillon a été réalisée par SPE avec des cartouches d'échange cationique étant donné que la plupart des hallucinogènes sélectionnés avaient un pKa supérieur à 7. Après l'évaporation du solvant SPE, les échantillons ont été reconstitués dans la phase mobile et analysés avec LC-MS/MS. La méthode a demandé 500 µL d'urine et a permis d'obtenir un LOQ de 0.05 ng/mL pour LSD, 1 ng/mL pour le 2-oxo-3-OH-LSD et de 0.5 ng/mL à 10 ng/mL pour les autres hallucinogènes. Il a été observé une régression linéaire et quadratique du LOQ pour chaque composé à 12.5 ng/mL pour LSD, 50 ng/mL pour le 2-oxo-3-OH-LSD et à 500 ng/mL pour les autres. La précision et le bias étaient <20% pour la plupart des hallucinogènes, sauf pour la bufotenine et la cathinone (bias(%)<24) et l'ibogaine (bias(%)<30). Les QCs externes qui contenaient le LSD ont été étudiés avec chaque série d'analyse. Nous avons obtenu d'excellentes récupérations (>87%) et il n'y a pas eu de problématique d'instabilité des échantillons extraits qui ont été conservés dans l'injecteur automatique pendant 24 h.

-La méthode a été appliquée à l'analyse des échantillons d'urine de deux jeunes qui avaient déclaré avoir pris des champignons hallucinogènes. Les échantillons d'urine ont été prélevés à 1, 4, 8 et 24 h. Les concentrations de psilocine plus élevées ont été retrouvées après 4 h et elle était présente dans l'urine pendant au moins 24 h. Nous avons également analysé des échantillons d'urine réels qui contaient la ketamine et la chlorpheniramine avec cette méthode.

6.2. Analyse de cannabis dans le sang

- Une méthode LC-MS/MS a été développée pour l'analyse du THC et de ses principaux métabolites, 11-OH-THC et THC-COOH, dans 250 µL de sang. Le nettoyage de l'échantillon a été réalisé avec une LLE avec hexane : éthyle acétate (90 :10,v/v). Il n'y a pas été constaté d'effet matrice et on a obtenu d'excellentes récupérations. L'intervalle de linéarité a été de 0.5-40 µg/L pour le THC, 1-40 µg/L pour le 11-OH-THC et de 2-160 µg/L pour le THC-COOH, avec une excellente précision intra et inter-jour, avec un RSD (%) <12 pour le LSD et le 11-OH-THC et <8% pour le THC-COOH pour les QCs externes certifiés. Les composés étaient stables dans les échantillons extraits et après 3 cycles de congélation/décongélation.

-Finalement, l'utilité de la méthode a été démontrée avec l'analyse de 63 échantillons réels de sang de consommateurs de cannabis.

6.3. Analyse de cannabis dans l'urine

-La technique SPE-LC-MS/MS on -line a été appliquée à l'analyse de THC-COOH dans l'urine (500 µL). Les échantillons ont été hydrolysés dans des conditions fortement basiques (KOH) et puis acidifiés avant leur injection. Les cartouches C8 ont prouvé leur efficacité pour réduire les interférences de la matrice et ils ont démontré une grande récupération. Le temps de l'analyse total était de 6 min sous conditions isocratiques. La méthode a démontrée une excellente précision intra et inter-jour (RSD(%)<7 et bias(%)<13) des 4 QCs externes et des 3 QCs internes. L'intervalle dynamique a été de 5 à 200 µg/L (r^2>0.99). Le LOQ et LOD ont été fixés à 5 µg/L et 0.25 µg/L, respectivement. En plus, les composés et les échantillons extraits n'ont pas présenté instabilité et on n'a pas trouvé de problèmes de contamination après l'analyse des urines fortement concentrées.

-Par la suite, la méthode a été appliquée à l'analyse de 34 échantillons authentiques dans une seule série. La médiane a été de 652 µg/L avec un intervalle minimum-maximum de [12.1-3681].

6.4. Analyse d'amphétamines dans le sang et l'urine

-Une méthode SPE-LC-MS/MS on-line a été développée pour l'analyse d'amphétamine, methamphétamine, MDMA, MDA, MDEA et éphédrine dans le sang et l'urine. La méthode a été complètement validée selon les normes internationales, en utilisant respectivement seulement 100 et 50 µL de sang et urine. On a obtenu des précisions intra et inter-jour très bonnes (RSD(%)<11.2 et bias(%)<13) pour les 2 QCs externes des deux matrices et pour les 3 QCs internes du sang et deux de l'urine. Le LOQ a été respectivement de 2.5 µg/L et 25 µg/L pour le sang et l'urine. La linéarité a été dans un intervalle de 2.5 à 400 µg/L pour le sang et de 25 à 1000 µg/L pour l'urine. Les composés et les extraits ont été stables (dans l'injecteur automatique pendant 72 h et après trois cycles de congélation/décongélation). On n'a pas constaté d'effet matrice, non plus.

-Finalement, la méthode a été appliquée à des échantillons de sang de 28 volontaires qui avaient consommé tant placebo comme une dose de MDMA. La médiane et l'intervalle minimum-maximum (en µg/L) pour le MDMA étaient respectivement de 48.3, [19.7-77.0],. La méthode a également été appliquée à l'analyse de sang et urine provenant des cas médico-légaux réels.

Conclusion générale

La toxicologie dans tous ses domaines d'application est largement dépendente des méthodes analytiques. Dans la nouvelle vague des développements analytiques, nous avons été témoins de l'introduction d'une variété de nouveaux avancements instrumentaux, particulièrement dans le domaine de la toxicologie médico-légale. L'essence de la discipline implique que l'on soit constamment confronté à de nouveaux cas inattendus pour lesquels il s'agit souvent de chercher une aiguille dans une botte de foin sans même savoir à quoi l'aiguille ressemble ! En termes scientifiques, le toxicologue n'a fréquemment aucune idée d'où chercher et se trouve dans des circonstances dans lesquelles il faut employer sa créativité et son expérience analytique. Le résultat est que de nouvelles tendances apparaissent comme les outils analytiques pour le toxicologue. Quelques unes ne restent pas beaucoup de temps dans le marché, d'autres devient les nouvelles techniques de référence.

Le LC-MS/MS offre une grande spécificité, une bonne précision et exactitude, un large intervalle dynamique et une haute sensibilité. Grâce à sa sélectivité de masses, on s'attend que le temps de développement d'une méthode et le flux d'analyse d'échantillons soit significativement réduit. Pourtant, l'effet matrice, exige que la plupart des constituants de la matrice biologique soient éliminés avant l'analyse avec le LC-MS/MS, faisant que la préparation de l'échantillon semble une perte de temps dans le développement d'un essai. Pendant le développement d'une méthode, la demande de sensibilité, précision et exactitude deviennent de plus en plus élevées, résultant en une augmentation du temps de développent de l'essai (passant d'un jour de façon rapide à plusieurs semaines pour valider complètement une méthode pour l'analyse des échantillons cliniques). C'est en particulier le cas des études médico-légales qui doivent répondre à des critères élevés de spécificité, exactitude et précision.

Dans cette thèse, les méthodes LC-MS/MS (qui utilisent le nettoyage de l'échantillon 'off-line et 'on-line') ont été appliqués à des échantillons réels de sang et urine provenant des cas médico-légaux qui couvraient un intervalle large de concentrations. On a obtenu une réduction du volume de l'échantillon par rapport aux analyses GC-MS actuellement utilisées dans le laboratoire De plus, ces méthodes LC-MS/MS sont déjà utilisés depuis plusieurs années dans le laboratoire. Elles se sont montrées robustes et exactes quand on utilise des QCs externes dans chaque série d'analyses.

Le SPE-LC-MS/MS on-line a été évalué du point de vue pratique, rapidité et efficacité par rapport aux systèmes LC-MS/MS classiques actuels.

La combinaison de la productivité du SPE on-line avec la sensibilité et sélectivité renommées assurées par le MS/MS a résulté en l'élimination du goulet d'étranglement associé aux exigences de la préparation de l'échantillon et il a fourni une haute sensibilité, exactitude et précision. Le Tableau 8 résume le temps approximatif employé à chaque étape pour les applications présentées dans cette thèse (en tenant compte d'une série de 15 échantillons).

Tableau 8. Temps nécessaire à chaque application présentée dans cette thèse

Préparation de l'échantillon	Application	Nombre de composés	Echantillons (n)	Préparation échantillon Hydrolyse (min)	SPE ou LLE (min)	Evaporation (min)	Reconstitution (min)	Analyse (min)
Off-line	Hallucinogènes dans l'urine	14	15	-	20	20	5	20
	Cannabis dans le sang	3	15	-	30	20	5	13
On-line	Cannabis dans l'urine	1	15	15	-	-	-	6
	Amphètamines dans le sang	7	15	-	-	-	-	20
	Amphetamines dans l'urine	7	15	-	-	-	-	20

*La surface grisée correspond au temps employé pour la préparation de l'échantillon

Comme on peut constater dans ce tableau, l'utilisation de la technique SPE-LC-MS/MS on-line a permis le développement de méthodes plus rapides en réduisant le temps de la préparation de l'échantillon et en augmentant la productivité. Le conditionnement, le lavage et l'élution ont été réalisés automatiquement et pendant qu'un échantillon était en train de d'être extrait, un autre était en train d'être analysé avec le LC-MS/MS. D'autres avantages importants de la SPE on-line sont le moindre risque de contamination de l'échantillon, l'élimination de la perte de composés du à l'évaporation ou la dégradation pendant la préparation de l'échantillon et l'augmentation de la précision et de l'exactitude. Ainsi, on a obtenu une plus grande sensibilité grâce au fait que la totalité de l'échantillon extrait est analysé par le système analytique, contrairement aux procédures off-line où seulement une aliquote de l'extrait est injecté dans la colonne. De plus, le système SPE on-line engendre une faible consommation de solvant et permet de diminuer les coûts de solvants.

Par ailleurs, le système on-line évite les erreurs de manipulation grâce à l'élimination de la préparation de l'échantillon. Comme tout est automatisé, on peut analyser successivement un grand nombre d'échantillons en donnant la possibilité d'inclure plus de QCs entre chaque série, ce qui est crucial pour les laboratoires accrédités.

Néanmoins, il y existe des limitations lorsque l'on travaille avec le système SPE-LC-MS/MS on-line. D'après notre expérience, quand on automatise une méthode SPE on doit garder à l'esprit qu'une méthode off-line ne peut pas être transférée sur le système on-line sans faire l'objet de quelques modifications. La pression et la composition du solvant d'injection doivent être optimalisés selon le type de SPE (attention à la stabilité !) . De plus, les composés doivent avoir des propriétés physico-chimiques similaires quand on développe une méthode on-line (moins de flexibilité qu'avec les procédures off-line). La contamination est aussi un paramètre à prendre en compte quand on travaille avec le système Symbiosis. Même si on utilise des cartouches jetables à chaque injection, des précipitations et/ou adsorptions du composé peuvent se produire dans les tuyaux et valves de l'appareil. La contamination est une question de bonne combinaison du hardware et de la chimie. De plus, la compatibilité du solvant d'élution du SPE avec l'intervalle de pH de la colonne est un autre paramètre à prendre en compte (par ex. si on travaille avec des cartouches d'échange cationique, on doit utiliser des solvants organiques à haut pH quand les colonnes génériques qui ne peuvent que travailler jusqu'un pH de 8). Il vaut mieux éviter les changements brusques de pH dans le système.

Conclusiones

Esta tesis ha pretendido demostrar la utilidad de las aplicaciones actuales con LC-MS/MS empleadas en los laboratorios forenses y el desarrollo instrumental, gracias a la gran evolución de la técnica LC-MS/MS.

6.1. Análisis de múltiples alucinógenos, clorofeniramina, ketamina, ácido ritalínico y varios metabolitos:

- Se ha desarrollado y validado un método con LC-MS/MS para el análisis simultáneo de múltiples alucinógenos, clorofeniramina, ketamina, ácido ritalínico y varios metabolitos en orina.

-La preparación de la muestra consistió en una SPE utilizando cartuchos de intercambio catiónico debido ya que la mayoría de los alucinógenos seleccionados tenían un pKa mayor de 7. Tras la evaporación del disolvente SPE, las muestras fueron reconstituidas en la fase móvil para su análisis con LC-MS/MS. El método ha requerido 500 µL de orina logrando un LOQ de 0.05 ng/mL para el LSD, 1 ng/mL para el 2-oxo-OH-LSD y de 0.5 ng/mL a 10 ng/mL para el resto de los alucinógenos. Se observó una regresión lineal y cuadrática del LOQ de cada compuesto hasta 12.5 ng/mL para el LSD, 50 ng/mL para el 2-oxo-3-OH-LSD y 500 ng/mL para los otros. La precisión y el bias fueron <20% para la mayoría de los alucinógenos, excepto para bufotenina y la catinona (bias(%)<24) e ibogaína (bias(%)<30). Con cada grupo de análisis se analizaron QCs externos que contenían LSD. Se obtuvieron excelentes recuperaciones (>87%) y no hubo inestabilidad de las muestras procesadas que se mantuvieron en el autocargador de muestras durante 24 h.

-El método fue aplicado al análisis de muestras de orina de dos jóvenes que habían declarado haber consumido setas alucinógenas. Las muestras de orina fueron recogidas tras 1, 4, 8 y 24h. Las concentraciones más altas de psilocina se obtuvieron a las 4 h tras la ingestión y estuvo presente en la orina al menos hasta durante 24 h. Con este método también se analizaron muestras de orina auténticas que contenían ketamina y clorofeniramina.

6.2. Análisis de canabis en sangre

-Se ha desarrollado un método LC-MS/MS para el analisis de THC y de sus principales metabolitos, 11-OH-THC y THC-COOH, en 250 µL de sangre. La limpieza consistió en una LLE con hexano:acetato de etilo (90:10,v/v). No se observó efecto matriz y se obtuvieron excelentes recuperaciones. El rango de linealidad fue de 0.5-40 µg/L para el THC, 1-40 µg/L para el 11-OH-THC y de 2-160 µg/L para el THC-COOH, con excelente precisión intra e inter-día, obteniendo un RSD (%) <12 para el LSD y el 11-OH-THC, y <8% para el THC-COOH para los QCs externos certificados. Los compuestos fueron estables en las muestras procesadas y tras 3 ciclos de congelado/descongelado.

-Finalmente, la utilidad del método fue demostrada con el análisis de 63 muestras de sangre auténticas de consumidores de canabis.

6.3. Análisis de canabis en orina

-La técnica SPE-LC-MS/MS fue aplicada al análisis de THC-COOH en orina (500 µL). Las muestras fueron hidrolizadas en condiciones fuertemente básicas (KOH) y acidificadas antes de su inyección. Los cartuchos C8 demostraron ser muy eficaces para reducir las interferencias de la matriz y dieron una gran recuperación. El tiempo de análisis total fue de 6 min trabajando en condiciones isocráticas. El método mostró una excelente precisión intra e inter-día (RSD(%)<7 y bias(%)<13) en los 4 QC externos y los 3 QC internos. El rango dinámico fue de 5 a 200 ng/mL (r^2>0.99). El LOQ y LOD fueron determinados a 5 µg/L y 0.25 µg/L, respectivamente. Además, el analito y las muestras procesadas no presentaron inestabilidad y no se observó contaminación (prueba de arrastre) tras el análisis de orinas altamente concentradas.

-Posteriormente, el método fue aplicado al análisis de 34 muestras auténticas en una serie de análisis. La mediana fue 652 µg/L con un rango mínimo-máximo de [12.1-3681].

6.4. Análisis de anfetaminas en sangre y orina

-Se ha desarrollado un método on-line SPE-LC-MS/MS para el análisis de anfetamina, metanfetamina, MDMA, MDA, MDEA, PMA y efedrina en sangre y orina. El método ha sido completamente validado según las normas internacionales, empleando solo 100 µL y 50 µL de sangre y orina, respectivamente. Se obtuvieron precisiones intra e inter-día muy buenas (RSD(%)<11.2 y bias(%) <13) para los 2 QCs externos para ambas matrices y para los 3 y 2 QCs internos para sangre y orina, respectivamente. La linealidad fue en el rango de 2.5 a 400 µg/L para la sangre y de 25 a 1000 µg/L para la orina. El LOQ fue de 2.5 µg/L y 25 µg/L para la sangre y orina, respectivamente. Los analitos así como las muestras procesadas fueron estables (en el autocargador de muestras durante 72 h y tras tres ciclos de congelado/descongelado). Tampoco se observó efecto matriz.

-Finalmente, el método fue aplicado a 28 voluntarios que habían consumido tanto placebo como una dosis de MDMA. La mediana y el rango mínimo-máximo (en µg/L) para el MDMA fueron 48.3, [19.7-77.0], respectivamente. El método también fue aplicado al análisis de sangre y orina autentica procedente de casos forenses.

Conclusión general

La toxicología en todas sus áreas de aplicación depende fuertemente de los métodos analíticos. En la ola de las nuevas tecnologías analíticas hemos sido testigos de la introducción de una variedad de nuevos avances instrumentales, especialmente en el campo de la toxicología forense. La esencia de la disciplina suscita que uno se enfrente continuamente a casos nuevos e inesperados en los cuales a menudo a uno se le pregunta buscar una aguja en un pajar, ¡incluso aun sin saber a que se parece esa aguja!. En términos científicos, el toxicólogo a menudo no tiene mucha idea de donde buscar y se encuentra en circunstancias en las que tiene que emplear la creatividad y sus habilidades analíticas. Como resultado, están apareciendo nuevas tendencias como herramientas analíticas para el toxicólogo. Algunas de ellas duran poco tiempo en el mercado, otras, se convierten en las nuevas técnicas de referencia.

LC-MS/MS ofrece gran especificad, buena precisión y exactitud, un amplio rango dinámico y alta sensibilidad. Debido a su selectividad de masas, se espera que el tiempo de desarrollo de un método y el flujo de análisis de muestra se reduzca significativamente. Sin embargo, la supresión iónica requiere que la mayoría de los constituyentes de la matriz biológica sean eliminados antes del análisis con el LC-MS/MS haciendo que la preparación de la muestra parezca más bien una perdida de tiempo en el desarrollo de un experimento. Durante el proceso del desarrollo de un método la demanda de sensibilidad, precisión y exactitud se vuelven cada vez más estrictos, resultando en un aumento del tiempo del desarrollo del método (desde un día 'de forma rápida y mal' hasta varias semanas para validar completamente un método para el análisis de muestras clínicas). Sobretodo en el caso de los casos forenses se debe cumplir con la gran demanda de especificidad, exactitud y precisión.

En esta tesis, los métodos LC-MS/MS (empleando limpieza de la muestra 'off-line' y 'on-line') han sido aplicados a muestras auténticas de sangre y orina obtenidas de casos forenses que cubrieron un amplio rango de concentraciones. Se ha obtenido una reducción del volumen de muestra, comparado con los análisis con GC-MS actuales que se emplean en nuestro laboratorio y además estos métodos LC-MS/MS se están utilizando actualmente en el trabajo de rutina de los casos forenses que llegan al INCC ya durante varios años. Han demostrado ser robustos y exactos al utilizar QCs externos encada serie de análisis.

El on-line SPE-LC-MS/MS ha sido evaluado desde el punto de vista práctico, rapidez y eficacia que se pueden logran comparado con los sistemas LC-MS/MS actuales.

La combinación de la productividad del SPE on-line con la conocida sensibilidad y selectividad asegurada con el MS/MS ha resultado en la eliminación del obstáculo asociado a los requerimientos de la preparación de la muestra y ha proporcionado una alta sensibilidad, exactitud y precisión. La Tabla 8 resume el tiempo aproximado empleado en cada etapa para las aplicaciones presentadas en este trabajo (considerando una serie de 15 muestras).

Tabla 8. Tiempo requerido en cada aplicación incluida en esta tesis

Preparación muestra	Aplicación	Número de compuestos	Muestras (n)	Preparación de la muestra				Tiempo análisis (min)
				Hidrólisis (min)	SPE or LLE (min)	Evaporación (min)	Reconstitución (min)	
Off-line	Alucinogenos en orina	14	15	-	20	20	5	20
	Canabis en sangre	3	15	-	30	20	5	13
On-line	Canabis en orina	1	15	15	-	-	-	6
	Anfetaminas en sangre	7	15	-	-	-	-	20
	Anfetaminas en orina	7	15	-	-	-	-	20

*El área sombreada corresponde al tiempo empleado en la preparación de la muestra.

Como se puede observar en esta tabla, el uso de la técnica SPE-LC-MS/MS on-line ha hecho posible el desarrollo de métodos más rápidos reduciendo el tiempo de la preparación de la muestra y aumentado la productividad. El condicionamiento, lavado y la elución han sido realizados automáticamente y de manera que mientras una muestra era extraída la otra era analizada por el LC-MS/MS. Otras ventajas importantes de la SPE on-line son el menor riesgo de contaminación de la muestra, eliminación de la perdida de analito debido a la evaporación o degradación durante la preparación de la muestra y el aumento de la precisión y la exactitud. También se logra una mayor sensibilidad gracias a que el total de la muestra extraída es analizada por el sistema analítico, al contrario de los procedimientos off-line donde solo una alícuota del extracto es inyectado en la columna. Además, el sistema SPE on-line tiene un bajo consumo de disolvente por lo tanto disminuye el gasto total del análisis.

Al mismo tiempo, el sistema on-line evita errores de manipulación debido a la eliminación de la preparación de la preparación de la muestra. Como todo está automatizado, se pueden analizar seguidas un gran número de muestras, dando la

posibilidad de incluir más QCs en cada serie, siendo de gran importancia en los laboratorios acreditados.

Sin embargo, existen varias limitaciones cuando se trabaja con el sistema SPE-MS/MS on-line. Según nuestra experiencia, cuando se automatiza un método SPE uno debe mantener en mente que un procedimiento manual no se puede transferir sin hacer varias modificaciones. Se deben optimizar la presión y la composición del disolvente de inyección, debiendo ser adecuados según el tipo de SPE (!cuidado con la estabilidad!). Asimismo, los compuestos deben poseer propiedades físico-químicas parecidas cuando se desarrolla un método (menos flexibilidad que en on procesos SPE off-line). La contaminación también es un parámetro a tener en cuenta cuando se trabaja con el sistema Symbiosis. Aunque se utilizan cartuchos desechables en cada inyección, pueden producirse precipitaciones y/o adsorciones del analito en los conductos y válvulas del instrumento. La contaminación es cuestión de una combinación adecuada de hardware y química. Además, la compatibilidad del disolvente de elución del SPE con el rango de pH de la columna es otro parámetro a tener en cuenta (por ejemplo, cuando se trabaja con cartuchos de intercambio catiónico se suelen utilizar disolventes orgánicos a pH muy básico cuando las columnas genéricas llegan solo hasta un pH 8). En la manera de lo posible, se deben evitar cambios bruscos de pH dentro del sistema.

VII. FUTURE PERSPECTIVES
Perspectives futures
Perspectivas futuras

Future perspectives

7.1. A new trend in Liquid Chromatography: Ultra Performance Liquid Chromatography (UPLC)

Analysis of benzodiazepines in biological matrices by UPLC-MS/MS:

As efficiency and speed of analysis has become of a great importance in many applications of liquid chromatography, especially in the field of toxicological analysis where it is important to increase throughput and reduce analysis costs, UPLC could play a significant role in the future of liquid chromatography. The recent commercialization of porous hybrid organic-inorganic silicon-based particles with a narrow size distribution in the range of 1.7 µm has enabled a new level of performance, but only through the use of newly developed technology that permits pumping and injection of liquids at pressures in excess of 10,000 psi. In addition, the use of porous, sub-2µm particles allowed a wide 'sweet pot' in the van Deemter curve for the increased flow rates without the loss of chromatographic resolution, and smaller particles increase the efficiency of separation because efficiency (N) is inversely proportional to particle size (dp). The linear velocity of the mobile phase (flow rate for a fixed column ID) at which the maximum efficiency occurs increases as dp reduces. Furthermore, with sub-2µm particles, the flow rate region at which the optimal efficiency is obtained is much wider. The overall result is higher efficiency at high flow rates, resulting in faster analytes and better sensitivity (1). Therefore, to keep up with the pace of data acquisition the mass spectrometer must acquire data quickly enough to match the chromatographic output. Mass spectrometers without enough scan speed could not keep up with this type of analytical strategy because they need a longer scan time to acquire high-resolution data, typically 1 sec to achieve a resolution of 60000 FWHM. As a result, few data points will be collected, which might result in the loss of information.

For some analyses, however, speed is of secondary importance, and peak capacity and resolution are priority, for example for the analysis of methods including more than 30 compounds, including isomers, that must be separated.

The next step in the section of toxicology of the NICC will be to completely validate an UPLC-MS/MS method for the analysis of 30 benzodiazepines and related compounds in biological matrices. During the past years, a conventional LC-MS/MS method for the analysis of 30 benzodiazepines in blood, urine and hair was used. The method comprises a LLE with 1-butylchlororide. Two injections onto the LC-MS/MS of 35 min were necessary due to the number of compounds. Recently, a new UPLC-MS/MS method is under development for the analysis of these compounds. The sample preparation has been modified in such a way that LLE is now carried out with n-butyl acetate. N-butyl acetate is a medium polarity solvent. The emulsion formation, which is generally considered to be the major shortcoming of LLE, was completely avoided. Furthermore, n-butyl acetate is also comparatively safe for health, especially compared to other alternatives, and has a strong and typical odor, which makes the presence of the solvent clearly recognizable and suitable for routine use. Only the relatively high boiling point of n-butyl acetate (126°C) is its minor shortcoming, but it is not a problem in the case of benzodiazepines and other hypnotics, which evaporate at significantly higher temperature.

The first results show that the chromatographic method has been shortened to one single injection of just 13 min for the analysis of the 30 compounds (prazepam is the last compound to elute at 10 min). Next step will be the complete validation of the method and the application to forensic blood, urine and hair samples.

7.2. Analysis of alternative matrices for driving under the influence of drugs (DUID): oral fluid

<u>Analysis of drugs in oral fluid by on-line SPE-LC-MS/MS:</u>

In Belgium, the law concerning DUID has changed very recently, i.e. blood and urine analysis will be replaced by oral fluid screening and analysis. Advantages of this matrix include the ease and non-invasiveness of specimen collection and reduced opportunity for specimen substitution and adulteration. Salivary glands have a high blood flow. It has been suggested that the concentrations of many drugs in oral fluid correlate well with their concentrations in blood, which suggests that the analysis of the former matrix may show value in the determination of the current degree of exposure to a particular drug at a time of sampling. Drug

concentrations in oral fluid reflect the free, unbound parent drug and lipophilic metabolites. Since these are the forms of the drug which cross the blood-brain barrier and affect performance and behavior, oral fluid is a good specimen for detecting drug involvement in driving behavior. However, the oral fluid sample volume is usually low (1 mL or less).

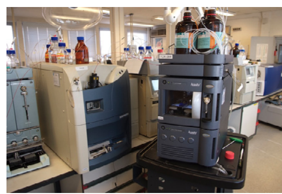
The section of toxicology of the NICC has already some experience in the analysis of drugs of abuse in oral fluid using off-line sample preparation procedures with LC-MS/MS (2-5). Our Institute has also participated in an external quality assessment scheme for drugs of abuse testing in oral fluid, ORALVEQ (6). During the 2007 and 2008 round of this program, the off-line SPE method and the under development on-line SPE procedure were carried out in parallel. Very comparable results were obtained assessing that on-SPE-LC-MS/MS is a good alternative tool for the analysis of drugs in oral fluid. Just a slight difference was observed for the results obtained for cocaine. One explanation could be that cocaine is a relatively instable compound (7). In terms of stability during the SPE procedure, one of the advantages of the on-line SPE is that the whole eluate is loaded directly onto the analytical column, thus there is a lesser risk of degradation of the compounds during the clean-up procedure.

Therefore, the next step will be the development and validation of an on-line SPE-LC-MS/MS method for the analysis of drugs in oral fluid, including MDMA, MDA, amphetamine and methamphetamine, morphine, codeine, 6-MAM, cocaine, benzoylegonine and THC, requiring just 250 µL of sample. As in on-line SPE everything is automated, all these compounds will be extracted and analyzed in the same run, with the highest sensitivity and lowest sample preparation (just dilution of the oral fluid directly in the LC vials).

Perspectives futures

7.1. La nouvelle tendance en Chromatographie Liquide : Ultra Chromatographie Liquide de Haute Performance

Analyse de benzodiazépines dans les matrices biologiques avec UPLC-MS/MS



Puisque l'efficacité et la vitesse d'analyse sont devenues cruciales pour diverses applications de la chromatographie liquide, surtout dans le domaine des analyses toxicologiques où il est important d'augmenter la productivité et réduire le coût d'analyse, l'UPLC pourrait jouer un rôle prédominant dans le futur de la chromatographie liquide. La commercialisation récente de particules poreuses composées d'un hybride organique – inorganique de silice avec une distribution de taille étroite dans l'intervalle de 1.7 µm a amélioré le rendement, mais uniquement avec l'utilisation de la nouvelle technologie UPLC qui permet de pomper et injecter des liquides à des hautes pressions qui dépassent les 10000 psi. Par ailleurs, l'usage de particules inférieures à 2 µm entraîne une d'amélioration de la courbe de Van Deemter avec l'augmentation de la vitesse de flux sans la perte de résolution chromatographique. Aussi, l'emploi de particules si petites augmente l'efficacité de la séparation grâce au fait de que l'efficacité (N) est inversement proportionnelle à la taille de particule (dp). La vitesse linéaire de la phase mobile (vitesse de flux pour une colonne avec un ID fixé) à laquelle nous avons la plus grande efficacité augmente à mesure que le dp diminue. De plus, avec des particules inférieures à 2 µm, la région de flux à laquelle on obtient l'efficacité optimale est plus large. Le résultat général est une plus grande efficacité à des flux plus élevés et permet des analyses plus rapides avec la plus grande sensibilité (1). D'autre part, pour maintenir le flux d'acquisition des données, le spectromètre de masses doit acquérir les donnés suffisamment rapides en fonction de la productivité chromatographique. Les spectromètres de masses qui n'ont pas la vitesse de scan nécessaire, typiquement d'un sec pour obtenir une résolution de 60000 FWHM, ne pourront pas bénéficier de ce type de technique analytique, du fait qu'ils n'enregistreraient que quelques points de chaque chromatogramme, générant une grande perte d'information.

Néanmoins, pour quelques analyses, la vitesse est d'importance secondaire, et la capacité de pic et résolution sont prioritaires, par exemple dans les méthodes

analytiques qui comprennent plus de 30 composés, avec des isomères, qui doivent être séparés.

La prochaine étape dans la section de toxicologie de l'INCC consistera à développer et valider entièrement une méthode UPLC-MS/MS pour l'analyse de 30 benzodiazépines et composés similaires dans les matrices biologiques. Au cours des dernières années, on a utilisé une méthode avec LC-MS/MS classique pour l'analyse des 30 benzodiazépines dans le sang, urine et cheveux. La méthode utilisait une LLE avec 1-chlorobutane. Du fait d'avoir tellement de composés, il était nécessaire d'injecter deux fois pendant une analyse de 35 min. Récemment, nous sommes en train de développer une méthode UPLC-MS/MS pour l'analyse de ces composés. La préparation de l'échantillon a été modifiée de sorte que la LLE est faite avec butyle acétate. Le butyle acétate est un solvant de polarité moyenne. On a éliminé la formation d'émulsion, qui est le plus grand inconvénient de la LLE. De plus, le butyle acétate est moins nocif pour la santé par rapport à d'autres alternatives et il a une odeur caractéristique qui permet de détecter sa présence, ce qui fait qu'il est plus conseillé pour le travail de routine. Le seul inconvénient est son haut point d'ébullition (126 °C), mais ce n'est pas un problème dans le cas des benzodiazépines et d'autres hypnotiques, puisqu'elles se' évaporent à une température relativement élevée.

Les premiers résultats montrent que la méthode chromatographique est plus rapide en effectuent une seule injection de 13 min pour l'analyse des 30 composés (prazepam est le dernier à sortir après 10 min). La prochaine étape sera la validation complète de la méthode et son application sur des échantillons médico-légaux dans le sang, urine et cheveux.

7.2. Analyse des matrices alternatives dans les cas de conduite sous l'influence des drogues (DUID).

<u>Analyse des drogues dans le fluide oral avec SPE-LC-MS/MS on-line</u>

En Belgique, la loi relative à la DUID a récemment changée de sorte que les analyses de sang et urine sont remplacées par le screening et l'analyse du fluide oral. Les avantages de cette matrice sont que le prélèvement de l'échantillon est simple, non invasif et la chance d'adultérer ou de remplacer l'échantillon est plus faible. Il est suggéré que la

concentration des différentes drogues dans le fluide oral est proportionnelle à la concentration dans le sang, c'est qui suggère que l'analyse de cette matrice permettrait une estimation du degré d'altération du comportement et l'effet de la drogue sur la personne au moment du prélèvement. Les concentrations de drogues dans le fluide oral représentent la drogue libre et les métabolites lipophiliques. Etant donné qu'il existe des formes de la drogue qui traversent la barrière du sang au cerveau et affectent le comportement de la personne, le fluide oral permet détecter l'effet de la drogue sur la conduite. Cependant, le volume de fluide oral est souvent bas (1 mL ou moins).

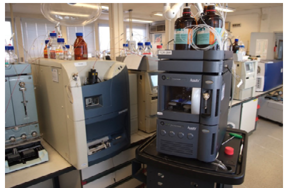
La section de toxicologie de l'INCC avait déjà un peu d'expérience dans le domaine de l'analyse des drogues dans le fluide oral en utilisant des procédures de préparation des échantillons off-line (2-5). Notre Institut a participé au concours externe de contrôle de qualité pour l'analyse des drogues dans le fluide oral ORALVEQ (6). Au cours des années 2007 et 2008 du concours, la méthode off-line employée habituellement dans notre laboratoire et la méthode on-line en cours de développement ont été appliquées en parallèle. On a obtenu des résultats très comparables ce qui démontre que la méthode on-line SPE-LC-MS/MS est une excellente alternative pour l'analyse des drogues dans le fluide oral. Cependant, on a trouvé une légère différence entre les concentrations obtenues pour la cocaïne. Une explication possible est que la cocaïne est relativement instable (7). En ce qui concerne la stabilité pendant la procédure du SPE, un des avantages de l'extraction on-line est que l'élution du SPE va directement à la colonne analytique. Il y a donc moins de risque de dégradation des composés pendant la préparation de l'échantillon.

C'est pourquoi la prochaine étape sera le développement et validation d'une méthode SPE-LC-MS/MS on-line pour l'analyse de drogues dans le fluide oral, en incluant le MDMA, MDA, amphétamine, méthamphétamine, cocaïne, benzoylecgonine et THC, en utilisant seulement 250 µL de fluide oral. Puisque tout est automatisé dans le système SPE on-line , ces composés pourraient être extraits et analysés dans la même analyse, en obtenant la plus grande sensibilité avec la une moindre préparation de l'échantillon (seulement la dilution du fluide oral directement dans les fioles LC).

Perspectivas futuras

7.1. La nueva tendencia en Cromatografía Líquida: Ultra Cromatografía Líquida de Alto Rendimiento (UPLC)

Análisis de benzodiazepinas en matrices biológicas mediante UPLC-LC-MS/MS



Como la eficacia y la rapidez de análisis se han vuelto de gran importancia en varias aplicaciones de la cromatografía líquida, sobretodo en el área de los análisis toxicológicos donde es importante aumentar la productividad y reducir el coste de análisis, UPLC podría jugar un rol substancial en el futuro de la cromatografía líquida. La reciente comercialización de partículas porosas hechas de un híbrido orgánico-inorgánico de sílice con una distribución de tamaño estrecha en el rango de 1.7 µm ha permitido un nuevo nivel de rendimiento, pero solo con el uso de la nueva tecnología UPLC que permite bombear e inyectar líquidos a presiones que sobrepasan los 10000 psi. Además, el uso de partículas de menos de 2 µm permite dar un toque de mejora a la curva de van Deemter con el aumento de la velocidad de flujo sin la pérdida de resolución cromatográfica. Igualmente, el uso de partículas tan pequeñas aumenta la eficacia de la separación ya que la eficacia (N) es inversamente proporcional al tamaño de partícula (dp). La velocidad lineal de la fase móvil (velocidad de flujo para una columna con un ID determinado) a la cual tenemos la mayor eficacia aumenta a medida que el dp reduce. Lo que es más, con partículas menores de 2 µm, la región de flujo a la que se obtiene la eficacia óptima es más amplio. El resultado general es una mayor eficacia a flujos más elevados, resultando en análisis más rápidos con la mayor sensibilidad (1). Sin embargo, hay que tener en cuenta que para mantener el flujo de adquisición de datos el espectrómetro de masas debe adquirir los datos lo suficientemente rápido de acuerdo a la producción cromatográfica. Los espectrómetros de masas que no tengan la velocidad de escaneo necesaria, generalmente 1 sec para obtener una resolución de 60000 FWHM, no podrán aprovechar este tipo de técnica analítica, ya que solo registrarían unos pocos puntos de cada cromatograma, resultando una pérdida importante de información.

Sin embargo, para algunos análisis, la velocidad es secundaria, y la capacidad de pico y resolución son prioridades, por ejemplo en los métodos de análisis que incluyen mas de 30 compuestos, incluyendo isómeros, que deben ser separados.

La próxima etapa en la sección de toxicología del INCC será desarrollar y validar completamente un método UPLC-MS/MS para el análisis de 30 benzodiazepinas y compuestos relacionados en matrices biológicas. Durantes estos años, se ha utilizado un método con LC-MS/MS clásico para el análisis de las 30 benzodiazepinas en sangre, orina y pelo. El método empleaba una LLE con 1-cloruro de butilo. Debido al gran número de compuestos eran necesarias dos inyecciones de 35 minutos. Recientemente, se esta desarrollando un método con el UPLC-MS/MS para el análisis de estos compuestos. La preparación de la muestra ha sido modificada de tal modo que la LLE se lleva a cabo ahora con acetato de butilo. El acetato de butilo es un disolvente de polaridad media. Se ha eliminado la formación de emulsión, que es el mayor inconveniente de las LLE. Además, el acetato de butilo es más seguro para la salud, comparado con otras alternativas, y tiene un olor característico que permite detectar su presencia y que lo hace adecuado para su uso en la rutina. El único inconveniente es su alto punto de ebullición (126°C), pero no es un problema en el caso de las benzodiazepinas y otros hipnóticos, ya que se evaporan a una temperatura relativamente alta.

Los primeros resultados muestran que el método cromatográfico es más corto realizando una sola inyección de solo 13 min para el análisis de los 30 compuestos (prazepam es el ultimo compuesto en salir a los 10 min). El próximo paso será la validación completa del método y su aplicación a muestras forenses de sangre, orina y pelo.

7.2. Análisis de matrices alternativas en casos de conducción bajo la influencia de las drogas (DUID)

Análisis de drogas en fluido oral con on-line SPE-LC-MS/MS

En Bélgica, la ley que trata el DUID ha cambiado recientemente, de modo que el análisis de sangre y orina serán remplazados por el screening (barrido) y análisis de fluido oral. Las ventajas de esta matriz es que la toma de la muestra es fácil, no invasiva y hay una menor posibilidad de adulterar o sustituir la muestra. Las glándulas salivales tienen un alto flujo de sangre. Se ha sugerido que la

concentración de varias drogas en el fluido oral se correlaciona bien con sus concentraciones en sangre, lo que indica que el análisis de esta matriz permitiría una estimación del grado de deterioro y efecto de la droga sobre en consumidor en el momento de la toma de la muestra. Las concentraciones de droga en fluido oral reflejan la droga madre libre y los metabolitos lipofílicos. Debido a que hay formas de la droga que cruzan la barrera de la sangre-cerero y que afectan al rendimiento y el comportamiento de la persona, el fluido oral es un espécimen para detectar el efecto de la droga cuando se conduce. Sin embargo, el volumen de fluido oral es normalmente bajo (1 mL o incluso menos).

La sección de toxicología del INCC ya tenía algo de experiencia en el campo del análisis de drogas en fluido oral empleando procedimientos de preparación de la muestra off-line (2-5). Nuestro Instituto ha participado al concurso externo de control de calidad para el análisis de drogas en fluido oral, ORALVEQ (6). Durante la ronda del 2007 y 2008 de este programa, el método off-line empleado habitualmente en nuestro laboratorio y el método en desarrollo on-line fueron aplicados ambos a las muestras. Se obtuvieron resultados bastante comparables con ambos métodos demostrando que el SPE-LC-MS/MS on-line es una buena alternativa para el análisis de drogas de abuso en fluido oral. Solamente se encontró una ligera diferencia en las concentraciones obtenidas para la cocaína. Una explicación posible es que la cocaína es relativamente inestable (7). Respecto a la estabilidad durante el proceso SPE, una de las ventajas de la extracción on-line es que la elución del SPE va directamente a la columna analítica, por lo que hay menor riesgo de degradación de los compuestos durante la preparación de la muestra.

Por lo tanto, la próxima etapa será el desarrollo y validación de un método on-line SPE-LC-MS/MS para el análisis de drogas de abuso en fluido oral, incluyendo MDMA, MDA, anfetamina, metanfetamina, cocaína, benzoyleconina y THC, empleando solo 250 µL de muestra. Como en el sistema SPE on-line todo está automatizado estos compuestos podrán ser extraídos y analizados en el mismo análisis, obteniendo la mayor sensibilidad con la mínima preparación de la muestra (solamente la dilución del fluido oral directamente el los viales LC).

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VIII. ANNEXES

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ORIGINAL PAPER

Detection of diazepam in urine, hair and preserved oral fluid samples with LC-MS-MS after single and repeated administration of Myolastan® and Valium®

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Abstract Sedative agents are used to facilitate sexual assault due to their ability to render the victim passive, submissive and unable to resist. The primary pharmacological effect of the benzodiazepine tetrazepam is muscle relaxation, whereas the benzodiazepine diazepam acts on the central nervous system (CNS) exerting mainly sedation effects. Therefore, contrary to tetrazepam, diazepam is an often-abused drug, which can potentially be used as a date-rape drug. In this study, we describe the detection of low amounts of diazepam in Myolastan® (Sanofi–Synthelabo S.A., Brussels, Belgium) and Epsipam® (Will-Pharma, Wavre, Belgium) 50mg tablet preparations by LC-MS-MS, GC-FID and HPLC-DAD. Considering the important forensic implication of this finding, a study was conducted with volunteers receiving a single or repeated dosage of Myolastan®. Urine, hair and preserved oral fluid samples were analysed using a previously described sensitive and specific LC-MS-MS detection method allowing for the simultaneous quantification of tetrazepam, diazepam, nordiazepam, oxazepam and temazepam. This study demonstrates that diazepam can be observed in urine samples even after a single dose of Myolastan®. In addition, maintaining therapy for 1 week results in the detection of both diazepam and nordiazepam in urine samples and of diazepam in the first hair segment. Importantly, comparing urine and hair samples after a single intake of diazepam versus the single and 1 week administration of Myolastan® shows that the possible metabolic conversion of tetrazepam to diazepam is a more plausible explanation for the detection of diazepam in biological samples after the intake of Myolastan®. As such, these results reveal that the presence of diazepam and/or nordiazepam in biological samples from alleged drug-facilitated assault cases should be interpreted with care.

Keywords LC-MS-MS · Tetrazepam · Diazepam · Urine · Hair · Oral fluid

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Introduction

In the past few years, an increase in the number of scientific publications on so-called date-rape drugs, drug-facilitated sexual assault (DFSA) and drug-facilitated crimes (DFC) has been observed [1–8]. The most renowned drug associated with sexual assault is Rohypnol® (flunitrazepam) [9]. However, many other drugs have been implicated in these crimes, including other benzodiazepines, GHB (γ-hydroxybutyric acid) and ketamine. These drugs are all characterized by having depressant effects on their users, resulting in confusion, drowsiness, impaired memory and reduced inhibition. In addition, they induce amnesia, presumably the principal reason for their selection as date-rape drugs. During a 3-year surveillance in the UK, the toxicology results from

1,014 cases of claimed DFSA showed a relatively high number of cases which contained benzodiazepines (9%), with diazepam being the most prevalent benzodiazepine [10].

However, several difficulties in the diagnosis of DFC are apparent. These crimes are often difficult to prove due to factors such as the low concentrations of drugs used or their rapid clearance from the body. In addition, many victims of DFC do not report an incident until several days later, often due to the amnesia caused by the drug. Hence, conventional specimens from alleged victims such as blood or urine may have limited value. Recently, hair samples have been successfully used to document cases of DFC [4, 8, 11–14]. Kintz and co-workers concluded that due to the extremely low concentrations of drugs typically encountered in hair analysis (in the lower ranges of picograms per milligram) the "sensitivity of LC-MS-MS appears to be a pre-requisite to document any case involving drug-facilitated sexual assault" [4]. However, they also added the caveat that hair analysis should not simply be considered as an alternative to blood and urine testing but as a complementary technique where possible.

Furthermore, the use of preserved oral fluid samples to document DFC has recently been reported [15–17]. Indeed, preserved oral fluid offers a series of advantages over traditional specimens since it is non-invasive, with an easy collection protocol and can be considered as an additional source of information. It has been suggested that the concentrations of many drugs in oral fluid correlate well with their concentrations in blood, which suggests that the analysis of the former matrix may show value in the determination of the current degree of exposure to a particular drug at the time of sampling [18].

Recently, we presented a validated and highly sensitive LC-ESI-MS-MS method for the quantification of 26 commonly encountered benzodiazepines and their metabolites, zolpidem and zopiclone in three different biological matrices, i.e. blood, urine and hair [13]. During the routine application of this method on a hair sample from a regular Myolastan® user, diazepam and traces of nordiazepam were also detected in several segments (concentrations ranging from 1.0 to 8.2 pg mg^{-1} for diazepam). Myolastan® is a pharmaceutical specialty available on the Belgian market (Sanofi–Synthelabo, Brussels) which contains the benzodiazepine tetrazepam. Whereas tetrazepam is only used for its muscle-relaxant properties, diazepam (and its metabolite nordiazepam) also exerts strong hypnotic and sedative activities. As such, diazepam also appears on the list of alleged date-rape drugs [9]. In view of the possible erratic conclusions regarding DFC in victims taking therapeutic doses of Myolastan®, we evaluated the diazepam and tetrazepam concentrations in urine, hair and preserved oral fluid after the intake of a single or sustained therapeutic dose of Myolastan® and compared these results with those obtained after a single administration of diazepam (Valium®, Roche, Brussels).

Experimental

Specimens

Six healthy subjects (4 females and 2 males, aged 27–38 years, with a body weight varying between 64 and 93 kg) received either 50 mg tetrazepam in the form of one tablet of Myolastan® (Sanofi–Synthelabo S.A., Brussels, Belgium) or 10 mg diazepam in the form of one tablet of Valium® (Roche, Brussels, Belgium) along with 100 mL of water. In addition, one healthy female (aged 27 years, 64 kg) received a single dose of 25 mg tetrazepam in the form of half a tablet of Myolastan® each day, during a 1-week therapy. Subjects participated in the experimental part of the study through written informed consent. Urine was collected over 280 h in plastic tubes, without any preservative, and frozen at −20 °C until analysis (performed within 15 days). Preserved oral fluid was collected at regular time intervals during 600 min following administration, using the Intercept® device (OraSure Technologies, Bethlehem, PA) according to the manufacturer's instructions. The device collects an average of 0.38±0.19 mL of oral fluid and a dilution factor of 1 in 3 is arbitrarily accepted [19]. All devices were weighed before and after collection. An average weight of 0.86±0.14 g of oral fluid was collected. The tubes were centrifuged and the preserved oral fluid was stored at −20 °C prior to analysis (within 15 days). Blank preserved oral fluid, used for the preparation of negative controls, calibrators and quality control (QC) samples, was obtained from healthy volunteers (also collected with the Intercept®). The data are expressed as nanograms per milliliter of diluted specimen.

A lock of hair the size of the diameter of a pencil (about 100 mg) was collected from the posterior vertex of the head 3 weeks after the last dose of Myolastan®/Valium®. Hair was clipped as close to the scalp as possible and stored at room temperature. Hair strands were aligned to keep root ends together.

Chemicals

Individual ampoules of 7-aminoclonazepam, 7-aminoflunitrazepam, bromazepam, clonazepam, flunitrazepam, clobazam, desmethylflunitrazepam, estazolam, nitrazepam, alprazolam, temazepam, desalkylflurazepam, oxazepam, nordiazepam, triazolam, lormetazepam, lorazepam, prazepam, tetrazepam, diazepam and flurazepam (at a concentration of 1 mg mL^{-1} in methanol) and 7-aminoclonazepam-d4,

7-aminoflunitrazepam-d7, clonazepam-d4, flunitrazepam-d7, alprazolam-d5, temazepam-d5, desalkylflurazepam-d4, oxazepam-d5, nordiazepam-d5, triazolam-d4, lorazepam-d4, prazepam-d5 and diazepam-d5 (0.1 mg mL^{-1} in methanol) were purchased from LGC Promochem (Molsheim, France). Brotizolam, clotiazepam, chlornordiazepam, loprazolam, cloxazolam, zolpidem and zopiclone were a gift from Dr. V. Maes (VUB, Brussels) (pure standards obtained from respective manufacturers).

Methanol (Biosolve, Valkenswaard, The Netherlands), water (Biosolve), dicholoromethane (Merck, Darmstadt, Germany) and 1-chlorobutane (Merck) were HPLC grade. Acetic acid (glacial) 100% anhydrous (Merck) and NaOH (Fluka, Buchs, Germany) were used for the preparation of the acetate buffer 3 M, pH 4.6. For the saturated ammonium chloride buffer pH 9.2, ammonium chloride from Fluka and ammonia solution 32% from Merck were used. β-Glucuronidase, Type HP-2 from *Helix pomatia* (111,480 units mL^{-1}) was obtained from Sigma (Steinheim, Germany). For the mobile phase of the HPLC-DAD method, phosphoric acid, 85% wt % solution in water (Aldrich, Steinheim, Germany) and potassium dihydrogen phosphate (Merck) were used.

Preparation of standard solutions

An internal standard (IS) working solution of the deuterated analytes was prepared (0.4 µg mL^{-1} in methanol), which was further diluted with methanol to yield appropriate concentrations to add to samples, calibrators and QC samples.

A benzodiazepine working solution of all analytes was prepared (4 µg mL^{-1} in methanol) and further diluted with methanol to yield working solutions at appropriate concentrations to add to calibrators and QC samples. Separate stock solutions of tetrazepam, diazepam, nordiazepam, oxazepam and temazepam were prepared (4 µg mL^{-1} in methanol) and further diluted with methanol for the stability and ion suppression experiments.

Working solutions were prepared monthly and stored at 4 °C.

Sample preparation and extraction for LC-MS-MS analysis

Sample preparation

Urine and hair samples were analysed by LC-MS-MS for 26 benzodiazepines and metabolites, zolpidem and zopiclone according to a published validated procedure [13]. Briefly, urine samples (250 µL) mixed with 50 µL of the IS stock solution were buffered to pH 4.6 with 200 µL acetate buffer (3 M) and then incubated for 1 h at 56 °C with 25 µL of β-glucuronidase.

Hair samples were decontaminated, dried and cut in segments of 1–3 cm each. Approximately 20 mg was powdered in a ball mill which allowed simultaneous pulverization of 48 segments (Precellys 48, Bertin Technologies, Montigny-Le-Bretonneux, France) and then 50 µL of a 1:20 dilution of the IS stock solution was added. After incubation of the pulverized samples with 1 mL of methanol at 45 °C for 2 h with orbital shaking, samples were centrifuged. The supernatants were subsequently transferred to 10-mL disposable screw top vials and concentrated under nitrogen to 100–200 µL.

Preserved oral fluid samples (500 µL) were mixed with 50 µL of a 1:20 dilution of the IS stock solution.

Extraction

Following sample preparation, samples were extracted with 4 mL of 1-chlorobutane after the addition of a saturated ammonium chloride buffer (pH 9.2); 500 µL was used for urine and preserved oral fluid samples and 1 mL was used for the pulverized hair samples. After mechanical shaking (10 min) and centrifugation (10 min at 3,000 *g*), the organic phase was transferred to a 5-mL disposable screw top vial and then evaporated to dryness at 40 °C in a vacuum centrifuge. For the extracted urine samples, the residue was reconstituted in 100 µL of 0.1% formic acid in water/methanol (70:30, v/v) and 10 µL was injected into the LC-MS-MS system. The residue of the preserved oral fluid and hair samples was reconstituted in 80 µL of 0.1% formic acid in water/methanol (70:30, v/v) and 20 µL was injected into the LC-MS/MS system. The limit of quantification (LOQ) in urine was 25 ng mL^{-1} for tetrazepam and 10 ng mL^{-1} for diazepam, nordiazepam, oxazepam and temazepam. For hair samples, the LOQ was established at 10 pg mg^{-1} for tetrazepam and 1 pg mg^{-1} for diazepam, nordiazepam, oxazepam and temazepam.

LC-MS-MS

Chromatography

LC was performed using a Waters Alliance 2690 separation module (Waters, Milford, MA, USA). Analytes were separated on a Gemini C18 column (150×2.0 mm, 3.5 µm) (Phenomenex, Torrance, CA), using a gradient elution with 0.1% formic acid (A) and methanol (B), at a flow rate of 0.2 mL min^{-1}. A gradient was applied starting from 10% B, and increased to 50% over the first 5 min. From 5 min to 20 min, B was linearly increased to 70% before returning to the initial conditions within 0.1 min and equilibrating for 14.9 min, which resulted in a total run time of 35 min.

Mass spectrometry

A Quattro Premier tandem mass spectrometer (Waters) was used for all analyses. Ionization was achieved using electrospray in positive mode (ESI+). The optimum conditions were capillary voltage, 1.0 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 270 °C and delivered at a flow rate of 700 L h^{-1}.

The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mbar) and the collision energy (eV) adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis. Quantification and a confirmation transition were selected for each compound, except for the deuterated analogs, for which only one transition was chosen. The optimized MRM transitions for diazepam, nordiazepam, oxazepam, temazepam, tetrazepam and the internal standards, diazepam-d5, nordiazepam-d5, oxazepam-d5 and temazepam-d5, are presented in Table 1. All aspects of data acquisition were controlled using MassLynx NT 4.1 software with automated data processing using the TargetLynx program (Waters).

LC-MS-MS assay validation for preserved oral fluid samples

Linearity, LOQ, precision, accuracy and recovery

The method was validated for the detection of diazepam, nordiazepam, oxazepam, temazepam and tetrazepam in preserved oral fluid samples. Selectivity was evaluated by analysing preserved oral fluid samples from eight healthy volunteers who did not take any of the targeted compounds for several days before preserved oral fluid sampling and checked for the absence of the compounds of interest by analysing the samples with the present technique before using them as blanks. Assay linearity was investigated by constructing calibration curves (n=5) using blank preserved oral fluid spiked with the analytes at concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10, 20 and 40 ng mL^{-1}. Quantification was achieved by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. Standard response curves were generated daily using a weighted ($1/x$) least-squares linear regression model.

The LOQ was defined as the concentration of the lowest calibrator which was calculated to be within ±20% of the nominal value and with a RSD (relative standard deviation) less than 20% [20, 21].

QCs were prepared for every run in blank matrix at a concentration of 0.75 ng mL^{-1} (low), 4 ng mL^{-1} (medium) and 15 ng mL^{-1} (high). Intra-assay precision was evaluated by analysis of five replicates of each QC in one run. Inter-assay precision was evaluated by replicate analysis of one set of QC samples in several experiments performed on five different days. The precision was expressed as the %RSD. A comparison of the calculated concentrations of the QC samples to their respective nominal values was used to assess the accuracy (bias) of the method.

Relative recoveries were estimated by comparing the ratio of the peak area of the low, medium and high QC samples when the non-deuterated compounds were added before extraction (n=3) divided by the peak area of the internal standards with the ratio of the peak area obtained when the non-deuterated analytes were added after the extraction (n=3) divided by the peak area of the IS. The deuterated standards were added before the extraction in all experiments.

Table 1 MRM transitions and conditions for tested benzodiazepines and the deuterated analogues

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Temazepam	300.90	255.00[a]	28	20
		283.00	28	15
Oxazepam	287.00	241.00[a]	27	22
		269.00	27	15
Nordiazepam	271.00	139.80[a]	43	28
		164.80	43	28
Tetrazepam	289.00	225.10[a]	45	28
		253.10	45	25
Diazepam	285.00	153.80[a]	40	28
		193.00	40	30
Temazepam-d5	305.90	260.10	25	23
Oxazepam-d5	292.00	246.10	40	23
Nordiazepam-d5	276.00	139.80	45	28
Diazepam-d5	290.00	153.80	40	28

[a] Transitions used for quantification

Stability of unprepared and prepared samples

Stability of tetrazepam, diazepam, nordiazepam, oxazepam and temazepam was monitored in non-extracted preserved oral fluid samples spiked at the initial concentrations of 0.75 and 15 ng mL^{-1}. Concentrations of the drugs in the samples were either determined (each in triplicate) immediately (control samples) or following incubation at room temperature for 24 h or at 4 °C for a period of 24 h, 48 h and 72 h after preparation. In addition, the stability of the same preserved oral fluid samples was evaluated after 2 weeks of incubation at −20 °C. Stability at each time point was tested against a lower acceptance limit corresponding to 90% of the mean of control samples by a one-sided *t*-test ($P<0.05$).

The stability of tetrazepam, diazepam, nordiazepam, oxazepam and temazepam in the extracted sample (preserved oral fluid initially spiked at 0.75 ng mL^{-1} and 15 ng mL^{-1}) was investigated by repeated injections of a mixture of six extracted samples (maintained in the autosampler at 4 °C) over a period of 24 h. Absolute peak areas were plotted as a function of injection time and the stability of the processed samples tested by regression analysis. Instability of the processed samples would be indicated by a slope that was significantly different from zero ($P<0.05$).

Assessment of matrix effects

To assess any potential suppression or enhancement of ionization from components present in the extracted biological matrix, a continuous post-column infusion was performed using a separate solution of tetrazepam, diazepam, nordiazepam, oxazepam and temazepam (10 ng mL^{-1} at a flow rate of 10 µL min^{-1}) to produce a constant elevated response in both MRM channels. The interference of this constant response was monitored following the injection of extracted samples and compared to the response following the injection of mobile phase only.

Quantification of creatinine

The concentration of creatinine in urine was determined using a Vitros 5,1 FS Chemistry System (Ortho-Clinical Diagnostics, Beerse, Belgium) and corresponding reagents according to the manufacturer's recommendations.

GC-FID

One tablet of Myolastan® was pulverized, weighed and dissolved in 10 mL of methanol. Tribenzylamine was added as internal standard at a concentration of 0.5 mg mL^{-1}. Samples were sonicated for 15 min and filtered using a Puradisc™ 25 NYL filter (0.45-µm pore size) (Whatman, Clifton, NJ), after which a 1-µL aliquot was injected into the chromatographic system for analysis. A Hewlett–Packard 6890 Series GC System was used furnished with a HP5-MS column (25 m, 0.32-mm i.d, 0.52-µm film thickness) (J&W Scientific, Folsom, CA) and a flame ionization detector at 300 °C. The injector was operated at 250 °C in the split mode, using a split ratio of 25:1. Helium was used as the carrier gas. The oven temperature programme was as follows: 150 °C (hold 1 min), 15 °C min^{-1} ramp to 300 °C (hold 3 min). The total running time was 14 min. For quantification, calibration curves with eight calibrators of diazepam and tetrazepam were prepared for each batch ranging from 2.88 µg mL^{-1} to 1.36 mg mL^{-1}. Internal QC samples were analysed with each batch of samples.

HPLC-DAD

One tablet of Myolastan® was dissolved in 10 mL of methanol. After addition of the internal standard (clobazam) and 500 µL of a saturated ammonium chloride buffer (pH 9.2), a 500-µL aliquot of this solution was extracted with 4 mL of 1-chlorobutane. Analyses were performed using a Hewlett–Packard HP 1100 Series HPLC (Agilent Technologies). Analytes were separated on a Lichrospher RP8ec column (250×4.0 mm, 5 µm) (Merck, Darmstadt, Germany), eluted isocratically with 100 mM phosphate buffer pH 2.3/acetonitrile (63:37, v/v), delivered at a flow rate of 1 mL min^{-1} with a run time of 30 min. Systematic toxicological analysis was performed using a UV spectra library [22]. Wavelength calibration and accuracy checks of the DAD were performed regularly.

Results

Validation of the analytical method for preserved oral fluid samples

The selectivity of the method was acceptable in terms of absence of interferences in the blank preserved oral fluid samples analyzed. The combination of retention time and two transitions (and their relative abundances) provided high specificity for all of the compounds [13]. The deuterated IS selected for each compound is shown in Table 2. No cross-talk interference with the deuterated IS was observed.

Calibration curves for all compounds tested were prepared in blank preserved oral fluid for each batch and ranged from the LOQ to 40 ng mL^{-1}. Linearity, LOQ, intra-assay and inter-assay precision and bias are summarized in Tables 2 and 3. The observed LOQ, ranging from 0.05 to 0.2 ng mL^{-1}, is consistent with previous reports [23, 24]. % RSD at the low (0.75 ng mL^{-1}), medium (4 ng mL^{-1}) and

Table 2 Equation of a typical calibration curve with coefficient of determination (r^2), and the estimated limits of quantification (LOQ) of the method for preserved oral fluid

Compound	IS	Equation	r^2	LOQ (ng mL^{-1})
Temazepam	Temazepam-d5	$y = 1.1626x - 0.0682$	0.9990	0.20
Oxazepam	Oxazepam-d5	$y = 1.5647x - 0.0317$	0.9988	0.05
Nordiazepam	Nordiazepam-d5	$y = 1.1832x + 0.0221$	0.9981	0.05
Tetrazepam	Diazepam-d5	$y = 0.8676x - 0.1062$	0.9989	0.20
Diazepam	Diazepam-d5	$y = 1.2846x - 0.0366$	0.9992	0.05

high (15 ng mL^{-1}) QC level were consistently below 15%. The extraction recovery of the analytes is presented in Table 4.

The stability of non-extracted spiked samples (0.75 and 15 ng mL^{-1}) was monitored at room temperature for 24 h or at 4 °C for a period of 24 h, 48 h and 72 h. No statistically significant differences could be observed for the two different concentrations under both conditions. Also no statistical differences could be noted for the stability of non-extracted spiked samples (0.75 and 15 ng mL^{-1}) during 2 weeks at −20 °C.

The potential for instability of the processed samples was also tested. To this end, the stability of the compounds was monitored by means of repeated injections of extracted samples (0.75 ng mL^{-1} and 15 ng mL^{-1}) over a period of 24 h, and by plotting the absolute peak areas as a function of time. The results indicated no significant instability over the course of the experiment.

Post-column infusion experiments (based on the method described by Bonfiglio et al. [25]) indicated no significant ion suppression or enhancement during the chromatographic run.

Analysis of the pharmaceutical specialty Myolastan®

To assess the origin of the diazepam and nordiazepam present in hair samples from a regular Myolastan® user, it was decided to initially analyse one tablet of the pharmaceutical specialty Myolastan® (50 mg tetrazepam). The presence of diazepam in this tablet was confirmed in two different lots by various techniques (LC-MS-MS, HPLC-DAD and GC-FID). Quantification with GC-FID showed a relative concentration of around 0.068% and 0.061% of the active ingredient for two different lots of Myolastan®.

Since tetrazepam is available as Myolastan® or Epsipam® 50 mg (Will–Pharma, Wavre, Belgium) tablets, an analysis of the latter was also performed. Diazepam was also present in this preparation at a relative concentration of 0.37% of the active ingredient.

Analysis of urine samples after the controlled administration of Myolastan® and Valium®

Urine samples were analyzed according to a previously published procedure [13]. In this report, a reduced stability for tetrazepam was observed during the enzymatic hydrolysis of the urine samples. Indeed, an experiment with spiked urine samples (n=3) showed a reduction of 94.3±0.3% and 96.8±0.2% for QCs at 150 ng mL^{-1} and 600 ng mL^{-1} respectively, when incubated at 56 °C for 1 h. However, no statistically significant difference was noted for diazepam, nordiazepam, oxazepam and temazepam under these conditions. Therefore, we decided to analyze all urine samples twice: once without enzymatic hydrolysis in order to have the optimal conditions for tetrazepam detection, and once using the described protocol for the quantification of diazepam, nordiazepam, oxazepam and temazepam. A limited validation including linearity, LOQ, precision and accuracy was performed (n=5). The obtained r^2 was in all cases >0.998 with an LOQ of 10 ng mL^{-1}. The %RSD and bias were

Table 3 Intra-assay and inter-assay precision (expressed as %RSD) and bias (%) of the QC samples spiked at a concentration of 0.75 ng mL^{-1} (low), 4.0 ng mL^{-1} (medium) and 15.0 ng mL^{-1} (high) in preserved oral fluid

Compound	Intra-assay precision (n=5)			Inter-assay precision (n=5)			Bias		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Temazepam	8.3	3.4	1.4	7.7	8.3	3.1	−12.9	−10.0	−1.7
Oxazepam	8.6	3.3	2.4	5.1	7.6	5.5	−9.3	−7.8	−2.6
Nordiazepam	5.7	2.7	5.1	12.2	7.6	6.3	13.9	9.6	−1.8
Tetrazepam	8.3	3.4	3.8	5.6	4.6	6.1	10.1	7.2	−5.1
Diazepam	7.9	2.3	2.3	7.7	7.0	4.6	−12.9	−7.3	−3.0

Table 4 Extraction recovery (%) of tested benzodiazepines for the QC samples spiked at a concentration of 0.75 ng mL^{-1} (low), 4.0 ng mL^{-1} (medium) and 15.0 ng mL^{-1} (high) in preserved oral fluid

Compound	Relative recovery (mean±1 SD)			
	Low	Medium	High	Average[a]
Temazepam	86.4±4.7	87.1±12.0	86.2±15.0	86.6±10.2
Oxazepam	72.6±9.3	92.8±15.1	88.3±6.0	84.6±9.3
Nordiazepam	88.3±8.8	95.1±8.5	94.0±1.3	92.5±6.6
Tetrazepam	80.8±7.8	78.9±6.5	79.8±12.3	79.8±8.4
Diazepam	81.0±8.1	87.2±9.3	87.7±13.1	85.3±9.9

[a] This column shows the average recovery (%) of all three concentration ranges

evaluated at 40, 150 and 600 ng mL^{-1} and were consistently <15%.

After oral administration of a single tablet of Myolastan® (50 mg tetrazepam) to three subjects, 17 consecutive specimens of urine were collected over 280 h (Fig. 1a). Benzodiazepine concentrations of all urine samples were interpreted with respect to the creatinine content to compensate for the diuretical state. Tetrazepam was present in all urine samples, with the highest concentration on average after 12 h. After 280 h, urine samples were still positive for tetrazepam at a LOQ of 10 ng mL^{-1}. In addition, diazepam could be detected in the first sample of two persons and was observed up to 36 h after intake. However, no metabolites of diazepam were noted in these samples. In order to rule out a possible formation of diazepam during the extraction process, blank urine samples, spiked with 600 ng mL^{-1} tetrazepam were analyzed following the described method. No diazepam was detected in these samples.

A typical therapy with tetrazepam includes a daily administration of 25–150 mg for 1 week. Therefore, half a tablet of Myolastan® (25 mg tetrazepam) was administered to one subject during 1 week. Urine specimens were collected for 280 h after the first intake and quantified with respect to creatinine concentrations in these samples (Fig. 1b). Diazepam was detected in these samples up to 210 h and a peak concentration was seen after 114 h. Furthermore, nordiazepam was additionally detected between 66 and 138 h. Further metabolites of diazepam could not be detected.

The concentrations detected in urine samples after single and repeated administration of Myolastan® were compared with those obtained after a single dose of Valium® (10 mg diazepam) to three persons (Fig. 1c). Traces of diazepam could only be detected in the first urine sample (8 h after intake) from one person. In the following samples from this person and all of the samples of the two other persons, only the metabolites of diazepam, i.e. nordiazepam, oxazepam and temazepam, were detected. Mean urine concentrations showed a large variation throughout the whole collection period. Whereas temazepam and oxazepam were alternately detected as the most abundant metabolite, nordiazepam was the minor metabolite in all urine samples analyzed.

Analysis of preserved oral fluid samples after the controlled administration of Myolastan® and Valium®

After administration of one tablet of Myolastan® (50 mg tetrazepam) to three volunteers, 20 specimens of oral fluid were collected from each subject with the Intercept® collection device (Fig. 2a). Tetrazepam was detectable 15 min after intake in all volunteers. Peak concentrations of tetrazepam were obtained after 105 min. Samples were still positive after 600 min. However, no diazepam or metabolites were detected in these samples.

The same study was conducted including three persons receiving one tablet of Valium® (10 mg diazepam) (Fig. 2b). Peak concentrations were noted in all subjects after 15 min, rapidly decreasing at the second time point (30 min), indicating a possible contamination of the oral cavity. A second peak was observed after 120 min. Oral fluid samples tested positive for diazepam over 600 min. The metabolites of diazepam could not be detected in these samples.

Analysis of hair samples after the controlled administration of Myolastan® and Valium®

The corresponding hair samples after the administration of a single dose or 1-week therapy with Myolastan® and a single dose of Valium® were collected 3 weeks after the last intake. After segmentation, the first three proximal segments were analyzed. Following a single dose of Myolastan®, the first segments of the three volunteers were positive for tetrazepam (17.3–59.7 pg mg^{-1}). No diazepam or metabolites could be detected in any of the segments tested. After 1 week of therapy with the same drug, tetrazepam was detected in the first (454.0 pg mg^{-1}) and second (13.0 pg mg^{-1}) segment (Fig. 3). In addition, the first segment of this person was also positive for diazepam (10.7 pg mg^{-1}). No nordiazepam was observed in any of the segments. After a single dose of

a

0.25
0.2
0.15
0.1
0.05
0

concentration (mg/g creatinine)

0 50 100 150 200 250 300

time after intake (hours)

Diazepam Tetrazepam

b

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

concentration (mg/g creatinine)

0 50 100 150 200 250 300

time after intake (hours)

Nordiazepam Diazepam Tetrazepam

c

0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0

concentration (mg/g creatinine)

0 50 100 150 200 250 300

time after intake (hours)

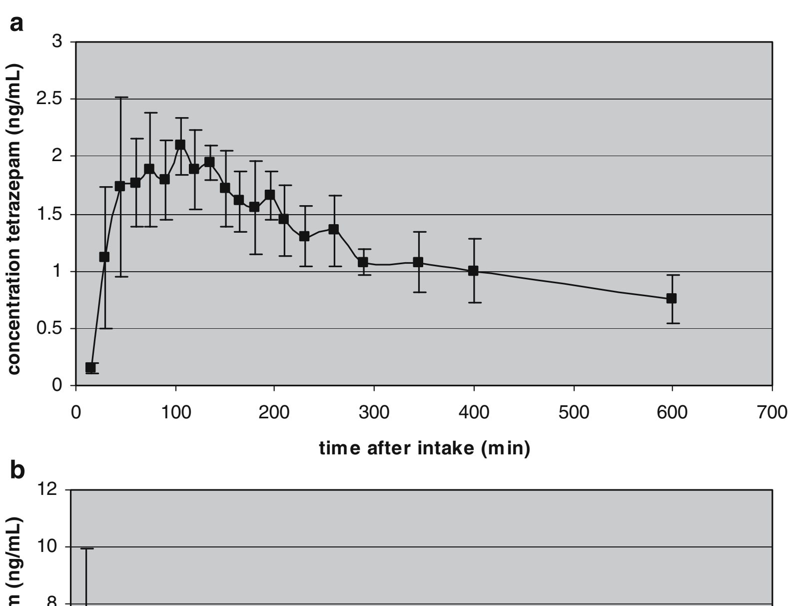
Temazepam Oxazepam Nordiazepam

◀ **Fig. 1** Mean excretion pattern in urine **a** after a single Myolastan® dose (50 mg tetrazepam, 3 subjects); **b** during and after a 1-week therapy with Myolastan® (25 mg tetrazepam, 1 subject); and **c** after a single Valium® dose (10 mg diazepam, 3 subjects). *Error bars* represent 1 SD

Valium®, the first and second segments were positive for diazepam (2.3–6.0 pg mg^{-1}) and nordiazepam (traces–5.4 pg mg^{-1}). The third segment of all volunteers was negative for all drugs tested.

Discussion

Whereas tetrazepam is a medication with primarily muscle-relaxant effects and is commonly prescribed for the treatment of muscle spasms, diazepam acts on the CNS with mainly sedation effects. Therefore, diazepam belongs to the class of abused drugs and shows a potential to be used as a date-rape drug [10]. The detection of the latter in all hair segments from a regular Myolastan® user, who denied the consumption of diazepam at any time in her life, led us to analyze the presence of diazepam in the pharmaceutical specialty Myolastan®. Confirmation of the presence of low quantities of diazepam in different lots of this preparation was obtained through GC-FID, LC-MS-MS and HPLC-DAD. Owing to the important forensic implication of this finding, a small-scale study with single and/or repeated administration of Myolastan® and Valium® was designed. Urine and hair samples were analyzed according to a previously published method [13]. In the present paper, the same liquid–liquid extraction protocol and LC-MS-MS method have been applied for the detection of diazepam, nordiazepam, oxazepam and tetrazepam in oral fluid samples, collected with the Intercept® device. The method



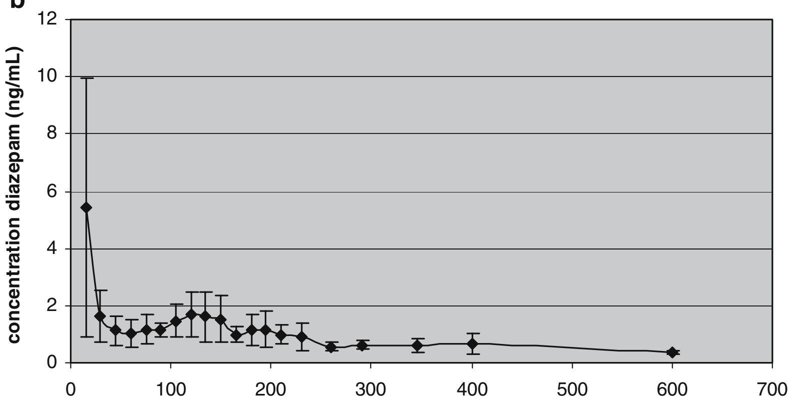


Fig. 2 Mean excretion pattern in preserved oral fluid **a** after a single Myolastan® dose (50 mg tetrazepam, 3 subjects); and **b** following a single Valium® dose (10 mg diazepam, 3 subjects). *Error bars* represent 1 SD

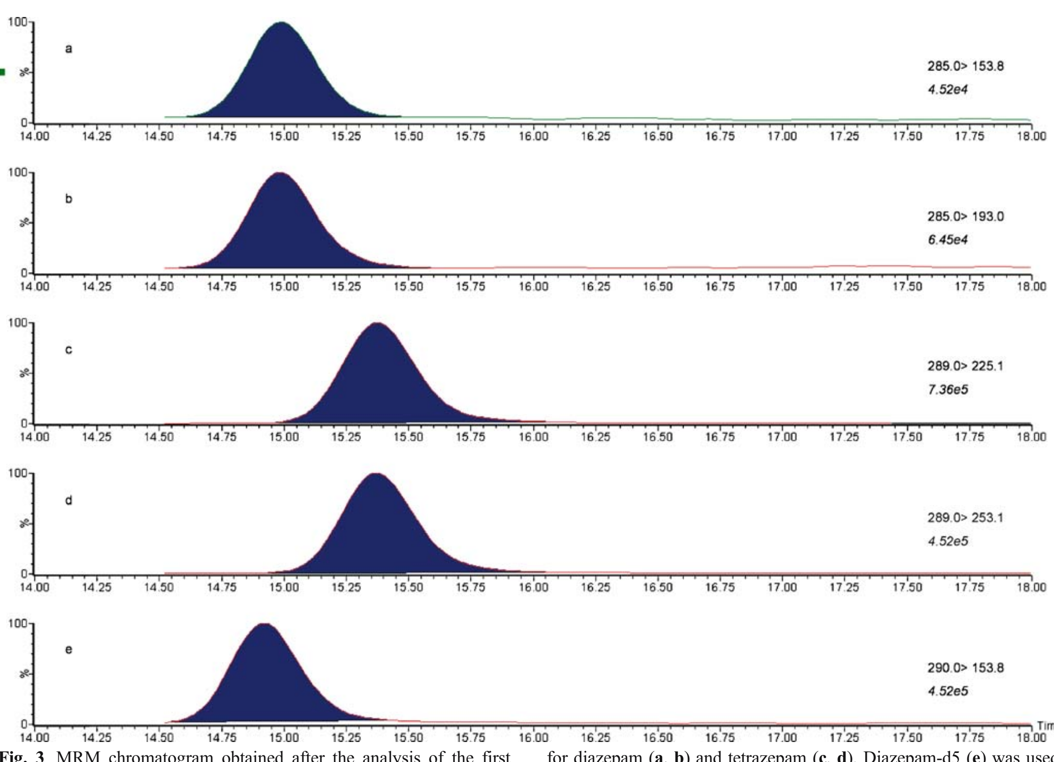


Fig. 3 MRM chromatogram obtained after the analysis of the first hair segment following a 1-week therapy with Myolastan® (25 mg tetrazepam). The figure shows the response (quantifier and qualifier) for diazepam (**a**, **b**) and tetrazepam (**c**, **d**). Diazepam-d5 (**e**) was used as internal standard. Peak intensity is shown in the *top right-hand corner* of each trace

was fully validated and the high sensitivity of the method permits the detection of very low concentrations of drugs in these samples.

Due to the instability of tetrazepam in urine samples during the enzymatic hydrolyzation process, we decided to analyze the urine specimens with and without a hydrolysis step. Indeed, whereas diazepam is extensively metabolized to oxazepam glucuronide, conjugated nordiazepam and conjugated temazepam, it appears that tetrazepam is not excreted in the glucuronidated form [26]. Thus, a hydrolysis step would not yield a higher concentration for tetrazepam.

After the administration of a single dose of one tablet of Myolastan® (3 subjects), the presence of diazepam in urine was observed for up to 36 h after the intake. Following a 1-week therapy with half a tablet of this preparation, both diazepam and nordiazepam were present at 8–210 h and 66–138 h, respectively, after the ingestion of the first dose. These results were compared with those obtained after a single dose of Valium®. However, in this case, only the metabolites of the parent drug, i.e. nordiazepam, oxazepam and temazepam, could be detected in these urine samples, indicating a complete metabolization of diazepam.

In contrast to an earlier report [27], diazepam could be detected in oral fluid samples throughout the whole collection period after the administration of a single dose of Valium®. However, the analysis of the preserved oral fluid samples showed the presence of only the parent benzodiazepines after a single dose of Myolastan® or Valium®. Similar concentrations of tetrazepam in oral fluid were observed in a previous study [15], taking into account a 1:3 dilution due to the use of the Intercept® collector. Peak concentrations were obtained after 105 min and 120 min for tetrazepam and diazepam, respectively, which is approximately the time of peak plasma concentration [26]. The absence of nordiazepam in the preserved oral fluid samples after the intake of Valium® can be explained by the fact that average peak concentrations of nordiazepam in blood are only reached 24 h following a single oral dose of 10 mg diazepam. In addition, approximately 97% of the nordiazepam in plasma is bound to proteins [26], consequently the concentrations in the preserved oral fluid samples are probably below the LOQ during the collection period. This is also likely to be the cause of the absence of diazepam in these samples after the administration of Myolastan®.

Finally, hair samples were also collected and analyzed. These results indicated that after a single dose of Myolastan®, no diazepam could be observed; however, the 1-week therapy resulted in a positive first segment for this compound. In addition, the observed concentration was higher than that observed following a single dose of Valium®. Under the latter condition, nordiazepam could also be detected. However, it is not clear why diazepam and nordiazepam could also be detected in the second segment of all persons after the intake of a single dose of Valium®. Hair strands were collected after 3 weeks of delay, in order for the region of drug incorporation to emerge above the scalp. Therefore, with an average growth of 1 cm $month^{-1}$, the administered drug should be present in the proximal segment, and not in the following segments. One possible interpretation is the variability in the incorporation of this drug into the hair shaft and axial migration after incorporation, leading to a further distribution, an observation already reported for bromazepam [7] and cocaine [28]. However, a decreased concentration would be expected in the second segment. Therefore, more research has to be done to study the incorporation of diazepam and nordiazepam in hair after controlled administration.

Another question remains as to whether or not the diazepam observed in the urine and hair samples after administration of Myolastan® is the result of the diazepam present in the pharmaceutical preparation. If this were true, diazepam should also experience complete metabolic conversion to nordiazepam, oxazepam and temazepam, as was observed after a single dose of Valium®. The presence of nordiazepam was however only noted after a repeated administration of Myolastan® with diazepam remaining the primary drug detected and the absence of other metabolites. In addition, after repeated administration of Myolastan®, only diazepam was detected in the first hair segment of this person, whereas after a single dose of Valium®, nordiazepam was also observed although lower concentrations of diazepam were present in comparison with the latter. Therefore, the results of this study may indicate that there is a metabolic conversion of tetrazepam to diazepam in the human body. In 1987, Maurer and Pfleger described the presence of the hydrolysis product of diazepam in urine samples collected after the ingestion of tetrazepam following acid hydrolysis [29]. Only one other recent report mentions the possibility of this metabolic conversion process [30]. In this report, plasma and urine were collected for 11 h and 72 h, respectively, after administration of 50 mg of tetrazepam. Nordiazepam was not detected in any of the plasma samples and diazepam could be found in only one sample. In all cases, diazepam was also detected in the first sample. In contrast to our observations, these authors also detected nordiazepam between 4 and 26 h after a single tetrazepam intake. However, no other matrices were analyzed and no correlation has been made after a single diazepam intake. Benzodiazepines are metabolized in the liver by enzymes of the cytochrome P-450 family of oxidoreductases. Therefore, the metabolic conversion of tetrazepam could be explained by a double hydroxylation of the unsaturated tetrazepam ring followed by subsequent elimination of two water molecules and the formation of the stable aromatic ring, and hence diazepam. However, further research is needed to elucidate the complete conversion process of tetrazepam.

In conclusion, detection of the presence of diazepam and nordiazepam in biological samples from suspected DFSA cases could lead to erratic conclusions. However, this study showed that, following the intake of diazepam, only diazepam metabolites were observed in urine, whereas the single or repeated administration of Myolastan® led mainly to diazepam in these samples.

Conclusions

Sedative agents are used to facilitate sexual assault due to their ability to render the victim passive, submissive and unable to resist. Most of these substances possess anterograde amnesic properties and can rapidly impair an individual. Benzodiazepines and related hypnotics are frequently observed in these cases. Diazepam is regarded as having a significant potential as a date-rape drug, whereas tetrazepam has only a low sedative effect and is commonly prescribed as a muscle relaxant. Tetrazepam is available as Myolastan® and Epsipam® 50 mg tablets in Belgium. However, the presence of low doses of diazepam in these preparations, as confirmed by LC-MS-MS, GC-FID and HPLC-DAD, could be the reason for the presence of diazepam and nordiazepam in the hair sample from a regular Myolastan® user. In the present paper, a sensitive, specific and reproducible method for the detection and quantification of tetrazepam, diazepam, nordiazepam, oxazepam and temazepam in preserved oral fluid samples is described and applied to a study involving the administration of Myolastan® and Valium®.

Our study shows that diazepam can be observed in urine samples even after a single dose. In addition, a 1-week therapy resulted in the detection of both diazepam and nordiazepam in urine samples and of diazepam in the first hair segment. However, a comparison with urine and hair samples after a single diazepam dose showed that the possible metabolic conversion of tetrazepam to diazepam is a more plausible explanation for the detection of diazepam in biological samples after the intake of Myolastan®. Due to the high protein binding property of diazepam, however, this compound was not observed in preserved oral fluid samples. These results have an important forensic implication and, therefore, the presence of diazepam and/or

nordiazepam in biological samples from alleged DFSA cases should be interpreted with care.

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High-throughput on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry method for the simultaneous analysis of 14 antidepressants and their metabolites in plasma

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Abstract

A rapid, sensitive and fully automated on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) method was developed and validated for the direct analysis of 14 antidepressants and their metabolites in plasma. Integration of the sample extraction and LC separation into a single system permitted direct injection of the plasma without prior sample pre-treatment. The applied gradient ensured the elution of all the examined drugs within 14 min and produced chromatographic peaks of acceptable symmetry. The total process time was 20 min and only 50 μL of plasma was required. Selectivity of the method was achieved by a combination of retention time and two precursor-product ion transitions for the non-deuterated compounds. The use of SPE was demonstrated to be highly effective and led to significant decreases in the interferences present in the matrix. Extraction was found to be both reproducible and efficient with recoveries >99% for all the analytes. The method showed excellent intra-assay and inter-assay precision (relative standard deviation (RSD) and bias <20%) for quality control (QC) samples spiked at a concentration of 40, 200 and 800 μg/L and the $r^2 > 0.99$ over the range investigated (10–1000 μg/L). Limits of quantification (LOQs) were estimated to be 10 μg/L. Furthermore, the processed samples were demonstrated to be stable for at least 48 h, except for clomipramine and norclomipramine, where a slight negative trend was observed, but did not compromise the quantification. The method was subsequently applied to authentic samples previously screened by a routine HPLC method with diode array detection (DAD).
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Keywords: Antidepressant; Plasma; On-line SPE; LC–MS/MS

1. Introduction

Major depressive disorder (MDD) is a condition characterized by a prolonged depression of mood or by a marked loss of interest or pleasure. Depression has received increased attention owing to the growing recognition of its prevalence. For many years, the prevailing hypothesis has been that the condition is caused by (or associated with) a deficiency of the monoamines, notably noradrenaline and serotonin; current theories also acknowledge that other factors may be involved in the pathogenesis of depression.

Pharmacological treatment for depression has advanced greatly since the development of the first therapies in the 1950s, with the introduction of monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs) [1]. Since the late 1980s, a whole new generation of chemically and neuropharmacologically unrelated agents have been introduced which appear to be safer and better tolerated [2]. These include: selective serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNaRIs), noradrenergic and specific serotonergic antidepressants (NaSSAs) and noradrenaline reuptake inhibitors (NaRIs) [3].

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A shared characteristic to the majority of the antidepressant drugs is the high interindividual variability in the plasmatic concentrations. It can be explained partly due to the existence of polymorphism in the genes that encode some of the CYP450 isoenzymes implicated in their metabolization [4,5]. These genetic differences have been well characterized mainly in the case of CYP2D6 and CYP2C19, and some studies about the recommendations on individual dose adjustment regarding it have been carried out [6]. These clinical consequences are more dramatic in the case of TCA since they are characterized by having a narrow therapeutic window with risk of cardiac and CNS toxicity [7,8]. Thus, therapeutic drug monitoring (TDM) for this class of antidepressant agents has become widely used because of its efficacy, safety and cost-effectiveness, the novel antidepressants show a less predictable concentration-efficacy relationship [9–12], in combination with a relatively low toxicity. Therefore, several authors also support the benefit of TDM for these drugs in special situations, such as to monitor compliance in the case of those patients who do not respond to an apparently adequate dose to identify those individuals who have particularly slow or rapid drug clearance, during pharmacokinetic studies and when administered to special populations (i.e., elderly or people with organic diseases) [10,13–15]. Although, there are some discrepancies about the therapeutic range for antidepressants, mainly the new generations, proposed therapeutic concentrations have been determined. They can be found in the list published by Winek et al., as well as the one elaborated by some recognised organizations such as TIAFT (The International Association of Forensic Toxicologist) [16,17].

Several methods have been published for the determination of one or more antidepressants in different biological matrices such as plasma or urine for monitoring or toxicological purposes [18–32]. In these reports, the use of gas chromatography (GC) coupled to nitrogen–phosphorus (NPD) [18,19], flame ionization (FID) [20], electron-capture (ECD) [21] and mass spectrometry (MS) [21–23] detection have been described. Liquid–liquid or solid-phase extraction (SPE) have been used for sample clean-up followed, in some cases, by a derivatization step [21–24]. However, in most of these reports, HPLC has been used in conjunction with UV [25–27] or fluorescence (FL) detection [27,28], thus necessitating an appropriate derivatization step to increase the fluorescence capacities of the compounds of interest. More recently, LC–MS or LC–MS/MS have been applied [29–32]. These techniques provide a high selectivity and sensitivity in combination with a good precision and accuracy over a wide dynamic range, allowing the development of very rapid and efficient analytical methods. Therefore, in many cases it is now the sample pre-treatment process that has become the bottleneck in method development and sample analysis [33,34].

High-throughput analysis is becoming increasingly important in all areas of science, the forensic sciences being no exception. Many efforts have been made to develop on-line extraction techniques, allowing automation and a high throughput of samples [35–37]. These procedures have been applied for the detection of only a few antidepressants.

The aim of this study was to develop a simple, rugged and high-throughput on-line SPE–LC–MS/MS method for rapid and simultaneous bio-analysis of the main antidepressants prescribed in Belgium and their metabolites in plasma. The method involves a fully automated SPE system (Spark Symbiosis Pharma), which allows the simultaneous extraction of the second sample in one clamp and the elution of the first sample in a second clamp, as such, achieving an optimal use of the extraction time. This system offers the entire process of conditioning, sample application, washing and elution taking place at constant flow rates, yielding better precision in comparison with off-line driven extraction procedures. Another important advantage is that no manual transfers are made and that the whole of the eluate is loaded onto the LC column without the need for a pre-concentration step.

2. Materials and methods

2.1. Reagents

Individual stock solutions of amitriptyline, nortriptyline, imipramine, desipramine, trazodone, fluoxetine, norfluoxetine, paroxetine, fluvoxamine, sertraline (all certified at a concentration of 1 mg/mL in methanol), and the internal standards (I.S.) [$^2H_3$]imipramine (imipramine-d_3), [$^2H_3$]desipramine (desipramine-d_3), [$^2H_3$]clomipramine (clomipramine-d_3), [$^2H_6$]fluoxetine (fluoxetine-d_6) and [$^2H_6$]paroxetine (paroxetine-d_6) (certified concentration of 0.1 mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX, USA). Venlafaxine as a solid was obtained from Lederle Labs. (New York, USA). Norclomipramine and citalopram in solid form were supplied by Sigma–Aldrich (St. Louis, MO, USA) and clomipramine as a solid was a gift from Novartis Farmacéutica (Barcelona, Spain).

Ammonium hydrogencarbonate (99% purity) and formic acid (mass spectroscopy grade) was purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile and methanol (both LC–MS grade) and water were purchased from Biosolve (Valkenswaard, The Nederlands). Isopropyl alcohol and ammonia solution (32%, extra pure) were from Merck (Darmstadt, Germany).

Oasis MCX (mixed-mode cation-exchange) Prospekt cartridges (30 mg, 1 mL) were from Waters (Milford, MA, USA).

2.2. Specimens

Pooled blank plasma samples were used for development and validation of the procedure and were obtained from a local blood bank. Authentic plasma samples were obtained from hospital cases.

2.3. Preparation of standard solutions

Separate working solutions of the drugs, for tuning and selectivity experiments, were prepared in the laboratory at a concentration of 1 mg/L in methanol. A mixed working solution of non-deuterated compounds at 10 mg/L in methanol was used for the preparation of calibrators and QC samples. A mixed I.S.

working solution of 1 mg/L was prepared in methanol. Working solutions were stored at −20 °C, and were prepared monthly.

To obtain the lower concentrations needed for internal standardization and validation of each experiment, further dilutions in water were prepared the same day.

2.4. SPE–LC–MS/MS

2.4.1. Sample preparation: XLC (on-line SPE)

Sample extraction was performed using the on-line SPE Symbiosis Pharma System (Spark Holland, Emmen, The Netherlands). It comprises two integrated units: the Reliance autosampler with two binary LC pumps integrated and the on-line SPE unit Prospekt-2 system [consisting of the automated cartridge exchange (ACE) unit and two high-pressure dispensers (HPD)]. The entire system was operated by SparkLink software (version 3.10, Spark Holland). The extraction procedure was carried out in total recovery screw top vials of high quality glassware (Waters). A 950 μL volume of 0.1% formic acid and 50 μL of the I.S. working solution (0.1 mg/L) were added to 50 μL of plasma. The following XLC program was subsequently used (Fig. 1): After conditioning with 1 mL of methanol, 1 mL of water and 1 mL of 0.1% formic acid (Fig. 1A), 100 μL of the diluted plasma sample was applied onto the SPE MCX cartridge (cation-exchange mode) using 1 mL of 0.1% of formic acid as transport solvent. Clean-up was accomplished with successive 1 mL washes of 0.1% of formic acid and methanol in order to wash out salts and endogenous interferences present in the biological sample (Fig. 1B). The cartridge was then physically moved with a robotic arm to the elution (right) clamp in line with the LC pumps, leaving the extraction (left) clamp ready to start with a new sample. Whilst the elution step was being performed, a new cartridge was conditioned, loaded and washed in the left clamp. The elution was performed with 300 μL of 5% ammonia in methanol (at 100 μL/min). Following the elution step, several automated clamp and valve washes were carried out to avoid contamination between samples.

2.4.2. Chromatographic conditions

Focusing of the eluate was simultaneously performed as the compounds were eluted from the SPE cartridge by the use of a focusing column, Gemini C_{18} guard column (4 mm × 2.0 mm, 5 μm) (Phenomenex, Torrance, CA, USA), and a gradient elution with 10 mM ammonium hydrogencarbonate (pH 10) (A) and acetonitrile (B) (Fig. 1C). A gradient was carried out starting from 0% B and a flow rate of 1 mL/min for 3 min, as the eluate was diverted to the waste using the MS/MS Rheodyne switching valve. At 3.01 min, a switch of the valve delivered the eluent to the analytical Gemini C_{18} column (150 mm × 2 mm, 5 μm) (Phenomenex) (Fig. 1D) to start with the separation of the compounds at a flow rate of 0.3 mL/min and 50% B over the next minute. From 4 to 5 min, B was subsequently increased to 70%, and then kept for 6.5 min. At 11.5 min, B was increased to 95% in 1.5 min before returning to 50% within 0.5 min and equilibrating for 4.5 min. At 18 min a switch of the MS/MS valve diverted the eluent again to the waste, returning to the initial conditions to be ready for the analysis of the following sample.

2.4.3. Tandem mass spectrometry

A Quattro Premier tandem mass spectrometer (Waters) was used for all analyses. Ionization was achieved using electrospray in positive ionization mode (ESI+). Nitrogen was used as nebulisation and desolvation gas at a flow rate of 800 L/h and heated to 350 °C. Capillary voltage and source block temperature were 1 kV and 120 °C, respectively.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards [200 μg/L, in 10 mM ammonium hydrogencarbonate (pH 10)–acetonitrile (50:50, v/v)] were infused into the mass spectrometer and the cone voltage (CV) optimised to maximise the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mBar) and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis.

All aspects of data acquisition were controlled using MassLynx NT 4.0 software with automated data processing using the TargetLynx software (Waters).

2.5. On-line SPE–LC–MS/MS assay validation

The analytical validation was performed according to the recommendations of Peters and Maurer [38] and Shah et al. [39].

2.5.1. Linearity, limit of quantification (LOQ), limit of detection (LOD), precision and accuracy

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. Freshly prepared working solutions of 0.01, 0.05, 0.25, and 1 mg/L in water were used to prepare plasma calibrators at a concentration of 10, 25, 50, 125, 250, 500 and 1000 μg/L. Standard curves, freshly prepared with each batch of QC and authentic samples, were generated using a least-squares linear regression, with a $1/x$-weighting factor for most of the compounds, except for trazodone, nortrityline and norclomipramine, for which a quadratic response was found to be more suitable.

The limit of quantification (LOQ) was defined as the concentration of the lowest calibrator that was calculated within ±20% of the nominal value and with a relative standard deviation (RSD) less than 20%.

The limit of detection (LOD) was estimated from blanc plasma samples, spiked with decreasing concentrations of the analytes, where the response of the qualitative ion could reliably differentiate from background noise and with signal to noise ratio (S/N) of the qualifier equal to or greater than three.

QCs were prepared for every run in blank plasma at a concentration of 40, 200 and 800 μg/L. Intra-assay precision was evaluated by replicate ($n = 5$) analysis of the three QC samples in one run. Inter-assay precision was evaluated by replicate

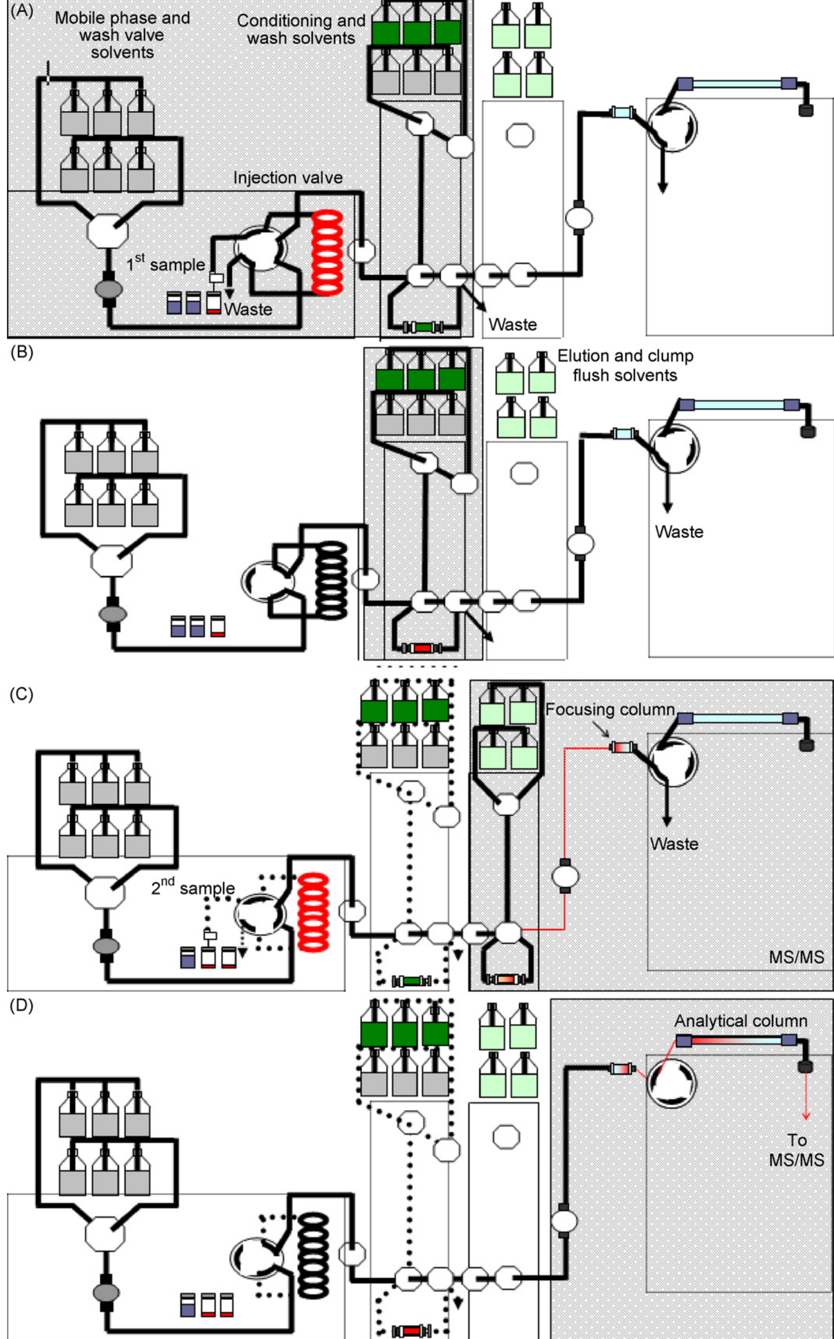

Fig. 1. Scheme of on-line SPE system-LC (Symbiosis Pharma) coupled to the MS/MS. (A) The sample is loaded into the sample loop while the first SPE cartridge is conditioned in the left clamp. (B) The sample is loaded onto the cartridge followed by a washing step. (C) The cartridge is moved to the right clamp, where the sample is eluted; the analytes are subsequently retained on the focussing column. Simultaneously, a second sample is subjected to the initial procedure of extraction in the left clamp. (D) After the elution step the MS valve is switched and the analytes are now directed to the analytical column in connection to the MS/MS. Following the analysis of the first sample, the second SPE cartridge is transferred to the right clamp in order to be eluted and analysed.

analysis of the QC samples in several experiments performed on six different days. Comparing the calculated concentrations of all calibrators and QC samples to their respective nominal values, provided data on the bias (accuracy) of the method.

2.5.2. *Selectivity and specificity*

The selectivity of the method against endogenous interferences was verified by examination of the chromatograms obtained after the extraction of six different blank plasma samples conserved in two different anticoagulants (sodium citrate ($n = 3$) and sodium fluoride ($n = 3$).

The combination of benzodiazepines with antidepressants potentiates the treatment of depression, thus, benzodiazepines are frequently encountered in these samples. For this reason, the specificity of the method was also assessed by the analysis of plasma samples spiked at 2 mg/L of a solution containing 27 benzodiazepines used for routine analysis in our laboratory.

2.5.3. *Stability of samples*

The stability of the drugs in plasma was monitored in diluted plasma samples as follows; 50 μL of blank plasma spiked at the initial concentrations of 40, 200 and 800 ($n = 9$, at each concentration) were diluted with 950 μL of 0.1% formic acid. The I.S. was added to the control samples ($n = 3$) and the concentrations were determined immediately. Another pool of samples was kept in the autosampler at 6 ± 2 °C and analyzed prior to the addition of the I.S., after 24 h ($n = 3$) and 48 h ($n = 3$). For an evaluation of freeze/thaw stability, the control samples at the concentrations of 40 and 200 μg/L ($n = 3$) were spiked with the I.S. and analysed immediately. The stability samples, spiked at the same concentrations ($n = 3$), were subjected to three freeze/thaw cycles. For each freeze/thaw cycle, the samples were frozen at −20 °C for

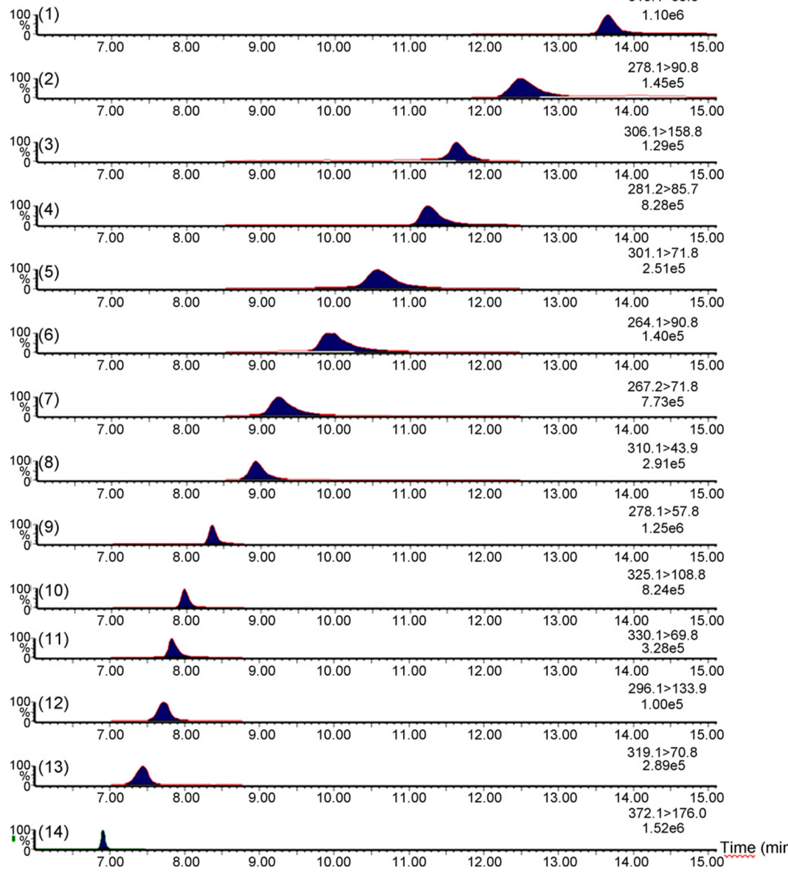

Fig. 2. MRM chromatograms obtained following the analysis of a spiked plasma sample with 10 μg/L of (1) clomipramine, (2) amitriptyline, (3) sertraline, (4) imipramine, (5) norclomipramine, (6) nortriptyliline, (7) desipramine, (8) fluoxetine, (9) venlafaxine, (10) citalopram, (11) paroxetine, (12) norfluoxetine, (13) fluvoxamine and (14) trazodone. Peak intensity is shown on the right-hand corner of each trace.

Table 1
MRM transitions and conditions for all the compounds and their deuterated analogues

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Trazodone	372.1	147.9	40	35
		176.0		25
Fluvoxamine	319.1	70.8	25	15
		86.8		15
Norfluoxetine	296.1	30.0	15	8
		133.9		6
Paroxetine	330.1	69.8	40	28
		192.1		20
Citalopram	325.1	108.8	30	25
		115.8		25
Venlafaxine	278.1	57.8	20	18
		260.1		12
Fluoxetine	310.1	43.9	20	12
		147.9		8
Desipramine	267.2	44.0	25	30
		71.8		15
Nortriptyline	264.1	90.8	25	20
		233.2		15
Norclomipramine	301.1	43.9	25	35
		71.8		20
Imipramine	281.2	57.7	25	35
		85.7		10
Sertraline	306.1	158.8	15	30
		275.0		12
Amitriptyline	278.1	90.8	30	25
		104.8		20
Clomipramine	315.1	57.9	30	30
		85.8		18
Paroxetine-d_6	336.1	75.8	35	32
Fluoxetine-d_6	316.1	43.9	20	12
Desipramine-d_3	270.2	74.8	25	15
Imipramine-d_3	284.2	88.8	25	18
Clomipramine-d_3	318.1	88.8	30	20

Underlined transitions were used for quantification.

24 h, thawed, and then maintained at ambient temperature for 1 h. After the three cycles, the samples were spiked with the I.S. and analyzed. Stability was tested against a lower percentage limit corresponding to 90% of the mean value of control samples by on-sided *t*-test ($P < 0.05$).

2.5.4. *Assessment of matrix effects*

To assess any potential suppression or enhancement of ionization due to the sample matrix, two different analyses were carried out. The first one involved a post-column infusion experiment. The study was based on a continuous post-column infusion of a mixture of the drugs and their internal standards (10 μg/L at a flow rate of 10 μL/min) to produce a constant elevated response in the MRM channels. The interference of this constant response was monitored following the injection of plasma samples (in two different anticoagulants: sodium citrate ($n = 3$) and sodium fluoride ($n = 3$) and compared to the response following

Table 2
Reported therapeutic range [16] and linearity data for 14 antidepressants and their metabolites

Compound	Therapeutic range (μg/L)	r^2	LOQ (μg/L)	LOD (μg/L)
Trazodone	500–2500	0.99995	10	0.5
Fluvoxamine	50–250	0.99848	10	1
Norfluoxetine	100–500	0.99890	10	1
Paroxetine	10–75	0.99939	10	0.5
Citalopram	20–200	0.99793	10	0.5
Venlafaxine	250–750	0.99727	10	0.5
Fluoxetine	150–500	0.99720	10	1
Desipramine	75–250	0.99945	10	1
Nortriptyline	50–250	0.99922	10	1
Norclomipramine	150–550	0.99702	10	1
Imipramine	45–150	0.99951	10	0.5
Sertraline	50–250	0.99900	10	1
Amitriptyline	50–300	0.99845	10	0.5
Clomipramine	20–250	0.99947	10	0.5

the injection of mobile phase only. A second type of experiment consisted of a comparison of the peak responses of the analysis of a blank plasma sample spiked at 1000 μg/L calibrator ($n = 3$) with those obtained from water spiked at the same concentration level.

2.5.5. Recovery

Recoveries were estimated by performing two experiments. In order to optimize the elution conditions i.e. elution volume and flow rate, a 1000 μg/L calibrator ($n = 3$) was injected in the absence of both the analytical and focusing column. As such the compounds achieve directly the MS/MS as they elute from the SPE cartridge. In a second experiment, performed to calculate de total recovery, a 1000 μg/L calibrator ($n = 3$) was loaded and washed in a first SPE cartridge while a second cartridge was placed in series to determine the breakthrough of the first one. Both cartridges where subsequently eluted independently. Recovery was considered to be the ratio between

Table 3
Intra-assay and inter-assay precision and bias of the QC samples prepared in plasma at a concentration of 40, 200 and 800 μg/L

Compound	Concentration of QC (μg/L)	Intra-assay precision ($n = 5$)			Inter-assay precision ($n = 6$)		
		Mean concentration found (μg/L)	RSD (%)	Bias (%)	Mean concentration found (μg/L)	RSD (%)	Bias (%)
Trazadone	40	43.6	6.1	9.0	41.0	11.5	2.5
	200	233.2	1.5	16.6	199.6	7.0	−0.2
	800	692.6	4.7	−13.4	673.4	13.2	−15.8
Fluvoxamine	40	41.9	7.2	4.8	36.5	10.9	−8.8
	200	216.5	3.3	8.3	189.4	7.6	−5.3
	800	843.8	3.8	5.5	807.0	8.7	0.9
Norfluoxetine	40	45.6	7.5	14.0	41.1	14.3	2.8
	200	236.1	3.8	18.1	193.2	15.0	−3.4
	800	880.9	7.5	10.1	801.5	8.6	0.2
Paroxetine	40	40.8	6.4	2.0	38.5	6.5	−3.8
	200	212.8	2.2	6.4	193.6	7.2	−3.2
	800	807.3	4.3	0.9	805.9	8.8	0.7
Citalopram	40	44.1	4.4	10.3	43.1	7.8	7.8
	200	222.0	2.0	11.0	201.3	7.8	0.7
	800	796.7	2.8	−0.4	780.7	10.1	−2.4
Venlafaxine	40	44.0	5.6	10.0	43.1	12.5	7.8
	200	227.4	1.7	13.7	198.3	10.4	−0.8
	800	780.8	5.5	−2.4	785.4	13.0	−1.8
Fluoxetine	40	43.8	7.3	9.5	40.0	10.7	0.0
	200	220.0	3.0	10.0	196.3	9.8	−1.8
	800	817.0	8.3	2.1	814.3	9.9	1.8
Desipramine	40	43.3	5.4	8.2	40.5	5.4	1.3
	200	213.7	3.1	6.9	194.4	6.2	−2.8
	800	830.6	3.2	3.8	791.0	11.1	−1.1
Nortriptyline	40	41.7	6.4	4.3	38.1	8.4	−4.8
	200	211.0	3.6	5.5	192.1	8.3	−4.0
	800	813.4	5.1	1.7	800.0	13.6	0.0
Norclomipramine	40	40.0	6.5	0.0	36.6	15.4	−8.5
	200	226.9	15.5	13.5	197.0	7.0	−1.5
	800	798.6	3.4	−0.2	808.9	5.5	1.1
Imipramine	40	43.4	2.3	8.5	40.1	6.4	0.3
	200	226.1	1.3	13.1	198.1	7.4	−1.0
	800	827.1	3.8	3.4	806.5	11.4	0.8
Sertraline	40	42.2	5.2	5.5	37.2	14.1	−7.0
	200	221.9	3.5	11.0	193.9	9.2	−3.1
	800	850.2	5.5	6.3	824.6	7.6	3.1
Amitriptyline	40	38.3	5.9	−4.3	35.2	10.1	−12.0
	200	203.1	3.1	1.6	181.8	9.2	−9.1
	800	805.6	4.2	0.7	780.1	9.7	−2.5
Clomipramine	40	43.9	6.5	9.8	41.5	9.9	3.8
	200	219.1	1.7	9.6	207.1	7.5	3.6
	800	764.4	5.1	-4.5	808.2	10.3	1.0

the response obtained after elution of the first cartridge and the total response (sum of both, the first and the second SPE cartridge).

3. Results and discussion

The method was validated for linearity, LOQ, precision and accuracy by the analysis of spiked plasma samples. In each case, a weighted (1/*x*) linear regression line was applied, except for trazodone, nortriptyline and norclomipramine, for which a quadratic response was found to be more suitable to obtain the best fit across the calibration range [38]. Correlation coefficient of $r^2 > 0.99$ was achieved in the range investigated: from 10 up to 1000 μg/L. The range investigated was considered according the therapeutic range elaborated by TIAFT [16] (Table 2) Fig. 2 shows the MRM chromatograms obtained following the analysis of the lowest calibrator (10 μg/L). At this concentration a signal-to-noise ratio (S/N) > 10:1 was observed for the qualifier and the criteria for LOQ were satisfied.

The applied gradient ensured the elution of all the drugs examined within 14 min and produced chromatographic peaks of acceptable symmetry. Since a focusing step is a crucial factor to obtain good peaks and sensitivity, many efforts were made to determine the optimal conditions.

Selectivity of the method was achieved by a combination of retention time, precursor and product ions.

The most prominent precursor-product transitions were used for quantification of the non-deuterated compounds and the next most abundant, used as qualifiers.

For the corresponding deuterated analogues, only one transition was monitored. Table 1 summarises the MRM transitions and conditions of all quantifiers and qualifiers for all analytes and I.S.

Injection of single analyte solutions did not produce interference in the other MRM channels. Linearity data, LOQ and LOD are shown in Table 2. No interference peaks were observed in the antidepressants MRM channels when blank plasma samples spiked with 27 benzodiazepines were analysed.

The intra-assay precision (repeatability) and inter-assay precision were satisfactory, with all relative standard deviations less than 20% (Table 3). Results indicated that the accuracy of the assay was >81%.

The stability of spiked samples (40, 200, 800 μg/L) was monitored at 24 and 48 h while kept in the autosampler at 6 ± 2 °C. No statistical significant difference could be observed for the three different concentrations, except for clomipramine and norclomipramine where a slightly negative trend was observed ($P > 0.05$), but it did not compromise de quantification. All compounds were stable ($P < 0.05$) after the three freeze thaw/cycles.

Insufficient sample clean-up can result in matrix effects, leading to either suppression or enhancement of the analyte

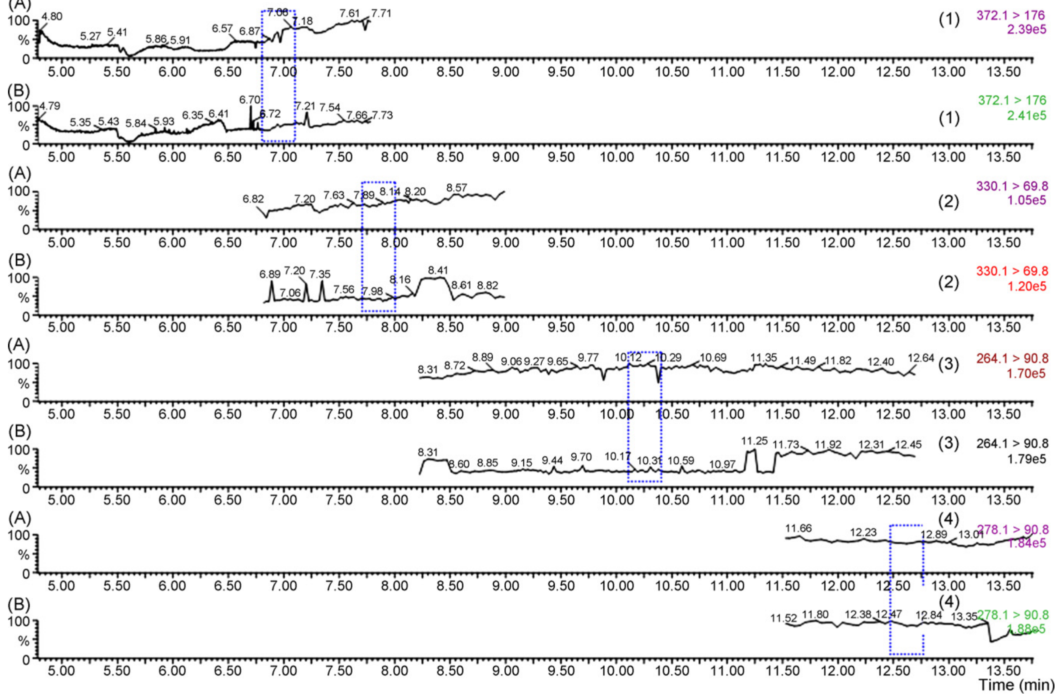

Fig. 3. Post-column infusion experiments: matrix effect. (1) trazodone, (2) paroxetine, (3) nortriptyline and (4) amitriptyline, of an injection of a mobile phase control (A) and a blank sample following the extraction of plasma (B). The shaded area indicates the elution position of the respective antidepressants.

response. This can lead to variable sensitivities and decreased precision and accuracy. Consequently, in the development of any LC–MS(/MS) method, the potential for any such ion suppression or enhancement should be assessed. Post-column infusion experiments (based on the method described by Bonfiglio et al. [40]) were performed to provide information of the effect of the matrix throughout the course of the elution time for the analytes. An example of the effect on drug response, obtained following the injection of a mobile phase control, is shown in Fig. 3A. As expected, no changes in response were observed. Another example of the effects obtained following the injection of a sample subjected to XLC are given in Fig. 3B. The results confirm the utility/benefits of the extraction as a sample clean-up before chromatography to obtain reproducible and reliable quantitative results for all the compounds without major interferences of matrix compounds. A second experiment was carried out and we compared peak responses obtained when the antidepressants were spiked to a blank plasma sample with the responses obtained when the antidepressants were added to a sample where the plasma was substituted with water. No statistically significant differences in peak areas were observed.

The Oasis MCX Prospekt cartidges (30 mg, 1 mL) utilise mixed-mode (cation-exchange) sorbents, which provide effective sample clean-up for basic drugs. The results of the extraction recovery study are presented in Table 4. Very high and reproducible recoveries were obtained with this SPE procedure for all analytes and all compounds were totally eluted from the SPE cartridge at the elution step conditions.

The validated SPE–LC–MS–MS method was applied to the analysis of 11 authentic samples from clinical cases and previously analysed by liquid chromatography with diode array detection (DAD) using a routine screening method. The results of both methods were compared qualitatively (Table 5). Fig. 4 shows the chromatogram obtained after the analysis of one of these samples, positive for trazodone and venlafaxine. The measured concentrations varied in a wide range and several samples had to be re-analysed after 1:10 dilution with blank plasma.

Several analytical methods for the determination of antidepressants have been published using LC or LC–MS/MS. However, most of them only allow the determination of one or few compounds with longer off-line SPE procedures [30,41–43]. Sauvage et al. [44] have also developed an innovative method for the determination of 13 antidepressants and some metabolites using turbulent-flow liquid chromatography (TFC). The

Table 4
Extraction recovery and matrix effect

	Recovery (%)	Estimated matrix effect after SPE (%)
	($n=3$)	($n=3$)
Trazodone	99.9	−10.9
Venlafaxine	99.8	−10.6
Citalopram	99.9	−5.9
Desipramine	99.8	−9.1
Imipramine	99.9	−7.1
Nortriptyline	99.6	−11.8
Amitriptyline	99.9	−12.8
Paroxetine	99.8	−6.7
Fluvoxamine	99.7	−16.8
Norfluoxetine	99.8	0.5
Fluoxetine	99.9	−12.1
Sertraline	99.9	−15.2
Clomipramine	99.7	−17.7
Norclomipramine	99.9	−10.4

Data represent the mean of three experiments with a 1000 μg/L calibrator.

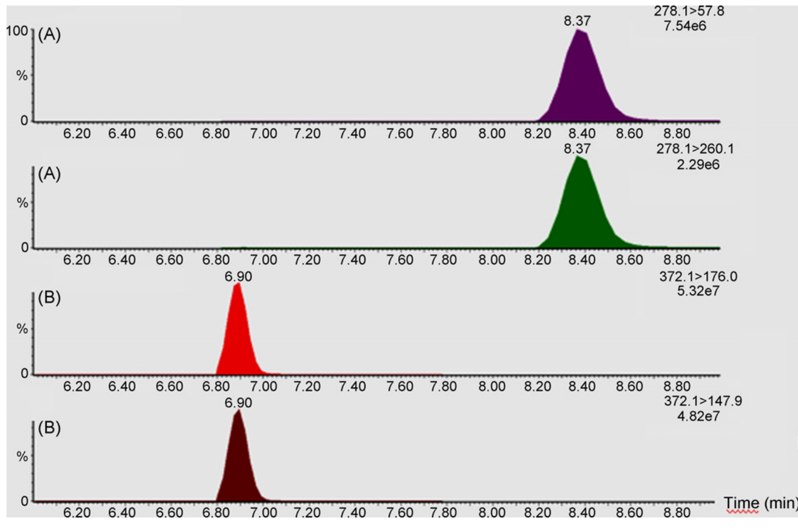

Fig. 4. Typical MRM chromatograms obtained following the analysis of one authentic plasma sample. Concentrations were 127 μg/L for venlafaxine (A) 1036 μg/L for trazodone (B). The figure shows the response for the two transitions of both compounds (quantifier and qualifier). Peak intensity is shown in the top right-hand corner of each trace.

Table 5
Comparative results when analysing the real samples with the on-line SPE–LC–MS/MS method and LC–DAD

Sample ID	Concentration (μg/L) SPE-LC-MS/MS	LC-DAD
1	Citalopram (14)	–
	Fluoxetine (114)	+
	Norfluoxetine (47)	+
2	Trazodone (1036)	+
	Venlafaxine (127)	+
3	Fluoxetine (214)	+
	Norfluoxetine (170)	+
4	Trazodone (1963)	+
5	Fluoxetine (381)	+
	Norfluoxetine (95)	+
6	Amitriptyline (132)	+
	Nortriptyline (90)	+
7	Trazodone (1284)	+
8	Fluoxetine (92)	+
	Norfluoxetine (47)	+
9	Amitriptyline (1542)	+
10	Fluoxetine (114)	+
	Norfluoxetine (160)	+
11	Fluoxetine (108)	+
	Norfluoxetine (183)	+

antidepressants were divided into two groups depending on their chromatographic properties, thus two injections were necessary to screen all the compounds. On the other hand, it shows lower sensitivity and longer duration of entire total process (sample preparation and analysis) when comparing to the present paper.

4. Conclusions

To our knowledge, this is so far the first on-line SPE method with single use cartridges, coupled to LC–MS/MS applied for the direct analysis of 14 antidepressants and metabolites in plasma. The combination of on-line SPE with MS/MS allowed the development of a high-throughput, fast and sensitive method with a 20 min total analysis time without compromising the method validation criteria. The method was successfully applied to 11 authentic plasma samples, proven to be appropriate for the quantification of low both dose as high dose of these compounds in plasma collected from forensic toxicology cases.

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Correlation of Δ^9-tetrahydrocannabinol concentrations determined by LC–MS–MS in oral fluid and plasma from impaired drivers and evaluation of the on-site Dräger DrugTest®

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Abstract

Oral fluid (collected with the Intercept® device) and plasma samples were obtained from 139 individuals suspected of driving under the influence of drugs and analyzed for Δ^9-tetrahydrocannabinol (THC), the major psychoactive constituent of cannabis, using a validated quantitative LC–MS–MS method.

The first aim of the study was to investigate the correlation between the analytical data obtained in the plasma and oral fluid samples, to evaluate the use of oral fluid as a 'predictor' of actual cannabis influence. The results of the study indicated a good accuracy when comparing THC detection in oral fluid and plasma (84.9–95.7% depending on the cut-off used for plasma analysis). ROC curve analysis was subsequently used to determine the optimal cut-off value for THC in oral fluid with plasma as reference sample, in order to 'predict' a positive plasma result for THC. When using the LOQ of the method for plasma (0.5 ng/mL), the optimal cut-off was 1.2 ng/mL THC in oral fluid (sensitivity, 94.7%; specificity, 92.0%). When using the legal cut-off in Belgium for driving under the influence in plasma (2 ng/mL), an optimal cut-off value of 5.2 ng/mL THC in oral fluid (sensitivity, 91.6%; specificity, 88.6%) was observed.

In the second part of the study, the performance of the on-site Dräger DrugTest® for the screening of THC in oral fluid during roadside controls was assessed by comparison with the corresponding LC–MS–MS results in plasma and oral fluid. Since the accuracy was always less than 66%, we do not recommend this Dräger DrugTest® system for the on-site screening of THC in oral fluid.
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Keywords: Oral fluid; Plasma; THC; Driving under the influence; Roadside drug test

1. Introduction

In Belgium, cannabis is the most frequently detected drug in cases of driving under the influence of drugs (DUID) and surveys show an increase in current and lifetime consumption [1]. As with many other CNS active drugs, experimental studies using instrumented cars have shown that the use of cannabis (100–200 μg/kg) significantly affects the standard deviation of lateral position (SDLP), which is somewhat higher than the impairment produced by 0.5 g/L ethanol [2]. Drummer et al. obtained epidemiological data for Δ^9-tetrahydrocannabinol (THC), the major psychoactive constituent of cannabis, in blood during an Australian responsibility study on 3398 fatally injured drivers [3]. Results confirmed that the probability of being responsible for a crash is significantly higher in drivers with THC in their blood than in the group of the drug-free drivers. An odds ratio of 2.7 was calculated for the group of THC-positive drivers. The risk of being culpable for a fatal crash increased to nearly seven-fold when THC blood concentrations exceeded 5 ng/mL.

For several years now, fast urine tests have been used to detect illegal drugs in different settings, e.g. workplace drug

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testing, treatment programs and roadside screenings. However, the detection of cannabinoids in oral fluid is a better indication of recent use than the detection in urine [4,5]. Despite the lack of contribution from blood THC to oral fluid concentrations, Niedbala et al. [4] and Huestis and Cone [6] showed that, after dissipation of the initial contamination of oral fluid during smoking (generally within 30 min), THC levels in oral fluid followed a similar time course as plasma THC following cannabis smoking under controlled dosing conditions.

The first on-site tests for drugs in oral fluid appeared in the late 1990s, allowing an immediate testing of the oral fluid specimen during roadside controls. At the initiative of the European Commission, the Rosita (acronym for ROadSIde Testing Assessment) study was started to evaluate the value of on-site roadside tests for oral fluid and sweat. This study showed that the on-site oral fluid screenings, particularly for testing of THC, required significant improvement [7]. These results are in concordance with other reports [8,9]. In 2003, the Rosita-2 study was started, involving six European countries and five US states, in order to evaluate new on-site oral fluid devices.

Recently, we have developed a rapid and sensitive LC–MS–MS method for the detection of THC in oral fluid collected with the Intercept® device [10]. The Intercept® is a Food and Drug Administration (FDA) approved sampling device that is used on a large scale in the U.S. for workplace drug testing [5]. It is also one of the devices currently being used to collect oral fluid samples for confirmation analyses in the Rosita-2 study [7]. The collection system contains a buffer with stabilizing salts, non-ionic surfactants for surface wetting and antibacterial agents, and guarantees a good stability for most illicit drugs and their metabolites during storage at 4 °C. However, these ingredients can also cause interferences, e.g. ion suppression or enhancement during LC–MS–MS analysis in the absence of a suitable clean-up method. The value for recovery from the Intercept® device reported by Kintz et al. was $80.1 \pm 4.6\%$ [11]. As stated by these authors, one of the drawbacks of this collection device, is that it is not possible to evaluate the amount of collected oral fluid, even by weighing the test tube before and after the centrifugation, as the volume of the blue buffer varies from device to device. However, it was reported that the device collects an average of 0.38 ± 0.19 mL (range 0.05–0.8 mL) of oral fluid [4] and a dilution factor of 1 in 3 is arbitrarily accepted [4,5,11]. The problems associated with the collection aspect of this device have been thoroughly discussed in previous reports [4,5].

In the present study, the validated LC–MS–MS method was used to determine concentrations of THC in oral fluid (Intercept® device) and plasma of impaired drivers, collected at the roadside. Correlations between THC levels in both biological matrices were established and the optimal cut-off for oral fluid was calculated using ROC curve analysis (using plasma as reference sample). In addition, the reliability of the Dräger DrugTest® for the detection of THC in oral fluid was assessed by comparing its on-site results with the confirmatory LC–MS–MS results in plasma and oral fluid.

2. Materials and methods

2.1. Biological samples

During the course of police controls, conducted over the period from February 2004 until April 2005, 139 subjects agreed to provide oral fluid samples on a voluntary basis (written informed consent) in addition to plasma and urine. In 127 cases, the collection of the blood sample was preceded by on-site analysis of oral fluid with the Dräger DrugTest® system.

Blood samples were collected in 7 mL glass Vacutainer tubes using sodium fluoride and potassium oxalate as anticoagulant. The tubes were cooled immediately to +4 °C (cool box) and centrifuged the next day. The corresponding plasma was frozen at −20 °C until analysis. Pooled blank plasma samples were used for development and validation of the procedure and were obtained from a local blood bank.

Oral fluid was collected with the Intercept® device (OraSure Technologies, Bethlehem, PA, USA) according to the manufacturer's instructions. The tubes were centrifuged and the oral fluid was stored at −20 °C prior to analysis. Blank oral fluid used for the preparation of negative controls, calibrators and quality control (QC) samples was obtained from healthy volunteers (also collected with the Intercept®). The data are expressed as nanograms per millilitre of diluted specimen. The exact volume of specimen collected by the individual devices was not determined.

2.2. Analysis of oral fluid and plasma samples

Oral fluid samples were analyzed by LC–MS–MS for THC according to a published validated procedure [10]. The limit of quantification (LOQ) for THC was 0.5 ng/mL using 100 μL of oral fluid.

The same procedure was applied to plasma samples. Initially, the internal standard (THC-d3) at a concentration of 5 ng/mL was added to 250 μL of specimen, calibrator or control sample. Each sample was subsequently extracted with 4 mL of hexane. After mechanical shaking (30 min) and centrifugation (10 min at $3000 \times g$), the organic phase was removed and evaporated to dryness at 40 °C under nitrogen. The extract was reconstituted in 100 μL of mobile phase and 20 μL was injected into the LC–MS–MS system.

Quantitative analyses were performed using a Waters Alliance 2690 separation module (Waters, Milford, MA, USA), interfaced with a Quattro Premier tandem mass spectrometer (Waters). Analytes were separated on a XTerra MS C_{18} column (150 mm × 2.1 mm, 3.5 μm) (Waters), eluted isocratically with 1 mM ammonium formate–methanol (10:90, v/v), delivered at a flow rate of 0.2 mL/min. Ionization was achieved using electrospray in positive ionization mode (ESI+). The optimum conditions were: capillary voltage, 2.0 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 280 °C and delivered at a flow rate of 700 L/h. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mbar) and the collision energy (eV)

adjusted to optimise the signal for the most abundant product ions, which were subsequently used for multiple reaction monitoring (MRM) analysis. The transitions were m/z 315.2 → 193.1 and m/z 315.2 → 259.3 for THC. The former (and most prominent precursor-product transition) was used for quantification and the latter transition used as a qualifier. The transition for THC-d3 was m/z 318.2 → 196.1.

2.3. Data analysis

The experimental data were processed to obtain the following parameters: the true positives (TP, positive oral fluid samples matching a positive plasma sample; positive drug tests matching a positive oral fluid/plasma sample), true negatives (TN, negative oral fluid samples matching a negative plasma sample; negative drug tests matching a negative oral fluid/plasma sample), false positives (FP, positive oral fluid samples that were not confirmed in plasma; positive drug tests that were not confirmed in oral fluid/plasma) and false negatives (FN, oral fluid samples that were negative but corresponding to a positive plasma result; drug tests that were negative but corresponding to a positive oral fluid/plasma result). Based on these results, the sensitivity, specificity and accuracy were calculated.

In addition, the LC–MS–MS results for oral fluid and plasma were used to establish receiver operating characteristic (ROC) curves: the sensitivity was plotted versus 100-specificity for each cut-off concentration using MedCalc® (version 7.3) statistical software. The optimal cut-off for oral fluid to 'predict' a positive plasma result is the value corresponding with the highest accuracy (minimal FN and FP results).

For the analysis of the results, the applied cut-off for oral fluid was always the LOQ of the LC–MS–MS method for oral fluid (0.5 ng/mL). For plasma, two different cut-off values were used: the LOQ of the LC–MS–MS method for plasma (0.5 ng/mL) and the legal limit in Belgium for DUID (2 ng/mL for THC).

2.4. The on-site Dräger DrugTest® system for oral fluid

As part of the Belgian participation in the Rosita-2 project, the Dräger DrugTest® for the on-site detection of THC in oral fluid was tested. This system combines the test strip method of immunological drug detection with an innovative signal technology known as UPT (Up-Converting Phosphor Technology). It consists of two main components: the Dräger DrugTest® Kit for Oral Fluids (comprising the collection device and the test cassette with inserted buffer cartridge) and the Dräger DrugTest® Analyzer (a portable analyzer for reading the test cassette and for data management). Different panel cassettes are available for detecting up to six substance classes in a single cassette. The test subject collects an oral fluid sample by gently moving the collection device in the mouth for about a minute. The sampling sponge swells in size as it soaks up the oral fluid, signaling to the tester that the sampling process is complete. The collection device is first inserted into the test cassette and pushed down to release some of the oral fluid into the buffer cartridge. The handle of the collection device is removed and the buffer cartridge is sealed. After a 4 min reaction time, the buffer cartridge is turned and lowered, triggering the immunological detection reaction within the test cassette. After 8 min, the test cassette can be inserted into the Dräger DrugTest® Analyzer, which displays the results of the analysis, distinct for each class of drugs, in the form of a qualitative reading (positive or negative). According to the manufacturer, the cut-off value for THC is 20 ng/mL.

3. Results and discussion

3.1. Validation of the analytical method for plasma samples

Selectivity of the method was achieved by a combination of retention time, precursor and product ions. Quantification was based on the most prominent product ion (i.e. quantifier); confirmation of THC was evaluated through the presence of the second product (i.e. qualifier). At the LOQ the qualifier had a signal to noise ratio (S:N) >10:1. The acceptance range for the peak area ratio quantifier/qualifier was 2.34 ± 0.28 for all analyses.

Calibration curves for THC were prepared in blank plasma for each batch and ranged from 0.5 to 100 ng/mL. Negative controls and low and high QC samples containing 2.5 and 25 ng/mL THC respectively, were included in each batch. In each case, a weighted ($1/x$) linear regression line was applied. Linearity with correlation coefficients $r^2 > 0.999$ were achieved within the range investigated. The back-calculated concentrations of all calibrators were compared with their respective nominal values and were within $100 \pm 15\%$ of the nominal value. The LOQ, defined as the concentration of lowest calibrator which was calculated to be within ±20% of the nominal value and with a % relative standard deviation (R.S.D.) less than 20%, was 0.5 ng/mL. The intra-assay precision (repeatability) and inter-assay precision (reproducibility) are shown in Table 1. Intra-assay variation was evaluated by

Table 1
Precision and accuracy data for the LC–MS–MS analysis of THC in plasma

Concentration of QC (ng/mL)	Intra-assay precision (n = 5)			Inter-assay precision (n = 10)		
	Mean concentration found (ng/mL)	R.S.D. (%)	Bias (%)	Mean concentration found (ng/mL)	R.S.D. (%)	Bias (%)
2.5	2.5	3.4	1.1	2.6	11.9	2.0
25.0	24.9	1.1	−0.5	25.3	11.1	1.1

Samples were prepared by the liquid–liquid extraction method as described in the text. QCs were prepared for every run in blank plasma. Intra-assay variation was evaluated by replicate (n = 5) analysis of both QC samples in a single run. Inter-assay variation was evaluated by replicate analysis of the QC samples in several experiments performed on 10 different days and by two operators. A comparison of the calculated concentrations of the QC samples to their respective nominal values, was used to assess the bias of the method.

replicate ($n = 5$) analysis of both QC samples in a single run. Inter-assay variation was evaluated by replicate analysis of these QC samples in several experiments performed on ten different days and by two operators. A comparison of the calculated concentrations of the QC samples to their respective nominal values, was used to assess the bias of the method. Results indicated that the bias of the assay was $\leq$2.0% with all R.S.D.s <12%. The recovery of the method was estimated by comparing the response of a 5 ng/mL calibrator when the non-deuterated compound was added before the extraction step ($n = 3$) with the response obtained when the non-deuterated analyte was added after sample preparation ($n = 3$). THC-d3 was added before the extraction step in both conditions. The recovery was 79.2 ± 0.9%. Matrix effects were evaluated by post-column infusion as described by Bonfiglio et al. [12]. No ion suppression or enhancement could be detected at the elution time of THC ($n = 3$).

3.2. Comparison of analytical data in oral fluid and plasma

The detection of the inactive metabolite 11-nor-9-carboxy-THC in plasma, which is considered not to be present in oral fluid [13], was not included in the method since only the indication of recent cannabis use (through the presence of THC) was of interest. In total, 139 combined oral fluid–plasma samples were obtained. These plasma samples included 114 positive samples (82.0%) for THC using the LOQ of the LC–MS–MS method at a cut-off value of 0.5 ng/mL. Using the legal limit for plasma as a cut-off value (2 ng/mL), 95 cases (68.3%) were positive for THC. The median concentration in plasma (8.8 ng/mL) clearly exceeded the legal cut-off level; concentrations ranged from 0.6 to 51.3 ng/mL. The analysis of the oral fluid samples from the same subjects showed a median THC concentration of 23.0 ng/mL with concentrations ranging from 0.5 to 1462 ng/mL. Table 2 presents the sensitivity, specificity and accuracy when comparing the analytical data obtained for oral fluid with those for plasma (i.e. the reference sample), using both cut-off values for the detection of THC in plasma. When using the LOQ of the LC–MS–MS method as cut-off value for plasma samples, the sensitivity and accuracy were >95%. A somewhat lower specificity of 84% was noted. These results indicate a good correlation of oral fluid and plasma samples and are in concordance with previous published reports, although other collection protocols and cut-off values were used [14–17]. However, when applying the legal cut-off value for plasma samples, the specificity decreased dramatically (51.2%) due to a high number of FP results. In this case, a high sensitivity was calculated since there were no FN results. An accuracy of 84.9% was noted. These results indicate a high probability of detecting recent cannabis use, however, a certain number of positive oral fluid samples may not be confirmed in plasma.

The usefulness of oral fluid testing for THC is dependent on its ability to serve as a diagnostic indicator of recent cannabis use. In this study, we determined what would be the optimal cut-off for THC in oral fluid, in order to ‘predict’ that the plasma sample would be positive for THC. This optimal cut-off was calculated using ROC curve analysis, with different cut-off values for plasma as reference sample: (A) the LOQ of the analytical method and (B) the legal limit in Belgium. Using the LOQ of the method, the analysis showed an optimal cut-off value at 1.2 ng/mL oral fluid, corresponding to a sensitivity of 94.7% and a specificity of 92%. This cut-off value was somewhat lower in comparison with the SAMHSA proposed confirmation cut-offs in oral fluid, i.e. 2 ng/mL THC [18]. When applying the legal cut-off for plasma samples, a higher optimal cut-off value of 5.2 ng/mL was calculated with a sensitivity of 91.6% and a specificity of 88.6%.

3.3. Evaluation of the on-site Dräger DrugTest® system for the detection of THC in oral fluid

The sensitivity, specificity and accuracy for on-site oral fluid analyses with the Dräger DrugTest® for THC are shown in Table 3, with either oral fluid or plasma (using the LOQ of the method or the legal cut-off value) as reference sample. In all cases, a low sensitivity and accuracy were noted (< 66%), due to a high number of FN results. However, there is no indication that the FN results correspond to the oral fluid samples with the lowest concentrations of THC. The application of higher confirmation cut-off values will therefore not alter the results significantly. On the other hand, the high number of FN results could be due to a state of dryness in the oral cavity, leading to insufficient sample volume in the sampling sponge. Based on previous reports,

Table 2
Prediction of the presence of THC in plasma by LC–MS–MS analysis of the corresponding oral fluid samples (collected with the Intercept® device)

	Plasma:LOQ (%)	Plasma:legal cut-off (%)
Sensitivity	98.2	100
Specificity	84.0	51.2
Accuracy	95.7	84.9

The sensitivity, specificity and accuracy were calculated based on the number of true positives, true negatives, false positives and false negatives, using plasma as the reference sample. The applied cut-off values were the LOQ of the method for oral fluid (0.5 ng/mL) and either the LOQ of the LC–MS–MS method for plasma (0.5 ng/mL) or the legal limit in Belgium for DUID for THC in plasma (2.0 ng/mL).

Table 3
Sensitivity, specificity and accuracy of the on-site Dräger DrugTest® system during roadside controls, calculated based on the number of true positives, true negatives, false positives and false negatives

	Oral fluid (%)	Plasma:LOQ (%)	Plasma:legal cut-off (%)
Sensitivity	49.5	50.9	57.8
Specificity	100	92.9	87.5
Accuracy	55.0	55.7	65.6

Either oral fluid or plasma were used as reference sample; the applied cut-off values were the LOQ of the method for oral fluid (0.5 ng/mL) and either the LOQ of the LC–MS–MS method for plasma (0.5 ng/mL) or the legal limit in Belgium for DUID for THC in plasma (2.0 ng/mL).

the detection of cannabis use in oral fluid appears to be a general problem for most on-site drug tests, due to low sensitivities [8–10,14].

4. Conclusions

The aim of the study was to evaluate (A) oral fluid as a predictor of positive plasma samples and (B) the use of the on-site Dräger DrugTest® system for the detection of THC in oral fluid at roadside controls. Oral fluid (collected with the Intercept® device) and plasma samples were analyzed with a validated LC–MS–MS method. The overall results of the study indicated a good accuracy (84.9–95.7% depending on the cut-off used for plasma analysis) when correlating THC detection in oral fluid and plasma, suggesting that oral fluid is a good predictor of actual cannabis influence. However, the Dräger DrugTest® system cannot be recommended for on-site oral fluid tests for THC due to the low accuracy (<66%). The search for an on-site screening method that can provide acceptable accuracies for the detection of THC in oral fluid remains a major hurdle.

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Review

Recent applications of liquid chromatography–mass spectrometry in forensic science

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Abstract

Recent years have seen the development of powerful technologies that have provided forensic scientists with new analytical capabilities, unimaginable only a few years ago. With liquid chromatography–mass spectrometry (LC–MS) in particular, there has been an explosion in the range of new products available for solving many analytical problems, especially for those applications in which non-volatile, labile and/or high molecular weight compounds are being analysed. The aim of this article is to present an overview of some of the most recent applications of LC–MS (/MS) to forensic analysis. To this end, our survey encompasses the period from 2002 to 2005 and focuses on trace analysis (including chemical warfare agents, explosives and dyes), the use of alternative specimens for monitoring drugs of abuse, systematic toxicological analysis and high-throughput analysis. It is not the intention to provide an exhaustive review of the literature but rather to provide the reader with a 'flavour' of the versatility and utility of the technique within the forensic sciences.
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Keywords: LC–MS; Forensic; Trace chemicals; Alternative specimens; Drugs of abuse; Systematic toxicological analysis

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1. Introduction

The term "forensic science" covers those professions which are involved in the application of the social and physical sciences to the criminal justice system. Forensic experts are required to explain the smallest details of the methods used, to substantiate the choice of the applied technique and to give their unbiased conclusions—all under the critical and often mistrustful gaze of the servants of the justice, as well as the general public and the media. The final result of the work of the forensic scientist exerts a direct influence on the fate of a given individual. This burden is a most important stimulus, and one which determines the way of thinking and acting in forensic sciences. Consequently, the methods applied in forensic laboratories should assure a very high level of reliability and must be subjected to extensive quality assurance and rigid quality control programs. The legal system is based on the belief that the legal process results in justice. This has come under some question in recent years. Of course, the forensic scientist cannot change scepticism and mistrust single-handedly. He or she can, however, contribute to restoring faith in the judicial processes by using science and technology in the search for facts in civil, criminal and regulatory matters.

Recent years have seen the development of powerful technologies that have provided forensic scientists with new analytical capabilities which were unimaginable only a few years ago. The ability of mass spectrometry (MS) to extract chemical fingerprints from microscopic levels of analyte is invaluable in this quest, enabling the legally defensible identification and quantification of a wide range of compounds. Gas chromatography (GC)–MS, liquid chromatography (LC)–MS, isotope ratio (IR)-MS and inductively coupled plasma (ICP)-MS have become routine tools to enable detection and characterization of minute quantities in what can often be very complex matrices.

In the case of LC–MS, the last two decades have seen some significant developments and improvements in instrumentation design. Particularly noteworthy has been the introduction of robust, user-friendly interfaces such as those based on atmospheric pressure ionisation techniques, e.g. electrospray (ESI) and atmospheric pressure chemical ionisation (APCI). Consequently, many analysts and laboratories are finally at the point where they are considering the acquisition of LC–MS capabilities. According to Willoughby et al. [1] LC–MS has progressed from the "innovators" stage through the "early adaptors", to the "early majority" stage and is now open to specialists from a variety of disciplines, especially for those applications where involatile, labile and/or high molecular weight compounds are being analyzed.

The purpose of this article is to review some of the most recent applications of LC–MS (/MS) to forensic analysis with special focus on the following; trace analysis, the use of alternative specimens for monitoring drugs of abuse, systematic toxicological analysis and high-throughput analysis.

2. Trace chemicals

2.1. Chemical warfare agents

Determining the use of chemical warfare agents (CWAs) in times of war or in acts of terrorism requires rapid and reliable methods. The sarin gas attacks by a Japanese cult in Matsumoto city (1994) and the Tokyo subway system (1995) represented the first cases in which a CWA was indiscriminately released against a civilian population [2]. The latter incident resulted in the deaths of 12 people and led over 5000 to seek medical attention. Nerve agents are extremely potent organophosphorus compounds that cause biological effects by irreversibly inhibiting the enzyme acetylcholinesterase (AChE). To confirm exposure, biological samples, e.g. urine, can be analysed for the agents themselves, their metabolites or their degradation products. Nerve agents are rather volatile compounds, thus analysis by GC–MS might be considered the obvious choice. However, in an aqueous environment, these agents readily hydrolyse to produce alkyl alkylphosphonates (RMPAs), these in turn can be further hydrolysed to methyl phosphonate (MPA) (Fig. 1). LC–MS is increasingly being used for these low molecular weight, highly polar compounds whilst exploiting the benefits over GC–MS, of reduced sample handling and no requirement for derivatisation [3–5].

Hayes et al. [6] recently developed LC–tandem MS (LC–MS/MS) methods for the analysis of the short-lived metabolites of several CWAs including; sulfur mustard, sarin, soman, cyclohexyl methylphosphonofluridate (GF) and *O*-ethyl *S*-2-diisopropylamino ethyl methylphosphonothioate (VX) in urine. These methods were also used to determine the feasibility of using saliva as a complementary or alternative matrix to urine; this could be a particularly valuable approach to assess the exposure of young children, where collection of a urine sample on demand is often difficult.

VX comprises a mixture of two enantiomers which demonstrate significant differences in the rate of AChE inhibition and overall toxicity. Thus, the ability to distinguish between them is desirable for toxicological studies and for the development of antidotes. Smith [7] has used normal-phase LC in conjunction with MS detection for this purpose.

LC–MS has also been used to investigate the longer-lived metabolites. Several groups have used LC–MS to determine the metabolites of sulfur mustard, i.e. the β-lyase metabolites in urine samples from human casualties after sulphur mustard poisoning [8,9].

In the case of large-scale attacks, analysis of the environment and other materials may also be required. Hancock and D'Agostino have developed a LC–ESI-MS (/MS) procedure which allows the identification of a munitions grade sample of tabun, sarin, soman, GF and the nerve agent stimulant triethyl phosphate (TEP) on manmade fibres [10]. Although this technique uses only minimal sample preparation the same group

Fig. 1. Hydrolysis pathway of nerve gases and the alkyl alkylphosphonates (RMPAs) namely, isopropyl (IMPA), pinacolyl (PMPA) and ethyl (EMPA) methyl phosphonates. In turn, these can be hydrolysed to methyl phosphonate (MPA).

have more recently experimented to omit sample preparation completely and to allow the direct analysis of TEP collected on solid-phase microextraction (SPME) fibres [11].

The biotoxin ricin originates from the seeds (castor beans) of the *Ricinus communis* plant and is extremely toxic (human LD_{50} estimated at 3–30 μg/kg by inhalation or ingestion, respectively) [12]. It has the unique position of being the only protein listed under the Chemical Weapons Convention and is of forensic interest due to its potential for terrorist use or as a homicide agent [13]. Due to the high molecular weight of this compound (66 kDa) absolute structural elucidation of the intact protein is not possible using nominal mass analysis. However, several groups have used a preliminary enzymatic digestion to convert the protein into intermediate molecular weight peptides followed by LC–MS (/MS) using a hybrid quadrupole time-of-flight (Q-TOF) instrument [12,14]. The methods were used to characterise purified ricin from several different varieties of *R. communis* and also from crude castor bean extracts.

2.2. Explosives

The analysis of trace levels of explosives is critical in crime scene forensic investigations, homeland security and environmental analysis. LC–MS is a well-established technique for explosives in associated complex matrices such as post-blast residues and in environmental samples such as soil and plant material extracts [15,16]. Although these compounds have a low vapour pressure they tend to be heat labile and can degrade at the high temperatures typically used in GC injectors. Thus, LC–MS is particularly well-suited to the analysis of these relatively polar molecules, heat labile compounds. Many of the methods rely on the formation of cluster or adduct ions for identification. Gapeev et al. [17] studied the formation of cluster ions of 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX), one of the most commonly used military explosives in both ESI and APCI. Results showed that in ESI, self-decomposition of RDX did not play a role in adduct formation; the adducts were produced from impurities present in the mobile phase at ppm levels. In contrast, with APCI, part of the RDX molecule decomposes yielding a NO_2^- species; this in turn clusters with other RDX molecules.

More recently, Mathis and McCord presented a comprehensive method to allow the screening of a panel of high explosives including; RDX, 2,4,6,-trinitrotoluene (TNT), pentaerythritol tetranitrate (PETN), 1,3,5,7-tetramethylene-2,4,6,8-tetranitramine (HMX), nitrogycerine (NG) and ethylene glycol dinitrate (EGDN). This method was based on the *competitive* formation of adducts following infusion of the high explosives with a mixture of four anions; chloride, formate, acetate and nitrate. Information relating to the relative extent of adduct formation (based on intensity ratios) in addition to adduct stability, was used to provide a multiplexed detection scheme (Fig. 2) [18].

Anti-personnel (AP) mines are currently in place in over seventy countries and are designed to maim or kill humans. In addition to the lives that are lost, the mere suspicion that they may be present, can prevent the use of large areas which could otherwise be utilised for agriculture or social infrastructure. Removal of landmines from such areas is known as humanitarian de-mining and relies on the accurate detection of the explosive. A potentially useful approach and one which is currently under investigation, is the detection of the chemical vapours which arise from the explosives and are transported into the surrounding atmosphere. High sensitivity is required since the

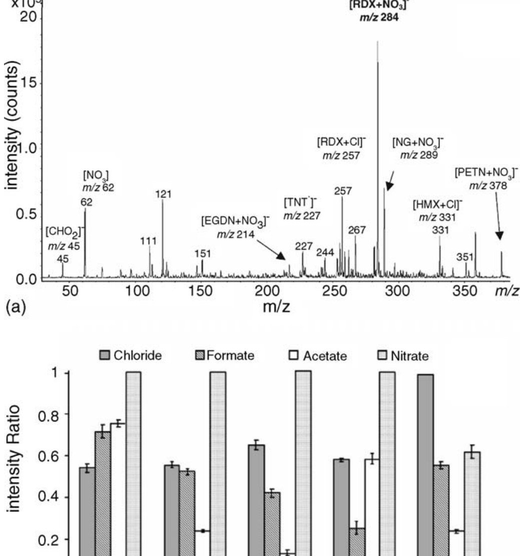

Fig. 2. (a) Mass spectrum of a mixture of high explosives containing EGDN, NG, TNT, PETN, RDX and HMX in 50% MeOH/50% aqueous mixture with 0.3 mM ammonium chloride, ammonium formate and ammonium nitrate. (b) Relative stability of high explosive adducts of chloride, formate, acetate and nitrate using negative ion ESI-MS. From Ref. [18] with permission.

concentration of molecules expected to reach the gas phase is low. Sanchez et al. [19] have developed a method for the sampling and identification of nitroaromatic explosives. Air was sampled at flow rates of up to 15 L/min using a holder fitted with a C_{18} solid-phase extraction (SPE) membrane. After sampling, trapped analytes were desorbed on-line and analysed by LC–MS/MS using an APCI interface. Storage stability studies indicated that the captured analytes were stable for 1 week or 3 weeks, when membranes were stored at room temperature or at −4 °C, respectively. The method allowed the identification and separation of most of the isomers of TNT and 2,4-dinitrotoluene (DNT); limits of detection were in the range of femtogram/L. The method is suitable for the chemical profiling of military-grade explosives and is valuable for both forensic identification and for de-mining purposes.

2.3. Dyes

Textile fibres found at a crime scene can be used as chemical evidence in a wide range of crimes; dye identification and comparison can be of particular importance. Recently, Huang et al. [20] have used LC–MS to enable unambiguous differentiation between structurally related textile dyes which were previously indistinguishable by UV–vis absorption profile or by microspectrophotometry. They concluded that where single-stage LC–MS fails to differentiate, analysis should be extended to include LC–MS/MS of the extracted dye mixture.

The group of colour additives known as the Sudan dyes are synthetically produced azo-dyes. Their degradation products are considered to be carcinogens and teratogens. Due to this fact their use as food additives is banned in the USA and the European Union (EU). However, in some countries they are still used to enhance the colour of bell pepper and chilli powders. The discovery of a batch of chilli powder contaminated with Sudan I in February 2005 resulted in the largest product recall in British history [21]. The widespread use of this batch of chilli powder led to the withdrawal of hundreds of food products including Worcester sauce, pizza and seafood sauces. Calbiani et al. [22] reported a LC–MS/MS (nominal mass/low resolution triple quadrupole) method for the simultaneous analysis of four Sudan dyes in foodstuffs (Fig. 3). More recently, this group have used capillary LC in conjunction with high-resolution MS instrumentation to further distinguish between isobaric ions and to further increase confidence by providing elemental composition [23]. Using exact mass in both MS and MS/MS experiments, they were able to provide unambiguous confirmation of Sudan I in authentic food samples.

Pepper sprays are readily available to law-enforcement personnel and to the general public for a variety of uses including riot control and self-defence. In these cases, the presence of pepper sprays, on clothing for example, may help to determine the facts of an incident. A common active ingredient of these sprays is capsaicin, an oily resin extracted from capsicum fruits. Some of the pepper sprays also contain a coloured dye or a UV-activated fluorescent marker to permit the localisation of the product. However, there are now a number of products on the market that do not contain such visible aids to analysis. Cavett et al. [24] have developed a method to initially visualise colourless pepper sprays on fabric and to subsequently confirm the presence of naturally occurring and synthetic capsaicinoid molecules. Visualisation was achieved by chemical derivatisation of the capsaicinoids using a diazonium salt. Identification of the capsaicinoids and their derivatives was then accomplished following methanolic extraction from the garment. Extracts were analysed within 6.5 min, using a YMC Basic column in conjunction with LC–APCI-MS detection. Work is on-going to confirm the spectra and proposed fragment ions of the derivatives via MS/MS and exact mass determination.

3. Drugs of abuse in alternative matrices

For the detection of illicit drugs, plasma and urine are currently the most common matrices investigated. However, over the past few years there has been an increased interest in the use of more convenient, less invasive specimens, e.g. hair, oral fluid and sweat, to document drug use and exposure [25,26]. Indeed in April 2004, the US Department of Health and Human Services proposed new guidelines for the use of these alternative specimens as an adjunct to urine, for the testing of employees in a number of situations including; pre-employment, random, reasonable cause and post-accident testing [27]. For these samples, collection is relatively easy to perform and requires no

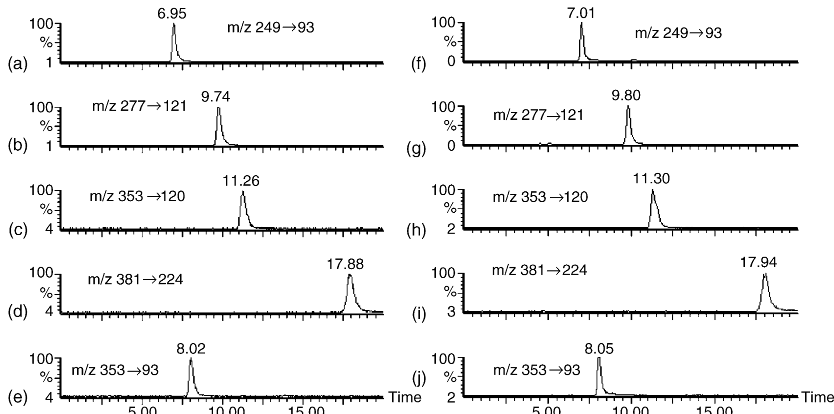

Fig. 3. LC–ESI–MS/MS MRM traces obtained from 125 μg/L standard solution of (a) Sudan I; (b) Sudan II; (c) Sudan III; (d) Sudan IV; (e) Disperse Orange 13 internal standard (100 μg/L; left column) and from a blank chilli tomato and cheese sauce sample spiked with 125 μg/L each (1685 μg/kg sample) of (f) Sudan I; (g) Sudan II; (h) Sudan III; (i) Sudan IV; (j) Disperse Orange 13 internal standard (100 μg/L; right column). From Ref. [22] with permission.

special equipment or facilities. Furthermore, collection can be supervised, thus reducing the opportunity for sample adulteration. One of the main disadvantages however, of using these alternatives is that the volume or amount of sample is usually limited, consequently highly sensitive confirmatory techniques such as LC–MS/MS become a necessity.

3.1. Hair

In addition to the convenience of sample collection, any drugs and metabolites incorporated into hair, tend to persist much longer than in conventional specimens. Recently, hair has been used to document drug exposure in a variety of scenarios such as forensic and workplace testing [28–30], to monitor compliance to drug therapy [31,32] and particularly for investigating cases of drug-facilitated crimes (DFC) [33–43]. The availability of standard reference materials for drugs of abuse in hair is vital and enables those laboratories performing hair analysis to check the accuracy of their methods [44].

Over the last few years DFC, e.g. sexual assault and robbery, have been increasing; these crimes are often difficult to prove due to factors such as the low concentrations of drugs used, or their rapid clearance from the body. In addition, many victims of DFC do not report an incident until several days later, often due to the amnesia caused by the drug. Hence, conventional specimens such as blood or urine may have limited value. Hair samples have been successfully used to document cases of DFC involving a variety of drugs including; benzodiazepines and the hypnotics (zolpidem and zopiclone), methadone and buprenorphine [33–43]. Kintz and co-workers concluded that due to the extremely low concentrations of drugs typically encountered in hair analysis (low pg/mg) the “sensitivity of LC–MS/MS appears to be a pre-requisite to document any case involving drug-facilitated sexual assault”. However, they also added the caveat that hair analysis should not simply be considered as an *alternative* to blood and urine testing but as a complementary technique where possible. The importance of this was revealed in a controlled study to investigate the window of detection for lorazepam in urine, oral fluid and hair [39]. Following a single (2.5 mg) dose, the drug could be still be detected in urine and oral fluid for 144 and 8 h, respectively, after dosing. However, they were unable to detect lorazepam in hair samples collected 4 weeks after administration.

Cheze et al. [42] used LC–MS/MS to conduct a survey into the drugs most commonly used to commit DFC in Paris over the period from June 2003 to May 2004. Out of the total of 128 cases investigated, 18% were proven DFC cases and they found a high prevalence of zolpidem and clonazepam, followed by bromazepam, nordazepam and midazolam (Fig. 4).

Laloup et al. [43] recently reported a LC–MS/MS method for the simultaneous analysis of 26 benzodiazepines and metabolites, zolpidem and zopiclone in blood, urine and hair. The

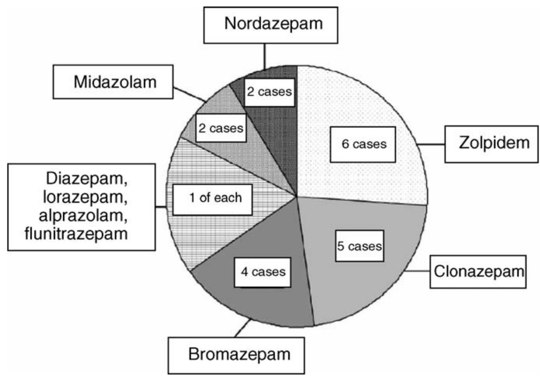

Fig. 4. Distribution of benzodiazepines and benzodiazepine-like hypnotics in 23 cases of proved DFC. From Ref. [42] with permission.

method was applied to authentic samples from both clinical and forensic cases, including the analysis of hair from a woman who claimed to have been drugged and sexually abused over a period of several years. Thirty-three centimetre lengths of hair were submitted for analysis and cut into 1–3 cm sections; all segments were found to be positive for more than one benzodiazepine, indicating multiple drug exposure, with higher concentrations closer to the root. These results demonstrated the utility of hair to provide a long-term drug history.

3.2. Oral fluid

The use of oral fluid as an alternative specimen is also increasing in popularity especially for monitoring recent drug use within the workplace, at the roadside, in prisons and to check compliance to medication.

Concheiro et al. [45] developed a method for the quantification of the active constituent of cannabis, i.e. Δ^9-tetrahydrocannabinol (Δ^9-THC) in oral fluid. Samples were collected by spitting into polypropylene tubes. Two hundred microlitres of sample was processed using liquid/liquid extraction (LLE) with hexane followed by analysis using LC–MS. Limits of detection of 2 μg/L were achieved. Wood et al. [46] reported a validated method for the simultaneous analysis of six amphetamines in oral fluid (also collected by expectoration). The procedure required only 50 μL of sample to achieve limits of detection of 2 μg/L or better and comprised rapid and simple sample preparation, i.e. protein precipitation (PPT) using methanol followed by LC–MS/MS. Dams et al. [47] described a method for methadone and multiple illicit drugs in addition to their metabolites in oral fluid. Their method also involved PPT using acetonitrile followed by LC–MS/MS analysis. The method proved useful for determining methadone concentrations in pregnant opiate and/or cocaine addicts.

Although the methods referenced above utilized oral fluid that has been collected by expectoration, it should be noted that the increased interest in oral fluid has also been accompanied by an increase in the availability of specialized collection devices; these promise a simplified, more controllable collection and sample stability. The final choice of oral fluid collection system, however, has been shown to have serious implications on drug analysis [48–50]. The Intercept is a US Food and Drugs Administration (FDA) approved sampling device that is used on a large scale in the USA for workplace drug testing and is one of the devices currently under investigation in a joint roadside study between the EU and the USA to detect driving under the influence of drugs [51]. The collection system contains additives which can cause problems, e.g. ion suppression during LC–MS/MS analysis in the absence of a suitable clean-up method. Several groups have employed LLE (with hexane) to prepare the so-called ‘preserved oral fluid’ specimen prior to analysis; drugs of interest have included Δ^9-THC, benzodiazepines and hypnotics [52,53]. A SPE method has also been developed, which is combined with LC–MS/MS to allow the simultaneous determination of a panel of common basic illicit drugs [50]. Work is underway to extend the current panel of analytes to include Δ^9-THC [54].

Other groups have used different devices to collect samples for the purpose of drug monitoring. Wylie et al. [55] developed a method for the analysis of 49 licit and illicit drugs in oral fluid collected using the Omni-Sal device. Samples were extracted using SPE and then analysed by LC–MS/MS and GC–MS.

Recently, Teixeira et al. [49] used the Salivette device to collect samples and to quantify Δ^9-THC in oral fluid samples following SPE and LC–MS.

A method was developed for the separation of the enantiomers of methadone and its metabolite EDDP in saliva [56]. Methadone is administered therapeutically as a racemic mix, i.e. a 50:50 mix of the enantiomers. There are significant differences between the enantiomers in terms of receptor affinity, analgesic potency and pharmacokinetic profiles. Thus, therapeutic monitoring of this agent and its metabolite requires an enantioselective technique. Samples were collected using the Salivette device. Following centrifugation, analytes were separated using an immobilized α1-acid glycoprotein chiral stationary phase (AGP-CSP) in conjunction with MS detection. The optimized and validated method was applied to the analysis of samples collected from patients following a methadone maintenance program.

3.3. Meconium

Drug abuse during pregnancy is a major problem and has been associated with prenatal complications and high morbidity and mortality rates of newborns. Some birth defects are thought to be related to fetal exposure to drugs. Detection of in utero drug exposure has traditionally been accomplished by urine drug testing. However, this only reflects maternal drug use over the last 3–4 days and abstinence of the mother for several days prior to delivery, may produce a negative result. Monitoring exposure through testing of alternative matrices, such as neonatal meconium and hair, offers advantages including non-invasive collection and detection earlier in gestation [57–59].

Meconium is the first fecal matter produced by the neonate typically within the first 5 days after birth. Since the formation of meconium starts between the 12th and 16th week of gestation and accumulates in the fetal bowel until birth, use of this specimen can extend the window of drug detection considerably, i.e. to approximately the last 20 weeks of pregnancy.

Pichini et al. have described methods for the analysis of opiates and cocaine and respective metabolites [60] and for the analysis of amphetamine derivatives in this specimen [61]. In both cases samples were prepared by SPE and analysis was achieved using LC–MS (three qualifying ions per compound). Sensitivity was sufficient to allow the detection of all drugs in the low nanograms per gram meconium. Another report describes the application of LC–MS/MS for the simultaneous quantification of methadone and its metabolites in meconium after methanolic extraction followed by SPE [62]. This method represents an improvement over previous methods in terms of sensitivity and specificity and was successfully applied for the quantification of these compounds in meconium from infants whose mothers were maintained on methadone during pregnancy.

3.4. Post-mortem alternative specimens

Clearly, alternative specimens can prove invaluable for the documentation of drug use in the living person. This can also be true for post-mortem investigations. Toxicological analysis of the usual post-mortem specimens can often pose special difficulties. This may because of the decomposed nature of the specimens themselves and/or the presence of putrefactive compounds. In the absence of any suitable tissues or fluids, insects have been proposed as reliable alternate specimens and indeed have been used to identify the presence of various drugs within the cadaver [63,64]. Although the involvement and contribution of the identified drugs to the actual death may be questionable, the insect tissues have, nevertheless, proved a useful sample.

Wood et al. [65] presented a method for the simultaneous analysis of 10 benzodiazepines in larvae and puparia of the *Calliphora vicina* (Diptera, Calliphoridae). Benzodiazepines are the most widely prescribed psychoactive active drugs in the world. However, they are frequently misused and are consequently often encountered in post-mortem analysis. Larvae were prepared by homogenization followed by precipitation using acetonitrile. Puparia were pulverized in a ball mill and then extracted by ultrasonification in methanol. All extracts were subsequently analysed using LC–MS/MS. The utility of this method was confirmed through its application to the analysis of larvae and puparia that had been reared on media spiked with a range of concentrations of nordiazepam. The concentrations were equivalent to those expected in skeletal muscle following fatal human overdoses. Both the parent drug and its metabolite oxazepam could be detected in single larvae or puparia.

Pien et al. [66] extended these preliminary studies to investigate the effects of different concentrations of nordiazepam on larval development and growth. Larval development can be used in the estimation of post-mortem interval. In some cases, the presence of drugs has been shown to affect development of the insect, consequently these disturbances can have serious implications on the accuracy of post-mortem interval calculations.

4. Systematic toxicological analysis using liquid chromatography–mass spectrometry

The ability to screen for a large number of unknown analytes in human samples is of interest to many areas of society including hospital emergency departments, forensic pathologists, police/prison officials and employers. Currently, the ‘gold standard’ for screening is GC–MS, owing in part, to the specificity and sensitivity of the technique, but also as a result of the availability of large libraries of standardized electron ionisation (EI) mass spectra. However, since GC–MS is limited to the analysis of volatile and thermally stable compounds, and because the technique usually requires a specific derivatisation for polar analytes, alternative methods have been investigated. One of the most important questions at present is: To what extent can LC–MS be applied to the search for unknown substances? Although its use as a confirmatory tool is becoming more widely accepted, its use for screening purposes is still not fully established. This is reflected in the many different LC–MS strategies currently being investigated for systematic toxicological anaysis (STA).

4.1. Targeted screening using liquid chromatography–tandem mass spectrometry

One approach for screening is to use LC-multiple (or selected) reaction monitoring (MRM or SRM) analysis. The clear advantage of MRM analysis is in its specificity (sensitivity); a precursor ion from the targeted molecule is selected by the first quadrupole or mass filter, fragmented in the collision cell and then a structurally significant or specific product ion (or ions) selected by the second quadrupole filter. For confidence in identification, several MRM channels should be included per compound and their ratios calculated (and compared to standards). The main drawback in terms of screening, is that the technique relies on the selection of a pre-defined precursor ion in the first quadrupole. Clearly, this is not applicable to the analysis of complete unknowns but rather is a method targeted against a panel of known/expected compounds. Nevertheless, this approach has been used successfully for single or multiple drug classes in a variety of biological matrices. However, due to the relatively low number of analytes often included, its utility for screening of real toxicological samples may be limited. In an attempt to circumvent this, investigators have attempted to create targeted methods for much larger panels of drugs. In 2003, Gergov et al. [67] reported a qualitative screening method for 238 drugs in blood. Following a two-step LLE procedure, compounds were separated on a C_{18} column. For each compound, identification was based on a single MRM channel and retention time (t_R). For 80% of the drugs investigated, the analytical sensitivity was sufficient to detect at therapeutic concentrations in blood. Although clearly a useful method, the high number of analytes meant that dwell times had to be low (25 ms), typically resulting in ∼4 data points across a chromatographic peak; whilst suitable for qualitative purposes, this is insufficient for accurate quantification which really requires 10–15 data points. Furthermore, such limitations on dwell times also meant that any attempts to try to increase the panel of drugs further, would be problematic without providing better chromatographic separation between compounds or by using instrumentation with faster scan capabilities.

Müeller et al. [68] recently presented a novel targeted method for the analysis of 301 compounds. In this procedure an initial ‘survey’ scan was performed which involved monitoring 301 MRM channels. An intensity threshold was set and if any MRM exceeded this pre-set threshold, the instrument was instantly switched into ‘enhanced parent ion’ (EPI) scan mode. Product ions were generated by acquisition at three different collision energies, i.e. 20, 35 and 50 eV and the resultant spectra subsequently searched against a database. The developed method was successfully applied to blood and urine from forensic cases. This procedure also met with the same limitations as the previous method, i.e. utilisation of the short dwell times means that this technique is currently limited to ∼300 analytes; better chromatographic separation will be required if this is to be increased in the future.

4.2. Liquid chromatography–mass spectrometry with reference libraries

An alternative to the use of MS/MS is single-stage MS. With this technique, collision-induced dissociation (CID) can be performed within the ion source region of the instrument and, by employing different voltage settings on the cone or orifice, can be used to generate reference libraries containing structural information.

In some cases, a targeted panel approach is still used. Maurer and co-workers have developed several LC–MS methods for the screening of separate panels of drugs in plasma including; benzodiazepines and benzodiazepine-receptor agonists, beta-blockers and neuroleptics [69–71]. Following sample clean-up and LC separation, analytes were detected using full MS scan in positive ionisation mode. Spectra were acquired throughout the chromatographic analysis at two fragmenter voltages, i.e. 100 and 200 V and compared then to the library spectra. Relative retention time (RRT) to the internal standard was used in cases of doubt.

A more general, less targeted approach has been used by some groups by employing larger spectral databases. In the creation of these databases two concepts have been used, i.e. the use of single or multiple spectral entries per compound. Marquet and co-workers have created libraries which are based on single composite spectra. Data were acquired throughout the chromatographic run in both positive *and* negative ionisation modes, and at both a low and a high cone voltage setting, i.e. 20 and 80 eV. Thus, for each ionization mode a single reconstructed spectrum was produced by summing the low and high spectra [72,73]. The method was evaluated by comparison to their other routinely used reference techniques including GC–MS (underivatised) and HPLC-diode array detection (DAD) and was shown to perform well in the identification of unknowns in 51 serum samples. Seventy-five percent of compounds were detected using the LC–MS method, compared to 66 and 71%, for GC–MS and HPLC-DAD, respectively. The authors concluded that the LC–MS technique performed at least as well as their other standard reference techniques and that in future all three analytical techniques would be employed simultaneously in their routine work.

Humbert et al. [74] created a LC–MS CID library for more than 500 toxicologically relevant compounds based on multiple spectral entries/analyte. Data were acquired using up to six different cone voltages in positive ionisation mode and six in negative ionisation mode during a 30 min chromatographic separation. Each analyte could be therefore be characterized by a maximum of 12 spectra in combination with t_R. The technique was assessed by the analysis of authentic serum and urine samples and performed well in comparison to their existing screening methods, i.e. LC-DAD and immunoassay. More recently, Humbert and co-workers have been investigating the feasibility of a generic sample preparation method. Currently they are evaluating the utility of LLE (both acid and basic) and an optimized SPE procedure using MCX cartridges. Future investigations are likely to include the possibility of an on-line version of the SPE protocol [75].

To date, the major obstacle for the wide-spread adoption of LC–MS in combination with reference libraries is considered to be the variability of spectra between instruments; not only those produced as a result of in-source CID but also those produced in the collision cell when performing classical MS/MS on a tandem MS instrument. Several groups have studied this phenomenon. Jansen et al. [76] showed relatively poor MS/MS spectral reproducibility both inter- and intra-instrument, despite an attempt to standardise the fragmentation using a reference compound. Spectra were studied on one instrument operated in EPI mode with the same instrument operated in standard product ion scan (PIS) mode. In addition, they studied PIS spectra obtained using instruments from two other manufacturers. Since the main differences appeared to be in relative ion intensities rather than the actual ions present, they concluded that in library searching algorithms it might be prudent to assign a heavier weight to the *m*/*z* ratio rather than the relative intensity in the mass spectra. These findings were in contrast to Gergov et al. [77] who compared MS/MS spectra from a total of four instruments. Two of the instruments were identical but installed in separate toxicology laboratories; the other two instruments were from two other manufacturers. All instruments were operated in PIS mode using three different collision energies. Identical instruments showed good agreement for MS/MS spectra. Following standardization of instrument parameters, very good agreement was also observed between the other brand instruments. Consequently, they concluded MS/MS spectral libraries were indeed suitable for inter-laboratory use after tuning.

4.3. Liquid chromatography–(tandem) mass spectrometry with exact mass

The undeniable appeal of using TOF-MS for drug analysis is related to its determination of the exact mass of the analytes, i.e. mass assignment accurate to four decimal places. The ability to determine the *m*/*z* of an ion to within 5 parts per million (ppm) allows the determination of a unique ionic formula based on the mass sufficiency of the constituent atoms. The ability to closely match the expected/theoretical mass with the observed mass greatly increases the confidence of identification; effectively removing matrix effects, reducing background signal from interferences and by reducing the number of other possibilities for identification. In other words, the better the accuracy and precision of the mass measurement, the fewer the number of compounds theoretically possible. Nowadays most LC-TOF instrumentation is capable of routinely offering mass accuracies of 5 ppm or better. Furthermore, when operated in full scan mode, these instruments can provide significantly better sensitivity compared to a quadrupole instrument. The last few years have seen some notable developments in both hardware and software which have led to an overall increased robustness of LC-TOF, e.g. the facility to allow the simultaneous introduction of a reference or ‘lock-mass’ which serves to ensure accurate mass assignment throughout the analytical run. In the past, one of the disadvantages of TOF-MS was its lack of dynamic range; modern instrumentation is now capable of offering linearity of response over 3–4 orders of magnitude.

One of the main limitations in building libraries based on nominal mass is that it requires the analysis of primary reference compounds. Such material is not always easy to obtain, particularly when dealing with metabolites, or the products of the ever-changing designer drugs scene. Owing to the advantage of the exact mass approach, any library can be created or updated without requiring such reference material. Information can be entered simply by provision of the molecular formula and a calculation of the theoretical accurate mass of the target compound. The utility of this was recently demonstrated by Laks et al. [78] who used full scan LC-TOF to analyse street drugs in seized material without reference standards being available. It should be noted, however, that exact mass *alone* still cannot always provide unequivocal identification of compounds, particularly in real forensic samples. This fact was illustrated in earlier work by the same group who developed a novel screening method based on LC-TOF analysis followed by reference to an exact mass database of 637 drugs, metabolites and pesticides [79]. Since a significant number of these compounds, i.e. 245 drugs, were metabolites, reference material (and consequently t_R information) was unavailable. Fifty autopsy urine samples were analysed and the results compared to the reference GC–MS screening method. Parent drugs were classed as positive by LC-TOF according to the following criteria; masses were accurate within 30 ppm and t_R values within 0.2 min. Due to the fact that t_R information for most of the metabolites was unavailable, positive metabolite findings were determined by a combination of theoretical accurate mass and the presence of a specific parent compound (matching the aforementioned criteria). Overall, the number of compounds identified by LC-TOF was higher than GC–MS but, due to some false positives, the authors concluded that a combination of accurate mass, t_R *and* metabolite profiles was really necessary for unambiguous identification of toxicological compounds.

Within the author's own laboratory, work is underway to create a rapid screening method for samples collected in suspected DFC cases [80]. The method is based on ultra performance liquid chromatography (UPLC) in conjunction with TOF analysis. Identification of analytes is based on a combination of exact mass, CID fragmentation patterns and t_R.

4.4. Data (or information)-dependent acquisition

Another attractive possibility for screening is the use of data (or information)-dependent acquisition (DDA or IDA) using LC-TOF instrumentation. This technique is capable of finding true unknowns since the method does not require any pre-selection of masses but rather operates 'on the fly'. Decaestecker et al. [81] investigated the use of a Q-TOF instrument for STA. During the chromatographic run the instrument is initially set to operate in MS mode, i.e. the quadrupole is operated in radio frequency (RF)-only mode and transmits all masses until an ion or ions exceed a certain threshold. Thereupon the instrument instantly switches into MS/MS mode with the quadrupole now selectively transmitting these ions of interest. Subsequent fragmentation within the collision cell leads to the generation of product ions. By limiting the TOF spectral accumulation time in MS/MS mode (to a statistically acceptable minimum) the instrument rapidly switches back into MS mode. Consequently, in a single acquisition, the exact mass of the initial precursor ions *and* any product ions are generated (Fig. 5). This method was used to provide a comprehensive profile of 17 common drugs. The limitations to this technique were the high and variable background signal seen with authentic samples which ultimately led to low overall sensitivity and a lack of linear dynamic range.

More recently, Marquet et al. [82] explored the use of a DDA-based method on a nominal mass instrument for STA. In this procedure the instrument is initially set to operate in full scan ('survey') mode until an ion exceeds a preset threshold at which point the instrument switches into EPI mode. Product ions were generated by acquisition two collision energies, i.e. 20 and 50 eV in positive mode or −15 and −40 eV in negative mode. Resultant spectra were subsequently searched against a database of composite spectra. The performance of the method was assessed by analysis of serum extracts spiked with eight test compounds and compared to the results obtained with their reference method. The method was successful in identifying all eight test compounds. The authors stated that the main difficulties with the technique were related to the settings of the threshold in survey mode; at that time it was only possible to use one threshold setting for all *m*/*z* ratios, thus too low a threshold meant that the method was not selective enough, too high a threshold meant that informative peaks could be lost.

4.5. Sample preparation: considerations for systematic toxicological analysis

Currently there is no established or generic protocol for the preparation of samples for STA; most investigators tend to use some form of LLE or SPE. When analysing samples by LC–MS techniques in particular, adequate sample clean-up has been demonstrated to be critical and close attention must be paid to the possibility of matrix effects, e.g. ion suppression or enhancement [83]. These effects may, or may not, be obvious, especially when taking into account the mode of data collection. For example, the use of exact mass instrumentation can truly reduce matrix effects by narrowing down the mass to such an extent that contaminating ions are 'removed' from the signal unless they fall within a defined ppm range. On the other hand, the use of MRM can essentially 'blind' us to, or mask, any contaminant which may be (and most likely *is*) present in the sample, quite simply because we are choosing to exclude them from our data in the first place.

In an attempt to find a suitable generic sample preparation procedure, Müller et al. [84] assessed the ion suppression effects associated with their own STA methodology (ESI in-source CID/library searching). In these experiments they used a post-column infusion of two test compounds, i.e. codeine and glafenine to determine matrix effects (in positive and negative mode, respectively), after several sample preparation protocols including; LLE, mixed-mode SPE, PPT-only and PPT in combination with polymer-based mixed-mode SPE. The results for these two compounds indicated that their two-step LLE protocol resulted in less ion suppression than the other methods investi-

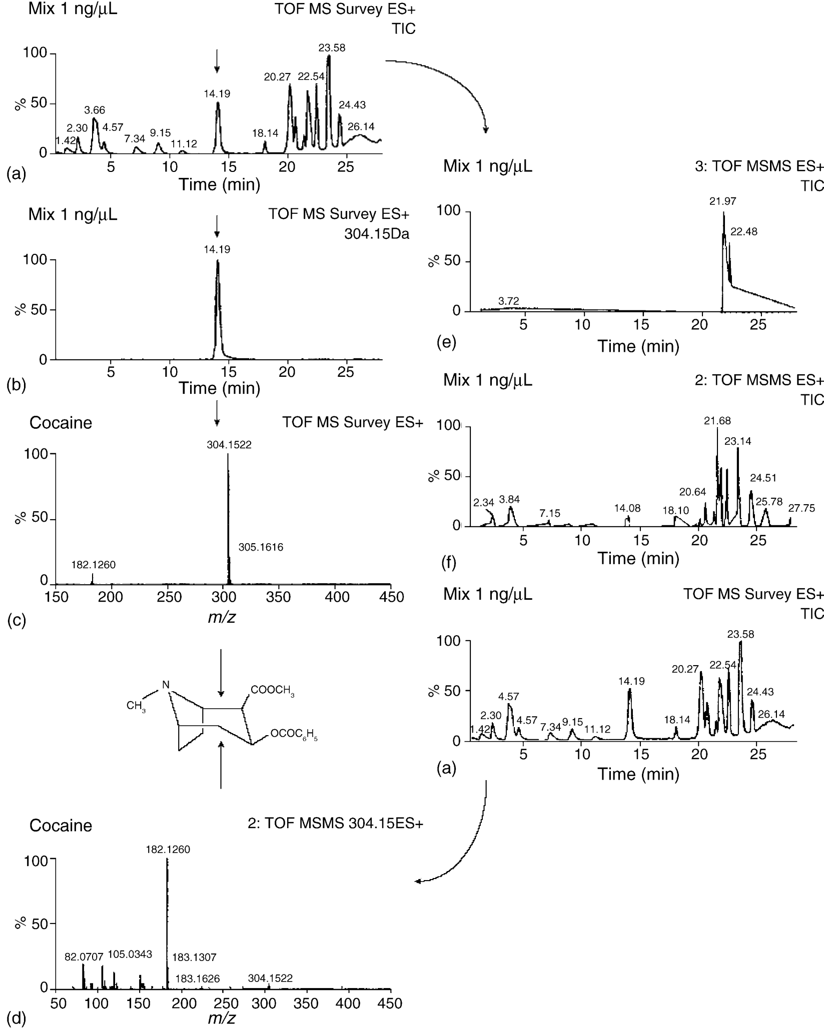

Fig. 5. (a) TIC in the MS mode, (b) MS extracted ion chromatogram of cocaine, (c) MS, (d) MS–MS spectra of cocaine and (e and f) TICs of the first two channels in the MS–MS mode. From Ref. [81] with permission.

gated and that the most critical period for suppression, regardless of the sample clean-up used, was just after the elution of the polar LC front (∼2.9 min). After this, no significant suppression was observed with any sample preparation method.

5. High-throughput liquid chromatographic–(tandem) mass spectrometric analysis

High-throughput analysis is becoming increasingly important in all areas of science; the forensic sciences being no exception. Moreover, due to the development of more potent drugs, drug concentrations in biological samples are often present at much lower levels than before. Therefore, fast analytical techniques with much higher sensitivity and selectivity are needed.

One of the main bottlenecks in bioanalysis is often associated with the sample preparation requirements, especially if the method requires manual extraction techniques. However, automation of off-line SPE is possible and the utility of "96-well SPE" was introduced some years ago. Nowadays an even more attractive approach is on-line SPE; the entire process of conditioning, sample application, washing and elution takes place at constant flow rates yielding better precision of quantitative methods in comparison with off-line vacuum driven extraction procedures. Another important advantage is that no manual

transfers are made and that the whole of the eluate is loaded onto the LC column without the need for a pre-concentration step.

A very elegant system for rapid analysis of complex samples can be obtained by the on-line coupling of SPE or short-column LC to MS detection. In this way, the sample is directly injected into the SPE-MS system and a rate-limiting step is eliminated.

However, some restrictions should be considered, such as the presence of endogenous matrix components, which can interfere in the different stages of the procedure. This is certainly true if no analytical column is present. Bruins et al. [85] developed a SPE-MS/MS procedure for the detection of clenbuterol in urine. They showed that the choice of sorbent was critical for the elimination of matrix effects. In addition, it was noted that, with single stage MS, the obtained sensitivity and selectivity were insufficient. The detection of various opioids using on-line SPE-LC–MS (/MS) has also been described; results were similar (or better) in comparison to their corresponding off-line methods [86,87]. Using the Prospekt-2 system (Spark, Emmen, The Netherlands), Kuhlenbeck et al. [88] developed an on-line SPE-LC–MS/MS method for the determination of dextromethorphan, dextrorphan and guaifenesin, the active ingredients of some cough medications, in human plasma. This system allows the simultaneous extraction of the second sample in one clamp and the elution of the first sample in the second clamp, thus leading to an optimal use of the extraction time.

Another rate-limiting step in bioanalytical analysis is the chromatography, especially when multiple drugs and metabolites are monitored simultaneously. Efforts to increase the throughput of chromatography are hindered by the inherent chromatographic efficiency of traditional columns packed with spherical silica particle phases and the pressure limit on conventional HPLC systems. One approach to resolve these restrictions was the introduction of monolithic columns, which have a bimodal pore structure, characterized by internal macropores and mesopores, providing extended surface area and high permeability for mobile phase solvents. As such, the mass transfer limitations inside the column are reduced, allowing higher mobile phase flows and lower backpressures on the LC system. Several approaches combining this monolithic chromatography with the advantages of on-line SPE have been presented [89–91].

The advent of UPLC technology promises significantly faster chromatography in addition to increased chromatographic resolution and sensitivity. The technology has resulted from a culmination of developments in several areas including LC columns based on smaller particle sizes (1.7 μm) and the necessary hardware to be able to cope with the associated increases in system pressure. Yu et al. [92] described a comparison of UPLC–MS/MS with the standard LC chromatography (also coupled with MS/MS), for the separation of five drugs (ibuprofen, alprazolam, naproxen, prednisolone, diphenhydramine) in rat plasma and noted that UPLC demonstrated improved sensitivity, sharper peaks and faster separation. UPLC–MS was also used for the analysis of seven β-blockers and results compared to those obtained using LC–MS. They concluded that UPLC gave superior separation performance and that the quality of the mass spectra, were at least as good as those obtained with their standard LC conditions [93]. A study conducted by Apollonio et al. [94], demonstrated the benefits of this technique for the determination of the commonly occurring "club" drugs and of the more novel analogues *p*-methoxyamphetamine (PMA), 4-methylthioamphetamine (4-MTA) and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB). Others have reported improved chromatographic resolution and a reduction in ion suppression with their UPLC based methods, ultimately this has led to increased sensitivity and lower limits of detection [95,96].

6. Conclusion

The combination of LC and MS has been used for many years. However, since the introduction of more user-friendly LC-interfaces, e.g. ESI and APCI, there has been a tremendous increase in the popularity of the technique amongst scientists from a wide variety of disciplines. LC–MS has evolved into a robust and reliable tool that also offers versatility, specificity and sensitivity.

For those involved in the forensic sciences in particular, the use of LC–MS has changed considerably. Where it was once a technique that was only used very infrequently, i.e. as an alternative to GC–MS for the more 'troublesome' analytes, it is now used extensively and has proved itself invaluable especially for the analysis of highly polar, involatile and thermolabile compounds.

The analytical environment is a dynamic one. Most technological advances are driven by analytical demands; e.g. the need for faster, more accurate/robust analysis. New and exciting technologies are constantly emerging. One of the most recent is UPLC, its advent promises significantly faster chromatography in addition to improved chromatographic resolution and sensitivity. It seems likely that over the next few years, we will see a significant increase in the use of this technology within the forensic sciences. The increased analytical capabilities afforded by UPLC, in turn drives the need for improved detection systems; in the case of MS detection, this means faster scan capabilities. Thankfully, continual developments in hardware and software lead to more robust, easier-to-use and more accurate instrumentation, all of which, help to establish what might initially be considered to be a 'novel' technique, and one which was only available to 'innovators', into one which is usable in a more routine setting and available to a wider group of analysts.

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Validation of a Liquid Chromatography–Tandem Mass Spectrometry Method for the Simultaneous Determination of 26 Benzodiazepines and Metabolites, Zolpidem and Zopiclone, in Blood, Urine, and Hair

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Abstract

A liquid chromatography–tandem mass spectrometry method was developed for the simultaneous quantification of 26 benzodiazepines and metabolites, zolpidem and zopiclone, in blood, urine, and hair. Drugs were extracted from all matrices by liquid–liquid extraction with 1-chlorobutane. Chromatography was achieved using a XTerra MS C18 column eluted with a mixture of methanol and formate buffer. Data were acquired using positive electrospray ionization and multiple reaction monitoring using one precursor ion/product ion transition per compound. Quantification was performed using 13 deuterated analogues. Further confirmation of the identity of the compounds was achieved through a second injection of positive samples, monitoring two transitions per compound. The limits of quantification for all benzodiazepines ranged from 1 to 2 ng/mL in blood, 10 to 25 ng/mL in urine, and 0.5 to 10 pg/mg in hair. Linearity was observed from the limit of quantification of each compound to 200 ng/mL, 1000 ng/mL, and 1000 pg/mg for blood, urine, and hair, respectively ($r^2 > 0.99$). Precision for quality control samples, spiked at three concentrations, was calculated (CV < 20% in most cases). Extraction recoveries for the three matrices ranged from 25.1 to 103.8%, except for one compound (cloxazolam in urine). Ion suppression was studied for all matrices. The validated assay was applied to authentic blood, urine, and hair samples from forensic cases.

Introduction

Benzodiazepines are a large class of prescribed psychoactive drugs used widely for different medical conditions such as the symptomatic treatment of anxiety and sleep disorders, the treatment of anxiety-related conditions, and as anti-convulsants (1). Benzodiazepines bind with high affinity to the β-subunit of the $GABA_A$ receptor in the central nervous system. Zolpidem and zopiclone are structurally different from the benzodiazepines, but also bind to the $GABA_A$ receptor. They provoke the same (side-) effects as the benzodiazepines, including sedation and hypnosis. Chronic use can generate tolerance to the effects.

Because of their wide therapeutic index, benzodiazepines have a low risk of serious adverse reactions and toxicity. Unfortunately, misuse of these compounds is often reported (1–4), and they are often implicated in connection with various types of crime, such as murder and drug-facilitated assault (5), in suicide attempts (6), and, due to their interference with cognitive and psychomotor functions, in road traffic accidents (7,8). Consequently, benzodiazepines are frequently encountered in clinical and forensic toxicology cases.

Blood and urine are the conventional specimens to document drug exposure. Hair analysis has proven to be a reliable indicator of past drug abuse, as a complement to blood or urine analysis, for proving or excluding chronic drug use, or, at least, exposure to drugs; therefore it becomes highly useful in monitoring long-term histories of drug abuse (9). As a biological matrix, hair offers particular advantages: it can be easily obtained without violating individual privacy and, due to its stability, it can be stored and transported without requiring specific precautions. However, the extraction and detection of benzodiazepines from hair samples is relatively difficult due to the low levels of drugs that are incorporated into the hair. Therefore, highly sensitive analytical techniques are required for trace-level quantification of benzodiazepines.

A number of studies have been reported on the qualitative and quantitative analysis of benzodiazepines and their metabo-

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Table I. MRM Transitions and Conditions for all Compounds and Their Deuterated Analogues*

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
7-Aminoclonazepam	286.00	120.80	40	28
		250.10	40	20
7-Aminoflunitrazepam	284.00	134.80	40	28
		227.00	40	25
Bromazepam	315.90	181.90	40	30
		209.00	40	25
Clonazepam	315.90	214.00	38	35
		270.00	38	25
Flunitrazepam	314.00	239.10	43	35
		268.10	43	25
Clobazam	301.00	224.00	35	33
		259.00	35	20
Desmethylflunitrazepam	300.00	198.00	40	38
		254.10	40	25
Estazolam	295.00	205.00	35	40
		267.10	35	23
Nitrazepam	282.00	180.00	38	35
		236.10	38	25
Alprazolam	309.00	205.00	45	43
		281.10	45	43
Chlornordiazepam	304.90	139.80	45	30
		206.00	45	35
Temazepam	300.90	255.00	28	20
		283.00	28	15
Desalkylflurazepam	289.00	139.80	43	28
		226.00	43	28
Oxazepam	287.00	241.00	27	22
		269.00	27	15
Nordiazepam	271.00	139.80	43	28
		164.80	43	28
Brotizolam	394.90	279.10	40	28
		314.00	40	23
Triazolam	342.90	308.10	45	25
		315.00	45	30
Lormetazepam	334.90	176.90	25	40
		289.00	25	22
Lorazepam	320.90	229.00	30	30
		275.00	30	22
Prazepam	325.00	139.80	40	38
		271.10	40	22
Clotiazepam	319.00	153.80	40	28
		291.10	40	23
Tetrazepam	289.00	225.10	45	28
		253.10	45	25
Diazepam	285.00	153.80	40	28
		193.00	40	30
Loprazolam	465.00	84.70	48	23
		110.80	48	25
Flurazepam	388.00	288.10	35	25
		315.10	35	23
Cloxazolam	349.00	139.80	40	38
		164.80	40	35
Zolpidem	308.10	91.70	45	50
		235.10	45	35

continued

* Underlined transitions were used for quantification.

lites in different biological matrices. Several techniques have been used for the toxicological analysis of benzodiazepines in blood, urine, or hair, including immunoassays (10–14), gas chromatography (GC) (11,14–19), and high performance-liquid chromatography (HPLC) (11,15,20–27). However, most methods covered only a single substance or mixtures of a few benzodiazepines in only one biological matrix.

Until now, only three reports dealing with the simultaneous analysis of a large series of benzodiazepines have been published. Pirnay et al. were the first to publish a GC–mass spectrometry (MS)–MS ion trap method covering 22 benzodiazepines in urine and blood extracts after trimethylsilylation of the drugs (28). However, due to partial or full thermal degradation of some benzodiazepines during the injection step [a phenomenon already reported by Japp et al. and Weston et al. (29,30)], decomposition products had to be taken into account in the method. Detection thresholds for this method were in the range of 10–500 ng/mL, except for the triazolo-benzodiazepines (alprazolam, estazolam, and triazolam), for which the detection threshold was 1000 ng/mL, due to poor chromatographic resolution.

The ability of HPLC to separate a large range of underivatized substances, coupled with the milder working conditions of the technique, makes it particularly valuable for the analysis of some of the more thermolabile benzodiazepines. Kratzsch et al. reported on a validated LC–atmospheric pressure chemical ionization-MS method for the screening of 23 benzodiazepines (their antagonist flumazenil and zaleplon), zolpidem, and zopiclone in plasma using liquid–liquid extraction (31). After screening and identification in the scan mode using an LC–MS library, the analytes were quantified in the selected-ion monitoring mode. The limit of quantification (LOQ) ranged from 0.5 to 200 ng/mL. Smink et al. described an LC–MS(–MS) ion trap method after solid-phase extraction for the determination of 33 benzodiazepines, metabolites, and benzodiazepine-like substances in whole blood (32). The LOQ for this method ranged from 0.4 to 41.9 ng/mL.

In this report, a validated and highly sensitive LC–electrospray ionization

Table I (continued). MRM Transitions and Conditions for all Compounds and Their Deuterated Analogues*

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Zopiclone	277.00	111.80	35	43
		<u>217.00</u>	35	25
7-Aminoclonazepam-d_4	290.00	120.80	40	30
7-Aminoflunitrazepam-d_7	291.10	137.90	40	28
Clonazepam-d_4	320.00	274.10	40	25
Flunitrazepam-d_7	321.10	275.20	40	25
Alprazolam-d_5	314.00	286.10	45	25
Temazepam-d_5	305.90	260.10	25	23
Desalkylflurazepam-d_4	293.00	139.80	45	30
Oxazepam-d_5	292.00	246.10	40	23
Nordiazepam-d_5	276.00	139.80	45	28
Triazolam-d_4	349.00	314.10	40	28
Lorazepam-d_4	326.90	281.00	30	20
Prazepam-d_5	330.00	276.10	40	22
Diazepam-d_5	290.00	153.80	40	28

* Underlined transitions were used for quantification.

(ESI)-MS–MS method is described for the quantification of 26 commonly encountered benzodiazepines and their metabolites, zolpidem and zopiclone, in three different biological matrices (i.e., blood, urine, and hair). This method covers more than 85% of all benzodiazepines and benzodiazepine-like substances registered for the Belgian market and was applied to authentic blood, urine, and hair samples from clinical and forensic cases.

Experimental

Chemicals

Individual ampoules of 7-aminoclonazepam, 7-aminoflunitrazepam, bromazepam, clonazepam, flunitrazepam, clobazam, desmethylflunitrazepam, estazolam, nitrazepam, alprazolam,

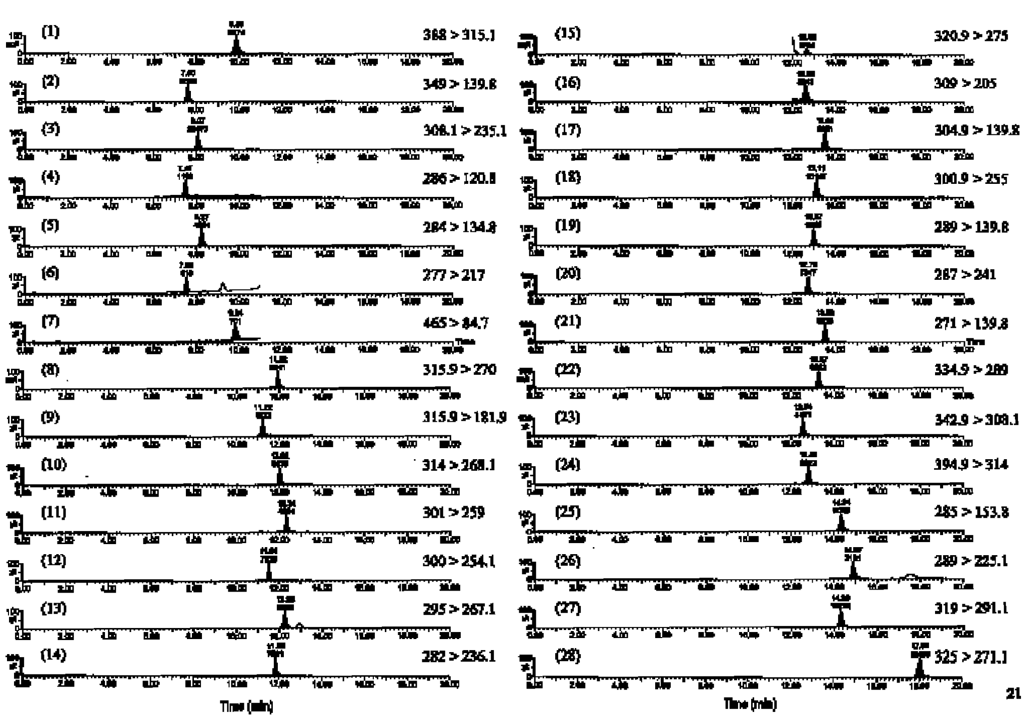

Figure 1. MRM chromatograms obtained following the analysis of a spiked hair sample with 10 pg/mg of flurazepam (1), cloxazolam (2), zolpidem (3), 7-aminoclonazepam (4), 7-aminoflunitrazepam (5), zopiclone (6), loprazolam (7), clonazepam (8), bromazepam (9), flunitrazepam (10), clobazam (11), desmethylflunitrazepam (12), estazolam (13), nitrazepam (14), lorazepam (15), alprazolam (16), chlornordiazepam (17), temazepam (18), desalkylflurazepam (19), oxazepam (20), nordiazepam (21), lormetazepam (22), triazolam (23), brotizolam (24), diazepam (25), tetrazepam (26), clotiazepam (27), and prazepam (28).

Table II. Intra-assay and Interassay Precision and Bias of the QC Samples, Prepared in Blood, Spiked at a Concentration of 4.0, 12, and 100 ng/mL

Compound	Concentration of QC (ng/mL)	Intra-assay Precision		Interassay Precision	
		RSD	Bias (%)	RSD	Bias (%)
7-Aminoclonazepam	4	2.3	–2.8	3.1	–1.9
	12	1.0	1.3	2.0	0.0
	100	1.6	2.3	3.1	0.5
7-Aminoflunitrazepam	4	2.6	0.0	2.6	2.7
	12	0.4	6.5	1.2	9.3
	100	1.1	2.2	2.5	4.0
Bromazepam	4	5.9	–5.4	10.4	–1.7
	12	2.8	–8.4	4.7	–4.0
	100	5.3	–7.6	4.9	–1.1
Clonazepam	4	1.0	–4.7	2.2	–0.2
	12	2.3	2.3	2.8	2.6
	100	1.6	4.4	2.5	3.7
Flunitrazepam	4	2.4	–0.9	5.1	–2.0
	12	2.4	0.9	2.9	–0.2
	100	2.1	1.7	2.1	0.6
Clobazam	4	4.0	–1.9	5.1	1.2
	12	2.6	6.3	4.7	5.6
	100	3.1	4.5	4.4	3.7
Desmethylflunitrazepam	4	2.8	–18.0	10.6	–11.0
	12	2.5	–12.9	10.6	–8.1
	100	3.4	–13.7	9.6	–7.6
Estazolam	4	4.0	–3.6	4.2	–1.4
	12	3.9	3.0	5.1	2.2
	100	2.2	2.8	3.0	3.0
Nitrazepam	4	1.7	–8.8	7.7	–4.0
	12	1.6	–6.2	5.8	–1.9
	100	5.0	–11.6	6.9	–4.9
Alprazolam	4	6.3	6.0	6.5	14.5
	12	3.8	–4.6	4.4	–1.9
	100	3.2	0.8	3.0	0.2
Chloornordiazepam	4	3.2	–0.3	5.1	–1.3
	12	4.9	4.2	6.0	0.5
	100	2.1	7.6	4.3	3.1
Temazepam	4	3.1	0.6	5.1	–1.0
	12	3.0	1.8	3.7	–0.3
	100	2.1	2.1	2.0	0.7
Desmethylflurazepam	4	1.1	–1.6	4.6	–1.5
	12	3.2	–0.6	3.5	–0.9
	100	1.3	1.4	2.2	0.9
Oxazepam	4	3.4	–2.8	5.3	1.0
	12	3.0	1.4	5.6	0.8
	100	1.7	0.9	2.7	1.4
Nordiazepam	4	5.4	–17.2	4.3	–2.8
	12	4.3	–2.6	5.2	–0.2
	100	3.7	1.8	4.1	1.4
Brotizolam	4	3.6	–1.8	7.1	0.3
	12	3.1	–0.1	9.4	–1.3
	100	1.3	1.0	6.2	1.6
Triazolam	4	3.4	0.8	6.3	–2.2
	12	6.1	2.1	5.4	0.5
	100	4.2	2.3	4.4	0.4
Lormetazepam	4	3.8	4.2	10.3	3.4
	12	3.6	3.5	9.5	3.0
	100	2.6	8.9	9.7	6.8

continued

temazepam, desalkylflurazepam, oxazepam, nordiazepam, triazolam, lormetazepam, lorazepam, prazepam, tetrazepam, diazepam, and flurazepam (at a concentration of 1 mg/mL in methanol) and 7-aminoclonazepam-d_4, 7-aminoflunitrazepam-d_7, clonazepam-d_4, flunitrazepam-d_7, alprazolam-d_5, temazepam-d_5, desalkylflurazepam-d_4, oxazepam-d_5, nordiazepam-d_5, triazolam-d_4, lorazepam-d_4, prazepam-d_5, and diazepam-d_5 (0.1 mg/mL in methanol) were purchased from LGC Promochem (Molsheim, France). Brotizolam, clotiazepam, chlornordiazepam, loprazolam, cloxazolam, zolpidem, and zopiclone were a gift from Dr. V. Maes (pure standards obtained from respective manufacturers). All solvents were HPLC grade.

Specimens

Pooled blank blood samples were used for development and validation of the procedure for blood and were obtained from a local blood bank. Blank urine and hair samples for the development and validation of the procedure were obtained from drug-free volunteers. Authentic blood, urine, and hair samples were collected during roadside controls for drugged driving, from drug-facilitated sexual assault (DFSA) cases (submitted as a part of the sexual assault kit), suicide cases, and (attempted) murder cases.

Preparation of standard solutions

An internal standard (IS) stock solution of each of the deuterated analytes was prepared (0.4 µg/mL in methanol), which was further diluted with methanol to yield appropriate concentrations to add to samples, calibrators, and quality control (QC) samples.

A benzodiazepine stock solution of all analytes was prepared (4 µg/mL in methanol) and further diluted with methanol to yield working solutions at appropriate concentrations to add to calibrators and QC samples.

Working solutions were prepared monthly and stored at 4°C.

Sample preparation and extraction

Preparation of blood samples. Blood samples (250 µL) were mixed with 50 µL of a 1:4 dilution of the IS stock solution.

Preparation of urine samples. Urine samples (250 µL) mixed with 50 µL of

the IS stock solution were buffered to pH 4.6 with 200 µL acetate buffer (3M) and then incubated for 1 h at 56°C with 25 µL of β-glucuronidase (*Helix pomatia*, 127,300 U/mL) (Sigma, St. Louis, MO).

Preparation of hair samples. After decontamination (twice with dichloromethane, once with water, and once with methanol, 15 min. each, under ultrasonication), hair samples were dried and cut in segments of 1–3 cm each. Approximately 20 mg was powdered in a ball mill, which allowed simultaneous pulverization of 48 segments (Precellys 48, Bertin Technologies, Montigny-Le-Bretonneux, France), and then 50 µL of a 1:20 dilution of the IS stock solution was added. After incubation of the pulverized samples with 1 mL of methanol at 45°C for 2 h with orbital shaking, samples were centrifuged. The supernatants were subsequently transferred to 10-mL disposable screw-top vials and concentrated under nitrogen to 100–200 µL.

Extraction. After sample preparation, samples were extracted with 4 mL of 1-chlorobutane after the addition of a saturated ammonium chloride buffer (pH 9.2); 500 µL was used for blood and hydrolyzed urine samples and 1 mL was used for the pulverized hair samples. After mechanical shaking (10 min) and centrifugation (10 min at 3000 × *g*), the organic phase was transferred to a 5-mL disposable screw-top vial and then evaporated to dryness at 40°C in a vacuum centrifuge. For the extracted urine and blood samples, the residue was reconstituted in 100 µL of 0.1% formic acid in water/methanol (70:30, v/v), and 10 µL was injected into the LC–MS–MS system. The residue of the hair samples was reconstituted in 80 µL of 0.1% formic acid in water/methanol (70:30, v/v), and 20 µL was injected into the LC–MS–MS system.

Table II (continued). Intra-assay and Interassay Precision and Bias of the QC Samples, Prepared in Blood, Spiked at a Concentration of 4.0, 12, and 100 ng/mL

Compound	Concentration of QC (ng/mL)	Intra-assay Precision		Interassay Precision	
		RSD	Bias (%)	RSD	Bias (%)
Lorazepam	4	2.4	–2.0	4.2	–3.7
	12	4.5	1.4	5.4	–0.3
	100	1.5	2.2	2.8	1.9
Prazepam	4	1.6	–8.9	2.3	–6.7
	12	2.7	–2.3	5.4	–4.2
	100	2.3	3.9	4.8	1.3
Clotiazepam	4	1.5	–2.4	4.9	–2.7
	12	2.9	–1.4	6.4	–3.3
	100	3.1	3.5	5.2	2.3
Tetrazepam	4	2.1	–5.5	4.2	–3.3
	12	3.1	–3.0	4.4	–4.6
	100	3.9	0.9	5.1	–0.3
Diazepam	4	2.7	–11.8	3.5	–3.0
	12	2.0	–0.5	4.0	3.3
	100	3.6	2.7	5.0	4.1
Loprazolam	4	2.0	–5.0	15.4	–9.7
	12	8.2	–15.6	19.4	–16.0
	100	9.6	–4.3	19.9	–3.2
Flurazepam	4	9.7	–3.2	12.1	–0.8
	12	7.3	–4.5	10.8	–5.5
	100	9.3	–11.8	9.4	–4.3
Cloxazolam	4	11.7	–11.3	11.5	–6.9
	12	13.4	–9.9	12.0	–9.1
	100	2.5	–6.6	16.6	–9.2
Zolpidem	4	1.3	–5.2	16.9	0.2
	12	3.4	–9.8	16.8	–5.3
	100	3.7	–4.5	13.1	1.7
Zopiclone	4	14.2	–12.5	17.3	–20.0
	12	12.6	–16.7	13.2	–15.0
	100	8.1	–15.0	9.7	–16.3

LC–MS–MS

Chromatography. LC was performed using a Waters Alliance 2690 separation module (Waters, Milford, MA). Analytes were separated on a XTerra MS C18 column (150- × 2.1-mm, 3.5 µm) (Waters), using a gradient elution with 0.1% formic acid (A) and methanol (B), at a flow rate of 0.2 mL/min. A gradient was applied starting from 10% B, and increased to 50% over the first 5 min. From 5 min to 20 min, B was linearly increased to 70% before returning to the initial conditions within 0.1 min and equilibrating for 14.9 min, which resulted in a total run time of 35 min.

MS. A Quattro Premier tandem MS (Waters) was used for all analyses. Ionization was achieved using electrospray in positive mode (ESI+). The optimum conditions were capillary voltage, 1.0 kV; source block temperature, 120°C; desolvation gas (nitrogen) heated to 270°C, and delivered at a flow rate of 700 L/h.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards [200 ng/mL, in 0.1% formic acid in water/methanol (70:30, v/v)] were infused into the MS and the cone voltage (CV) optimized to maximize the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mbar) and the collision energy adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis.

All aspects of data acquisition were controlled using MassLynx NT 4.0 software with automated data processing using the QuanLynx program (Waters).

LC–MS–MS assay validation

Linearity, LOQ, precision, accuracy, and recovery. Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of the deuterated analogues. The different IS were assigned to the different analytes in

Table III. Intra-assay and Interassay Precision and Bias of the QC Samples, Prepared in Urine, Spiked at a Concentration of 40, 150, and 600 ng/mL

Compound	Concentration of QC (ng/mL)	Intra-assay Precision		Interassay Precision	
		RSD	Bias (%)	RSD	Bias (%)
7-Aminoclonazepam	40	1.5	0.3	3.8	5.6
	150	2.5	–4.6	3.0	–3.3
	600	3.8	–4.5	4.6	–5.9
7-Aminoflunitrazepam	40	2.3	8.4	2.8	14.1
	150	2.3	3.2	4.0	11.7
	600	1.6	–1.9	3.2	5.0
Bromazepam	40	5.8	3.5	5.3	7.7
	150	9.4	4.8	10.0	–7.5
	600	8.1	5.0	7.6	–1.1
Clonazepam	40	2.0	5.4	1.9	4.8
	150	0.9	–0.6	1.2	1.8
	600	1.4	–1.3	3.0	–5.2
Flunitrazepam	40	2.7	3.9	2.6	3.7
	150	2.9	–1.5	3.0	–1.7
	600	0.9	–0.8	3.5	–0.5
Clobazam	40	1.8	8.5	1.6	6.8
	150	3.5	4.3	3.2	1.1
	600	2.8	–0.3	2.8	–4.1
Desmethylflunitrazepam	40	4.9	–1.1	6.5	10.0
	150	4.3	–0.1	4.2	6.2
	600	7.9	–5.0	7.7	–1.9
Estazolam	40	1.8	4.0	2.6	9.0
	150	2.3	–2.8	1.9	–2.6
	600	0.8	0.4	3.2	–7.6
Nitrazepam	40	2.8	1.9	2.3	3.3
	150	1.2	–3.3	1.0	–2.9
	600	1.6	–2.7	3.2	–9.8
Alprazolam	40	5.0	1.9	4.1	1.0
	150	3.9	–4.9	3.4	–5.7
	600	2.3	3.4	3.4	–2.2
Chloornordiazepam	40	2.9	5.2	3.6	10.7
	150	2.2	0.1	2.3	1.8
	600	3.4	–1.9	4.0	1.6
Temazepam	40	2.2	1.2	2.5	5.4
	150	2.4	–4.5	2.2	–5.3
	600	2.1	–3.9	3.9	1.4
Desalkylflurazepam	40	1.9	2.2	2.6	3.2
	150	1.7	–2.6	1.7	–1.1
	600	3.3	2.4	3.2	–1.8
Oxazepam	40	3.5	–0.1	4.1	0.3
	150	3.1	–6.5	3.6	–8.6
	600	3.1	–2.0	2.8	0.5
Nordiazepam	40	0.9	3.7	2.3	6.6
	150	3.5	–1.1	3.0	–0.4
	600	1.6	1.0	2.0	–0.2
Brotizolam	40	3.1	3.8	4.1	8.2
	150	3.1	–1.8	3.0	–1.5
	600	3.2	3.8	4.1	4.7
Triazolam	40	2.1	3.5	3.2	–1.1
	150	1.3	–4.3	1.5	–6.3
	600	2.7	–1.2	3.0	–5.4
Lormetazepam	40	2.1	1.6	2.9	–0.2
	150	2.7	–4.0	3.6	–10.8
	600	3.6	5.0	3.7	–1.3

continued

the following combinations: 7-aminoflunitrazepam-d_7 was used for the calculations of peak-area ratios and concentrations of 7-aminoflunitrazepam, loprazolam, flurazepam, cloxazolam, zolpidem, and zopiclone; 7-aminoclonazepam-d_4 for 7-aminoclonazepam; flunitrazepam-d_7 for bromazepam and flunitrazepam; clonazepam-d_4 for clonazepam, clobazam, desmethylflunitrazepam, estazolam and nitrazepam; temazepam-d_5 for temazepam; desalkylflurazepam-d_4 for desalkylflurazepam and lormetazepam; nordiazepam-d_5 for chlornordiazepam, nordiazepam and brotizolam; triazolam-d_4 for triazolam; lorazepam-d_4 for lorazepam; alprazolam-d_5 for alprazolam; prazepam-d_5 for prazepam; diazepam-d_5 for clotiazepam, tetrazepam, and diazepam; oxazepam-d_5 for oxazepam.

Calibration curves ranged from 1 to 200 ng/mL (1, 2, 5, 10, 20, 40, 80, 160, and 200 ng/mL) for blood samples, from 10 to 1000 ng/mL (10, 25, 50, 100, 250, 500, 750, and 1000 ng/mL) for urine, and from 0.5 to 1000 pg/mg (0.5, 1, 2, 5, 10, 50, 100, 200, 500, and 1000 pg/mg) for hair samples. Standard response curves were generated daily using a weighted ($1/x$) least-squares linear regression model.

The LOQ was defined as the concentration of the lowest calibrator, which was calculated to be within ± 20% of the nominal value and with a relative standard deviation (RSD) less than 20% (33,34). QCs were prepared for every run in blank matrix at a concentration of 4, 12, and 100 ng/mL for blood samples, at a concentration of 40, 150, and 600 ng/mL for urine and at a concentration of 7.5, 75, and 750 pg/mg for hair samples. Intra-assay precision was evaluated by analysis of 5 sets of the QC samples in one run for each of the three biological matrices. Interassay precision was evaluated by replicate analysis of one set of QC samples in several experiments performed on five different days. The precision was expressed as the RSD. A comparison of the calculated concentrations of the QC samples to their respective nominal values was used to assess the accuracy (bias) of the method.

For the three matrices, relative recoveries were estimated by comparing the ratio of the peak area of the medium QC sample when the non-deuterated compounds were added before any pretreat-

ment or extraction (n = 3) divided by the peak area of the internal standards with the ratio of the peak area obtained when the nondeuterated analytes were added after the extraction (n = 3) divided by the peak area of the internal standards. The deuterated standards were added before the extraction in all experiments.

Assessment of matrix effects. To assess any potential suppression or enhancement of ionization from components present in the extracted biological matrix, a continuous post-column infusion was performed using a mixture of all benzodiazepines and deuterated analogues (10 ng/mL at a flow rate of 10 µL/min) to produce a constant elevated response in both MRM channels. The interference of this constant response was monitored following the injection of extracted samples and compared to the response following the injection of mobile phase only.

Results and Discussion

The applied gradient ensured the elution of all the drugs examined within 20 min and produced chromatographic peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, precursor, and transitions. During the first injection of an extracted sample, the most prominent precursor-product transitions (except for alprazolam, for which an elevated background was noticed in this transition) were used for quantification. Further confirmation of the identity of the compounds was achieved through a second injection of positive samples and by monitoring two transitions (i.e., a quantifier and a qualifier) per compound. For the deuterated internal standards, a single MRM transition was used. Table I summarizes the MRM transitions and conditions of all quantifiers and qualifiers for all analytes and IS. For all compounds investigated, peak-area ratios quantifier/qualifier were found to be very reproducible with variation (as RSD) less than 10%.

The method was validated for linearity, LOQ, precision, accuracy, and analytical recovery by the analyses of spiked blood, urine, and hair samples. In each case, a weighted ($1/x$) linear regression line was applied. Linearity with correlation coefficients $r^2 > 0.99$ were achieved in the range investigated (i.e., from the LOQ to 200 ng/mL for blood samples, from the LOQ to 1000 ng/mL for urine samples, and from LOQ to 1000 pg/mg for hair samples). The back-calculated concentrations of all calibrators were compared with their respective nominal values and were within 100 ± 20% of the nominal value.

The obtained LOQ in blood was 1.0 ng/mL for all analytes, except for lorazepam, loprazolam, zolpidem, and zopiclone, where an LOQ of 2.0 ng/mL was observed. For urine samples, the LOQ was established at 10 ng/mL for all analytes, except for brotizolam and tetrazepam with an LOQ of 25 ng/mL. Finally, for hair samples, LOQs ranging from 0.5 pg/mg (prazepam) to 10 pg/mg (tetrazepam, loprazolam, and zopiclone) were observed. Figure 1 shows the MRM chromatograms obtained following the analysis of a hair sample spiked with 10 pg/mg of each of the compounds. At the LOQ, the qualifier had a signal-to-noise ratio > 10:1. The intra-assay precision (repeatability), interassay precision (reproducibility), and bias were < 20% in most cases for all matrices tested (Tables II–IV).

The obtained LOQ was comparable with previous reports dealing with a large series of benzodiazepines (28,31,32). The reported sensitivities for hair samples were comparable with the values for other LC–MS–MS methods dealing with only one or a limited number of benzodiazepines (35–39). The LOQs were slightly higher than the values obtained with previous negative-ion chemical ionization-GC–MS methods detecting one or a few benzodiazepines in hair (40,41).

Table III (continued). Intra-assay and Interassay Precision and Bias of the QC Samples, Prepared in Urine, Spiked at a Concentration of 40, 150, and 600 ng/mL

		Intra-assay Precision		Interassay Precision	
Compound	Concentration of QC (ng/mL)	RSD	Bias (%)	RSD	Bias (%)
Lorazepam	40	2.5	2.8	3.0	0.5
	150	3.5	–3.9	3.1	–0.9
	600	2.8	–1.7	2.7	–5.4
Prazepam	40	2.2	5.2	2.4	6.4
	150	1.1	–2.2	1.0	–1.3
	600	1.0	0.4	1.7	–1.4
Clotiazepam	40	3.0	5.9	2.5	5.3
	150	1.0	–2.1	1.7	–4.5
	600	1.5	4.3	5.1	–8.9
Tetrazepam	40	7.3	9.8	17.8	12.7
	150	4.9	5.8	18.0	–20.6
	600	6.5	–0.2	20.6	–6.5
Diazepam	40	2.7	3.7	2.9	7.1
	150	2.1	–1.7	1.8	–1.7
	600	1.2	–0.1	2.0	–0.6
Loprazolam	40	10.2	19.0	12.0	13.0
	150	8.3	18.6	9.1	–12.5
	600	8.6	10.1	13.0	–6.8
Flurazepam	40	9.7	19.4	10.9	–10.1
	150	4.5	6.7	9.7	–19.3
	600	3.4	5.0	5.7	–7.9
Cloxazolam	40	4.5	11.2	16.5	–3.8
	150	2.3	–0.4	20.8	–5.9
	600	4.9	–3.1	19.5	–9.7
Zolpidem	40	9.6	13.5	10.4	–18.1
	150	1.3	6.3	6.3	–2.7
	600	2.4	4.5	2.3	3.7
Zopiclone	40	9.1	18.0	12.6	–7.8
	150	2.5	7.1	10.7	7.9
	600	4.1	1.2	10.3	11.2

Table IV. Intra-assay and Interassay Precision and Bias of the QC Samples, Prepared in Hair, Spiked at a Concentration of 7.5, 75, and 750 pg/mg

Compound	Concentration of QC (ng/mL)	Intra-assay precision		Interassay precision	
		RSD	Bias (%)	RSD	Bias (%)
7-Aminoclonazepam	7.5	5.5	9.4	6.1	5.0
	75	2.5	0.8	2.2	2.5
	750	4.4	–2.7	4.2	–2.3
7-Aminoflunitrazepam	7.5	4.4	7.9	4.4	6.3
	75	1.6	0.1	2.7	2.2
	750	0.4	2.3	2.5	2.2
Bromazepam	7.5	16.8	–7.8	13.6	8.6
	75	8.1	12.7	7.4	9.8
	750	13.1	12.7	10.6	3.8
Clonazepam	7.5	3.5	12.7	4.3	9.7
	75	2.2	10.3	1.9	10.0
	750	2.0	4.9	4.5	2.8
Flunitrazepam	7.5	3.3	5.4	3.4	5.2
	75	1.1	2.2	5.3	5.6
	750	1.3	3.7	3.9	6.8
Clobazam	7.5	2.9	3.6	4.9	–1.3
	75	2.8	9.5	13.9	1.2
	750	5.6	5.2	14.5	–3.8
Desmethylflunitrazepam	7.5	9.1	10.3	9.0	15.1
	75	9.9	13.1	11.1	8.5
	750	9.0	4.9	9.0	10.0
Estazolam	7.5	5.0	10.4	9.9	15.2
	75	4.7	3.0	16.6	14.2
	750	2.9	0.4	12.6	10.2
Nitrazepam	7.5	5.6	8.8	4.9	11.8
	75	3.6	8.3	8.3	15.9
	750	2.6	2.2	3.4	8.1
Alprazolam	7.5	17.8	19.4	16.6	17.9
	75	3.8	–0.5	3.6	–0.3
	750	7.3	6.9	1.2	4.5
Chlornordiazepam	7.5	6.4	3.5	6.2	2.7
	75	2.7	6.5	4.5	5.8
	750	5.8	3.2	6.7	1.9
Temazepam	7.5	8.2	4.0	7.9	2.6
	75	0.8	3.7	2.1	5.0
	750	4.5	4.7	5.4	4.0
Desalkylflurazepam	7.5	7.4	8.9	6.7	9.1
	75	1.1	5.2	2.3	7.3
	750	2.7	6.5	2.3	8.0
Oxazepam	7.5	7.2	8.6	9.3	10.8
	75	3.5	2.7	16.5	9.1
	750	2.7	0.5	5.0	2.5
Nordiazepam	7.5	7.0	4.3	6.6	3.6
	75	3.4	4.1	4.0	7.2
	750	5.7	6.4	4.9	7.0
Brotizolam	7.5	7.0	5.8	8.2	6.3
	75	7.0	4.8	6.2	4.2
	750	8.6	4.2	7.6	3.0
Triazolam	7.5	12.3	2.4	11.0	4.7
	75	1.9	–1.6	3.9	2.0
	750	4.3	3.0	3.8	4.8
Lormetazepam	7.5	7.1	10.2	8.1	2.4
	75	8.0	8.9	7.5	1.6
	750	4.2	9.4	4.3	4.1

continued

After sample preparation, the same extraction procedure was applied for the three biological matrices. Reproducible recoveries were obtained, ranging from 49.2 to 103.7% in blood, from 25.1 to 98% for urine (except for cloxazolam, with an extraction recovery of 3.8%), and from 53.0 to 103.8% for hair. The extraction recoveries are similar for the three matrices, except for tetrazepam and cloxazolam, where significantly lower recoveries were noted for the extraction of spiked urine samples. To demonstrate that these compounds show a reduced stability when incubating at higher temperatures in aqueous solutions (during the hydrolysis of urine samples), an additional experiment was performed, adding the nondeuterated compounds after sample preparation, but before extraction with 1-chlorobutane. In this experiment, these extraction recoveries for tetrazepam and cloxazolam were similar as those obtained for blood, indicating a decomposition during the preparation of urine samples. The extraction recoveries obtained are comparable with previous reports (15,22,26–28,31,32).

Insufficient sample clean up can result in matrix effects, leading to either suppression or enhancement of the analyte response (42–44). This can lead to variable sensitivities and decreased precision and accuracy. Consequently, in the development of any LC–MS(–MS) method, the potential for any such ion suppression or enhancement should be assessed. To test this, post-column infusion experiments [based on the method described by Bonfiglio et al. (42)] were performed for the three biological matrices to provide information of the effect of matrix throughout the course of the detection window. A decrease in response ranging from 20 to 90% was observed starting from 1.7 min to maximal 6.0 min for the three matrices tested (data not shown). However, this suppression had diminished and normal baseline responses were restored before the elution time of the compounds. In the rest of the chromatographic run, no significant changes in responses were observed. The results confirm the usefulness of the liquid–liquid extraction procedure as a sample clean up before chromatography to obtain reproducible and reliable quantita-

tive results for all compounds without major interference of matrix compounds.

The validated LC–MS–MS method has been successfully applied to the analysis of external quality control samples in serum (organized by the Gesellschaft für Toxikologische und Forensische Chemie). A certificate was obtained for all compounds tested (diazepam, nordiazepam, bromazepam, clonazepam, flunitrazepam, desmethylflunitrazepam, 7-aminoflunitrazepam, and oxazepam).

In addition, the method was used for the analysis of authentic blood, urine, and hair samples. Blood and urine samples were collected during roadside controls for drugged driving, from DFSA cases, suicide cases, and (attempted) murder cases. Samples with a concentration above the linear range of the calibration curve were appropriately diluted in water (before extraction), and reanalyzed. To check the validity of the quantification for this modified sample preparation, five different added concentrations above the calibration range for blood and urine (n = 3 for each concentration) were analyzed with different dilutions. The bias was always < 20%.

Hair analysis was applied to two authentic cases. In the first case, a young woman claimed to have been sexually abused over a period of several years while being drugged. Because of the increased detection window, toxicological analysis of hair yields long-term information about drug consumption and allows the personal history of drug use to be established. As such, the laboratory was requested to analyze the victim's hair (33 cm). Starting from the root, 16 segments of 1–3 cm were analyzed. All segments were positive for more than one benzodiazepine, with generally higher concentrations in segments closer to the root. Benzodiazepines detected above the LOQ were 7-aminoclonazepam (in 5 segments, ranging from 2.2 to 11.7 pg/mg), bromazepam (in all segments, ranging from 19.1 to 1405 pg/mg), clonazepam (in 2 segments, ranging from 16.0 to 32.6 pg/mg), clobazam (in 1 segment, 11.8 pg/mg), oxazepam (1 segment, 8.5 pg/mg), nordiazepam (in 5 segments, ranging from 2.3 to 6.9 pg/mg), lormetazepam (in 5 segments, ranging from 5.0 to 28.1), lorazepam (in 4 segments, ranging from 7.6 to 13.9 pg/mg), tetrazepam (in 2 segments, 55.6 and 123.6 pg/mg), loprazolam (in 1 segment, 24.7 pg/mg), and zolpidem (in all segments, ranging from 8.5 to 177.5 pg/mg). These results indicate long-term multiple drug exposure.

In the second case, a middle-aged woman ingested one tablet of Lexotan (containing 3 mg of bromazepam) on two consecutive days, followed by one tablet on three consecutive days a month later. Hair (7 cm) was cut 6 weeks later and 5 segments of 1–1.5 cm were analyzed. Figure 2 shows the chromatogram obtained after the analysis of the first two proximal segments, positive for bromazepam at 24.2 pg/mg (segment 1, 1.5 cm) and 11.7 pg/mg (segment 2, 1.5 cm). In the third segment (1 cm) traces of bromazepam were detected, probably because of the variability in the incorporation of this drug into the hair shaft and axial migration after incorporation, leading to a distribution over a small region (35). This result is consistent with the doses taken and is in accordance with concentrations of bromazepam found in hair in earlier reports (35,37).

Table IV (continued). Intra-assay and Interassay Precision and Bias of the QC Samples, Prepared in Hair, Spiked at a Concentration of 7.5, 75, and 750 pg/mg

Compound	Concentration of QC (ng/mL)	Intra-assay Precision		Interassay Precision	
		RSD	Bias (%)	RSD	Bias (%)
Lorazepam	7.5	7.4	–0.8	15.0	–5.3
	75	4.0	3.3	16.0	–1.7
	750	6.4	4.8	18.0	–1.1
Prazepam	7.5	3.2	7.7	4.2	6.3
	75	1.3	3.8	2.4	7.1
	750	4.2	6.2	5.8	6.5
Clotiazepam	7.5	12.4	2.1	15.7	0.2
	75	3.1	–5.8	13.6	–4.8
	750	7.1	7.4	17.6	5.7
Tetrazepam	7.5	NA*	NA	NA	NA
	75	6.8	–5.5	19.4	–10.4
	750	7.1	6.4	16.3	0.2
Diazepam	7.5	4.7	5.2	4.8	5.2
	75	2.0	3.1	4.0	6.1
	750	7.5	1.2	7.1	2.6
Loprazolam	7.5	NA	NA	NA	NA
	75	8.9	15.1	19.7	–1.1
	750	7.9	14.1	6.3	9.8
Flurazepam	7.5	25.0	–6.1	20.0	18.1
	75	8.9	–15.2	14.3	11.2
	750	15.7	–4.3	17.3	15.2
Cloxazolam	7.5	12.6	4.3	11.9	11.5
	75	6.3	9.5	5.0	–3.9
	750	10.4	18.2	8.6	–0.4
Zolpidem	7.5	17.2	8.6	15.7	–3.1
	75	7.6	0.0	17.9	–3.4
	750	12.9	–6.2	14.2	10.8
Zopiclone	7.5	NA	NA	NA	NA
	75	11.9	–3.2	13.1	15.9
	750	11.2	14.0	10.2	15.0

*NA = not analyzed < LOQ.

Conclusions

In this report, a validated and highly sensitive LC–ESI-MS–MS method is described for the simultaneous quantification of 26 commonly encountered benzodiazepines and their metabolites, zolpidem and zopiclone, in three different biological matrices (i.e., blood, urine, and hair). Because of the low LOQs, the method was demonstrated appropriate for

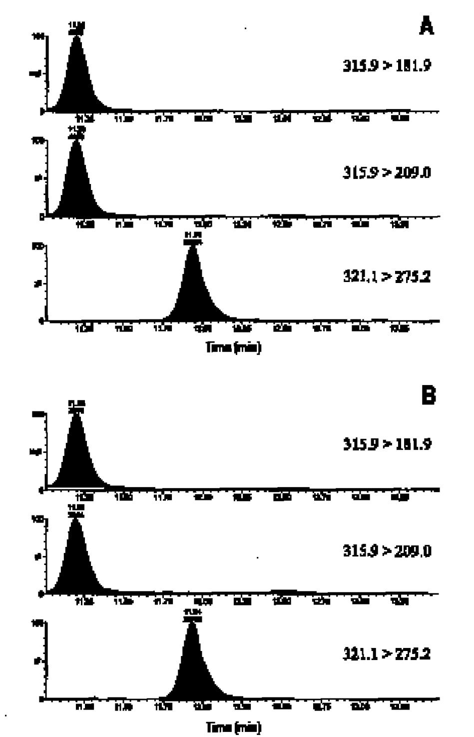

Figure 2. Chromatograms of a hair extract [segment 1 (A) and segment 2 (B)] from a subject exposed to one tablet of bromazepam on two consecutive days, followed by one tablet on three consecutive days a month later (top trace: quantifier, middle trace: qualifier, bottom trace: flunitrazepam-d_7). Bromazepam concentrations were 24.2 pg/mg (A) and 11.7 pg/mg (B).

the quantification of low doses of these compounds in authentic blood, urine, and hair samples collected from forensic toxicology cases.

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Quantitative analysis of Δ^9-tetrahydrocannabinol in preserved oral fluid by liquid chromatography–tandem mass spectrometry

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Abstract

A rapid and sensitive method for the analysis of Δ^9-tetrahydrocannabinol (THC) in preserved oral fluid was developed and fully validated. Oral fluid was collected with the Intercept, a Food and Drug Administration (FDA) approved sampling device that is used on a large scale in the U.S. for workplace drug testing. The method comprised a simple liquid–liquid extraction with hexane, followed by liquid chromatography–tandem mass spectrometry (LC–MS-MS) analysis. Chromatographic separation was achieved using a XTerra MS C_{18} column, eluted isocratically with 1 mM ammonium formate–methanol (10:90, v/v). Selectivity of the method was achieved by a combination of retention time, and two precursor-product ion transitions. The use of the liquid–liquid extraction was demonstrated to be highly effective and led to significant decreases in the interferences present in the matrix. Validation of the method was performed using both 100 and 500 μL of oral fluid. The method was linear over the range investigated (0.5–100 ng/mL and 0.1–10 ng/mL when 100 and 500 μL, respectively, of oral fluid were used) with an excellent intra-assay and inter-assay precision (relative standard deviations, RSD <6%) for quality control samples spiked at a concentration of 2.5 and 25 ng/mL and 0.5 and 2.5 ng/mL, respectively. Limits of quantification were 0.5 and 0.1 ng/mL when using 100 and 500 μL, respectively. In contrast to existing GC–MS methods, no extensive sample clean-up and time-consuming derivatisation steps were needed. The method was subsequently applied to Intercept samples collected at the roadside and collected during a controlled study with cannabis.
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Keywords: LC–MS-MS; Oral fluid; THC

1. Introduction

Currently, there is a strong interest in monitoring drug use through oral fluid testing in the context of driving under the influence, drug treatment, criminal justice, and workplace drug-testing [1–5]. Advantages of this matrix include the ease and non-invasiveness of specimen collection and reduced opportunity for specimen substitution and adulteration. However, two main limitations of oral fluid are apparent: the specimen volume is often small and the analyte concentration is lower than in urine. As such, oral fluid testing is a greater analytical challenge and highly sensitive techniques are required.

Due to the high specificity and the increased signal-to-noise in combination with short chromatographic run times, liquid chromatography–tandem mass spectrometry (LC–MS-MS) allows for specific, selective and sensitive analysis of compounds with a wide polarity range in samples of various nature. It offers the possibility to simplify sample preparation, although this approach should be treated with caution due to the possibility of ion suppression or enhancement as a result of the matrix. Consequently, attention must be paid to the choice of the sampling method and the influence of the collected matrix on the LC–MS-MS analysis. Several meth-

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ods of oral fluid collection have been used. The Intercept is a Food and Drug Administration (FDA) approved sampling device that is used on a large scale in the U.S. for workplace drug testing [6]. It is also used to collect oral fluid samples for confirmation analyses in the joint roadside study between the European Union and the U.S. to detect driving under the influence of drugs [5]. The collection system contains stabilising salts, non-ionic surfactants for surface wetting and antibacterial agents, and guarantees a good stability for most illicit drugs and their metabolites during storage at 4 °C. However, these ingredients can also cause interferences, e.g. ion suppression or enhancement, during LC–MS-MS analysis in the absence of a suitable clean-up method.

Drugs may appear in oral fluid via multiple pathways. Δ^9-tetrahydrocannabinol (THC), the major psychoactive constituent of cannabis, is deposited in the oral cavity during cannabis smoking. This "depot" represents the primary or sole source of THC detected when oral fluid is collected and analysed [7]. Despite the lack of contribution from blood THC to oral fluid concentrations, Huestis and Cone [7] and Niedbala et al. [8] showed that, after dissipation of the initial contamination of oral fluid during smoking (generally within 30 min), THC levels in oral fluid followed a similar time course as plasma THC following smoked cannabis administration under controlled dosing conditions. Within 12 h, both oral fluid and plasma THC concentrations generally declined below 1 ng/mL.

With the exception of a report by Schramm et al. [9], no other studies have revealed evidence of 11-hydroxy-THC or carboxy-THC in oral fluid after smoking of cannabis. However, it appears that in addition to THC, cannabidiol (CBD) and cannabinol (CBN) may be detected in oral fluid after smoking of hashish or marijuana cigarettes [10].

Most laboratories analyse THC in blood and oral fluid by GC–MS(-MS) after extraction and derivatisation [7,8,11–13]. Recently, LC–MS(-MS) has been successfully used to analyse cannabinoids in urine and blood [14–17]. However, only one paper reported on the application of LC–MS to detect THC in oral fluid [18]. These authors reported on the determination of THC in 200 μL of oral fluid, which was obtained by spitting. The limit of detection achieved was 2 ng/mL.

Our aim was to develop a fast and sensitive LC–MS-MS method for the confirmation of THC in preserved oral fluid samples collected with the Intercept. Validation of the method was performed using both 100 and 500 μL of preserved oral fluid. The method was subsequently applied to Intercept samples collected at the roadside and collected during a controlled study.

2. Experimental

2.1. Chemicals

Individual ampoules of THC (at a concentration of 1 mg/mL in methanol) and [$^2H_3$]THC (THC-d_3) (0.1 mg/mL in methanol) were purchased from LGC Promochem (Molsheim, France). Cannabinol and cannabidiol were from Lipomed (Arlesheim, Switzerland). All solvents were HPLC-grade and from Merck (Darmstadt, Germany).

2.2. Specimens

Blank preserved oral fluid, used for the preparation of negative controls, calibrators and quality control (QC) samples was obtained from healthy volunteers and collected with the Intercept collection device (OraSure Technologies, Bethlehem, PA, USA) according to the manufacturer's instructions. Briefly, after gently wiping the collector pad between gum and cheek for approximately 2 min (as a kind of toothbrush), the device is placed in the supplied vial, which contains a stabilising buffer solution, and sealed. After centrifugation, the recovered fluid is transferred in cryotubes and represents a mixture of the collected oral fluid and the buffer in a proportion of approximately 1 to 2. The device collects an average of 0.38 ± 0.19 (SD) mL with a range of 0.05 to 0.8 mL of oral fluid and a dilution factor of 1 in 3 is arbitrarily accepted [6]. The tubes were sealed and stored at −20 °C prior to analysis.

Authentic preserved oral fluid samples were collected by the police at the roadside during roadblocks to intercept drivers under the influence of drugs, using the same procedure as described for the blank samples.

A third series of preserved oral fluid samples was obtained with a similar protocol from nine healthy volunteers with a history of cannabis use. Once a week and for two consecutive weeks, subjects received either a placebo cigarette (containing cannabis where the THC had been previously extracted) or a marijuana cigarette (containing 300 μg THC per kilogram weight). Oral fluid samples were collected 0.5 h before and at various times after drug administration (0.25, 0.5, 1, 1.25 and 1.5 h). Thus, we obtained from each volunteer six oral fluid samples in the placebo condition and six in the THC condition. The study protocol was approved by the ethics committee of the University Hospital of Maastricht in The Netherlands.

2.3. Preparation of standard solutions and sample extraction

An internal standard (IS) working solution of THC-d_3 at a concentration of 10 ng/mL was prepared in methanol. Working solutions of THC at different concentrations (1, 2, 4, 5, 8, 16, 32, 50, 100, 200 ng/mL in methanol) were used for the preparation of calibrators and QC samples. Working solutions were stored at −20 °C, and were prepared monthly.

The extraction procedure was carried out in 10 mL disposable screw top vials of high quality glassware (Chromacol, Herts, UK) with 100 or 500 μL of preserved oral fluid specimen collected with the Intercept device. The pH of the preserved oral fluid samples ranged between 6.0 and 7.0. Fifty microliters of the IS working solution and 4 mL

of hexane were added; when only 100 μL was used, an additional 400 μL of deionised water was added.

After mechanical shaking (30 min) and centrifugation (10 min at 3000 × *g*), the organic phase was transferred to a 5 mL disposable screw top vial (Chromacol) and then evaporated to dryness at 40 °C under nitrogen. The extract was reconstituted in 100 μL of mobile phase and 20 μL was injected into the LC–MS-MS system.

2.4. LC–MS-MS

2.4.1. Chromatography

LC was performed using a Waters Alliance 2690 separation module (Waters, Milford, MA, USA). Analytes were separated on a XTerra MS C_{18} column (150 mm × 2.1 mm, 3.5 μm) (Waters), eluted isocratically with 1 mM ammonium formate–methanol (10:90, v/v), delivered at a flow rate of 0.2 mL/min. The total run time of the method was 8 min. All aspects of system operation and data acquisition were controlled using MassLynx NT 4.0 software.

2.4.2. Mass spectrometry

A Quattro Premier tandem mass spectrometer (Waters) was used for all analyses. Ionisation was achieved using electrospray in positive ionisation mode (ESI+). The optimum conditions were: capillary voltage, 2.0 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 280 °C and delivered at a flow rate of 700 L/h.

In order to establish the appropriate multiple reaction monitoring (MRM) conditions for the individual compounds, solutions of standards [500 ng/mL, in 1 mM ammonium formate–methanol (10:90, v/v)] were infused into the mass spectrometer and the cone voltage (CV) optimised to maximise the intensity of the protonated molecular species $[M+H]^+$. Collision-induced dissociation (CID) of each protonated molecule was performed. The collision gas (argon) pressure was maintained at 0.35 Pa (3.5×10^{-3} mBar) and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis. The transitions were m/z 315.2 → 193.1 and m/z 315.2 → 259.3 for THC. The former (and most prominent precursor-product transition) was used for quantification and the latter transition used as a qualifier. The transition for THC-d_3 was m/z 318.2 → 196.1.

All aspects of data acquisition were controlled using MassLynx NT 4.0 software with automated data processing using the QuanLynx program (Waters).

2.5. LC–MS-MS assay validation

2.5.1. Linearity, limit of quantification (LOQ), precision, accuracy and recovery

Quantification was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of its respective deuterated analogue.

Linearity was assessed when either 100 or 500 μL of the sample, collected with the Intercept device, was processed and analysed using LC–MS-MS. When 100 μL was used, calibration curves ranged from 0.5 to 100 ng/mL (0.5, 1, 2, 4, 8, 16, 25, 50, 100 ng/mL) and from 0.1 to 10 ng/mL (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5, 10 ng/mL) when 500 μL of preserved oral fluid was used. Standard response curves were generated daily using a weighted (1/*x*) least-squares linear regression model.

The limit of quantification (LOQ) was defined as the concentration of the lowest calibrator which was calculated to be within ±20% of the nominal value and with a % relative standard deviation (RSD) less than 20% [19,20].

QCs were prepared for every run in blank preserved oral fluid at a concentration of 2.5 and 25 ng/mL for 100 μL of sample and at a concentration of 0.5 and 2.5 ng/mL for 500 μL of preserved oral fluid. Intra-assay precision was evaluated by replicate ($n = 4$) analysis of the two QC samples in one run for each of both volumes of preserved oral fluid. Inter-assay precision was evaluated by replicate analysis of the QC samples in several experiments performed on eight different days by two operators. A comparison of the calculated concentrations of the QC samples to their respective nominal values, was used to assess the accuracy (bias) of the method.

Recovery was estimated by comparing the response of a 5 ng/mL calibrator when the non-deuterated compound was added before the extraction step ($n = 3$) with the response obtained when the non-deuterated analyte was added after sample preparation ($n = 3$). THC-d_3 was added before the extraction step in both conditions.

2.5.2. Stability of unprepared and prepared samples

Stability of THC in preserved oral fluid collected by the Intercept device was monitored in preserved oral fluid samples spiked at the initial concentrations of 1, 10 and 100 ng/mL. THC concentrations in the samples were either determined immediately (control samples, $n = 3$) or following incubation at room temperature or at 4 °C for a period of 24 h ($n = 3$) or 48 h ($n = 3$) after preparation. Stability at each time point was tested against a lower acceptance limit corresponding to 90% of the mean of control samples by a one-sided *t*-test ($P < 0.05$).

For an evaluation of freeze/thaw stability, a calibrator at 5 ng/mL was analysed before (control samples, $n = 3$) and after three freeze/thaw cycles (stability samples, $n = 3$). For each freeze/thaw cycle, the samples were frozen at −20 °C for 24 h, thawed, and then maintained at ambient temperature for 1 h. Stability was tested against a lower acceptance limit corresponding to 90% of the mean of control samples by a one-sided *t*-test ($P < 0.05$).

The stability of THC in the extracted sample (preserved oral fluid initially spiked at 5 ng/mL) was investigated by repeated injections of a mixture of five extracted samples (maintained in the autosampler at 4 °C) over a period of 15 h. Absolute peak areas were plotted as a function of injection

time and the stability of the processed samples tested by regression analysis. Instability of the processed samples would be indicated by a slope that was significantly different from zero ($P < 0.05$).

2.5.3. Assessment of matrix effects

To assess any potential suppression or enhancement of ionisation due to the sample matrix, two types of experiments were performed. In the first experiment, THC (5 ng/mL) was added after extraction of either water or preserved oral fluid i.e. before evaporation, and the peak responses obtained in both conditions were compared. A two-sided *t*-test was used to identify any significant differences ($P < 0.05$).

The second type of experiment involved a continuous post-column infusion of a mixture of THC and THC-d_3 (10 ng/mL at a flow rate of 10 μL/min) to produce a constant elevated response in both MRM channels. The interference of this constant response was monitored following the injection of samples either prior to or after extraction of 100 or 500 μL of preserved oral fluid and compared to the response following the injection of mobile phase only.

3. Results and discussion

The method was validated for linearity, LOQ, precision, accuracy and analytical recovery by the analysis of spiked preserved oral fluid samples, collected using the Intercept device. Two sets of calibration standard samples (in 100 and 500 μL of preserved oral fluid) were prepared for validation of linearity. The linearity data are summarised in Table 1. In each case, a weighted (1/*x*) linear regression line was applied. Linearity with a correlation coefficient $r^2 = 0.999$ was achieved in the range investigated: from 0.5 up to 100 ng/mL when 100 μL of preserved oral fluid was used and from 0.1 up to 10 ng/mL for 500 μL of sample. Fig. 1 shows the MRM chromatograms obtained following the analysis of a sample spiked with THC and THC-d_3 when either 100 and 500 μL of preserved oral fluid was used. For both calibration curves, the lowest calibrators, i.e. 0.5 ng/mL and 0.1 ng/mL when using 100 or 500 μL, respectively, of collected oral fluid, satisfied the criteria for LOQ. It should be noted that the mean resultant specimen volume, following dilution with the preservative solution, varied around 1.2 mL even under controlled conditions [8]. However, in reality, the volume is often reduced in driving under the influence (DUI) cases due to the stimulation of sympathetic nerves which results in the production of a viscous and less abundant oral fluid. This is the case particularly true for regular users of amphetamines [10]. In addition, in these cases the possibility of other drugs should be tested for. This necessitates optimal usage of the minimal amounts of specimen provided. For these cases, the LOQ when using only 100 μL of oral fluid was sufficiently low to meet the requirements of SAMSHA for oral fluid testing (i.e. 2 ng/mL THC in undiluted oral fluid) [21]. However, in pharmacokinetic studies, where the detection of THC over time often necessitates increased sensitivity and a lower LOQ, this can be achieved very simply, by using larger volumes of oral fluid. For example, when using 500 μL of collected oral fluid, the LOQ was determined to be 0.1 ng/mL. Thus, the choice of sample volume will largely depend on the application in addition to the requirements for sensitivity.

These results are comparable with previous GC–MS-MS reports [7,8]. The obtained LOQ for THC was lower than the one reported by Concheiro et al. [18], primarily due to the use of *tandem* MS instead of *single* MS. These authors used undiluted oral fluid collected by spitting. Hence, when using diluted oral fluid, collected with the Intercept device, a lower LOQ is needed to meet the requirements of SAMSHA.

Selectivity of the method was achieved by a combination of retention time, precursor and product ions. Quantification was based on the most prominent product ion (i.e. quantifier); confirmation of THC was evaluated through the presence of the second product (i.e. qualifier). At the LOQ the qualifier had a signal to noise ratio (S:N) > 10:1. The acceptance range for the peak area ratio quantifier/qualifier was 2.36 ± 0.35 for all analyses.

The intra-assay precision (repeatability) and inter-assay precision (reproducibility) were highly satisfactory with all relative standard deviations less than 6% (Table 2). Results indicated that the accuracy of the assay was > 93%. Recovery of the method was $85.6 \pm 0.5\%$.

The stability of spiked samples (1, 10 and 100 ng/mL) was monitored at 24 and 48 h at 4 °C and at room temperature. No statistical significant differences could be observed for the three different concentrations in both conditions. Also no statistical differences could be noted for the stability of spiked samples (5 ng/mL) during three freeze/thaw cycles.

In addition, the potential for any undesired stability of the processed samples was tested. To this end, the stability of THC was monitored by means of repeated injections of extracted samples (5 ng/mL) over a period of 15 h, and by plotting the absolute peak areas as a function of time. The

Table 1
Linearity and sensitivity data for THC in preserved oral fluid

Linearity data					Sensitivity data
Volume oral fluid (μL)	Slope[a]	Intercept[a]	RSD of slope[a]	r^2 (range of five consecutive days)	LOQ (ng/mL)
100	1.0635	0.0209	2.9	0.9993–0.9999	0.5
500	5.3976	−0.0009	4.1	0.9992–0.9999	0.1

Samples were prepared by the liquid–liquid extraction method as described in the text.
[a] Reported values are the mean of five determinations over five consecutive days.

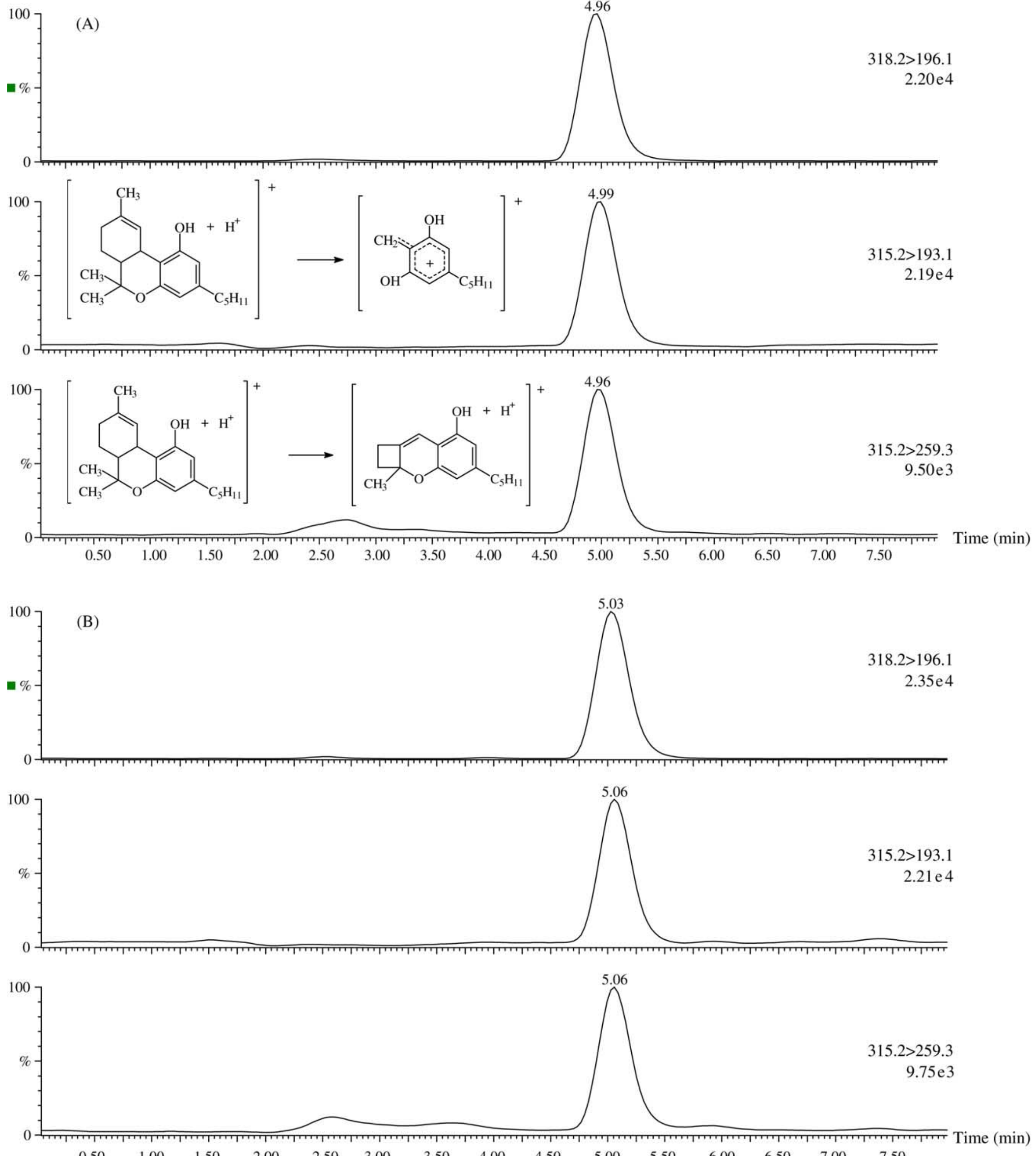

Fig. 1. MRM chromatograms obtained with a single injection of a 100 μL extracted preserved oral fluid sample enriched with 5 ng/mL THC and 5 ng/mL THC-d_3 (A) and of a 500 μL extracted preserved oral fluid sample enriched with 0.8 ng/mL THC and 1 ng/mL THC-d_3 (B). The figure shows the response for THC-d_3 (top trace) and for the two transitions of THC (quantifier and qualifier, middle and bottom traces, respectively). Peak intensity is shown in the top right-hand corner of each trace.

results indicated no significant instability over the course of the experiment.

Insufficient sample clean-up can result in matrix effects, leading to either suppression or enhancement of the analyte response [22–24]. This can lead to variable sensitivities and decreased precision and accuracy. Consequently, in the development of any LC–MS(-MS) method, the potential for any such ion suppression or enhancement should be assessed.

Table 2
Precision[a] and accuracy data for THC for the extraction of 100 and 500 μL of spiked preserved oral fluid samples

Volume oral fluid (μL)	Concentration of QC (ng/mL)	Intra-assay precision			Inter-assay precision		
		Mean concentration found (ng/mL)	RSD (%)	Bias (%)	Mean concentration found (ng/mL)	RSD (%)	Bias (%)
100	2.5	2.5	3.6	−1.0	2.4	2.9	−2.5
	25.0	24.8	5.4	−0.7	24.0	5.4	−4.1
500	0.5	0.5	2.5	−2.4	0.5	4.1	−5.5
	5.0	4.9	0.4	−2.0	4.7	3.8	−6.8

[a] Intra-assay precision was evaluated by the preparation and analysis of four replicates of a low and a high QC in a single assay for both volumes of oral fluid used. Inter-assay precision was evaluated by the preparation and analysis of each QC over eight consecutive days.

The Intercept collector contains a variety of chemicals, i.e. sodium chloride, sodium benzoate, potassium sorbate, bovine gelatin, Tween 20, chlorhexidine digluconate and a blue dye, some of which can interfere with the LC–MS-MS detection signal. To assess this, we compared peak area responses obtained when THC was added after the extraction of blank preserved oral fluid with the responses obtained when THC was added to an extract where the preserved oral fluid was substituted with water. No statistically significant different peak areas were observed.

Post-column infusion experiments (based on the method described by Bonfiglio et al. [22]) were performed to provide information of the effect of matrix throughout the course of the whole chromatographic run and not just at the elution time for the analytes. The effect on THC response obtained following the injection of a mobile phase control is shown in Fig. 2A. As expected, no changes in response were observed. The effects on THC response obtained following the injection of a sample prior extraction and after extraction of 100 and 500 μL of preserved oral fluid are given in Fig. 2B, C and D, respectively. The results confirm the usefulness of the liquid–liquid extraction as a sample clean-up before chromatography: a decrease of 100% in response starting from ∼1.7 min was observed when no sample clean-up was performed. A reduction of 50% was still noted at the moment of elution of THC, probably due to the elution of endogenous components. When injecting extracted samples, this suppression was still apparent but restored by the elution time of THC. In addition to THC, cannabidiol and cannabinol are two components that are also present in the

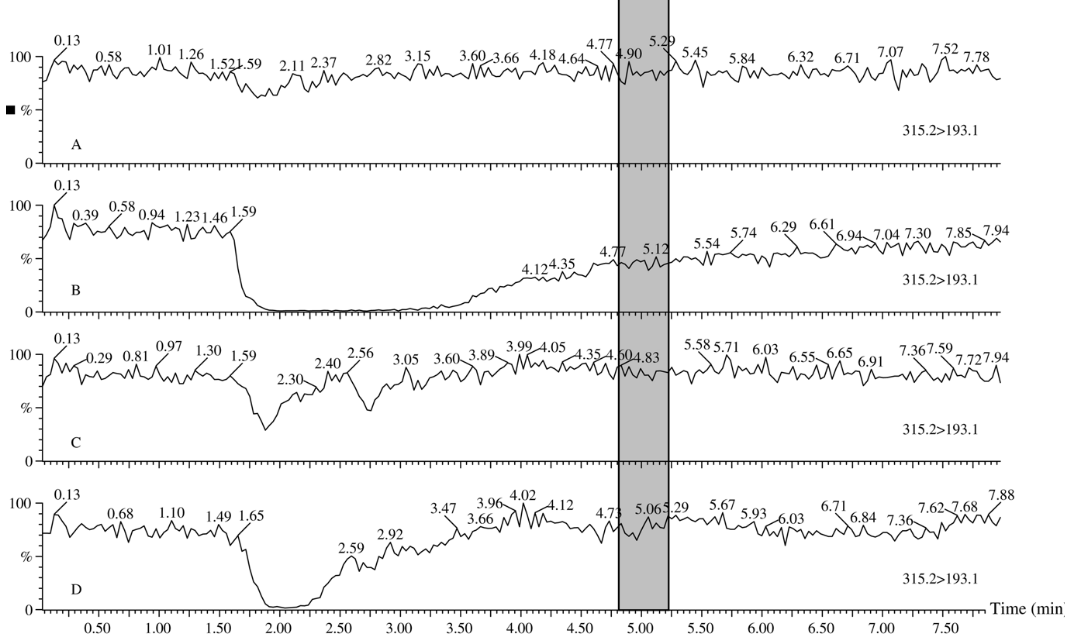

Fig. 2. Evaluation of the effect on THC response of an injection of a mobile phase control (A), a blank sample prior to extraction (B) and the same sample following the extraction of 100 and 500 μL of preserved oral fluid (C and D, respectively). The shaded area indicates the elution position of THC.

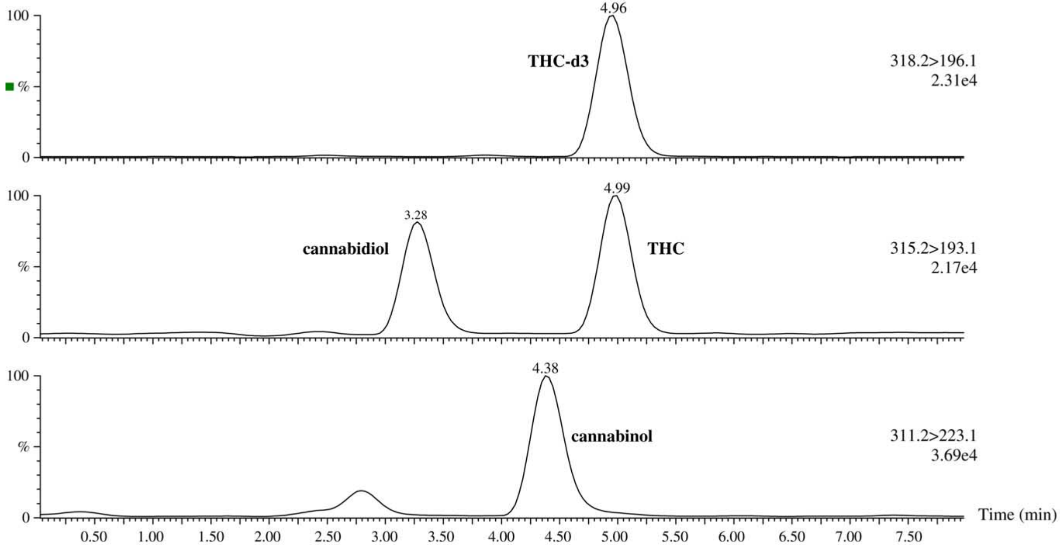

Fig. 3. LC–MS-MS analysis of an extracted 100 μL blank oral fluid sample enriched with 5 ng/mL THC-d_3 (top trace), THC and cannabidiol (middle trace) and cannabinol (bottom trace). Peak intensity is shown in the top right-hand corner of each trace.

Cannabis sativa plant and may also be detected in oral fluid. To evaluate their potential for interference, standards were analysed using the developed LC–MS-MS method. This is particularly important in the case of cannabidiol since this component has the same molecular mass (and thus the same protonated species) as THC and shows the same product ions after CID. Cannabidiol eluted at 3.28 min and was chromatographically resolved from THC. In contrast, cannabinol did not produce any response in the monitored MRM channel due to a different molecular mass. The appropriate MRM transition for this component was m/z 311.2 → 223.1, as determined by direct infusion experiments. Cannabinol was demonstrated to elute at 4.38 min. Fig. 3 shows the MRM chromatograms obtained following LC–MS-MS analysis of an extracted 100 μL blank oral fluid sample enriched with 5 ng/mL THC-d_3, THC, cannabidiol and cannabinol.

The validated LC–MS-MS method was applied to the analysis of 102 oral fluid samples collected with the Intercept from volunteers who had received either a placebo cigarette or a marijuana cigarette. THC concentrations obtained after smoking a single marijuana cigarette are shown in Fig. 4. For these cases only the presence of THC had to

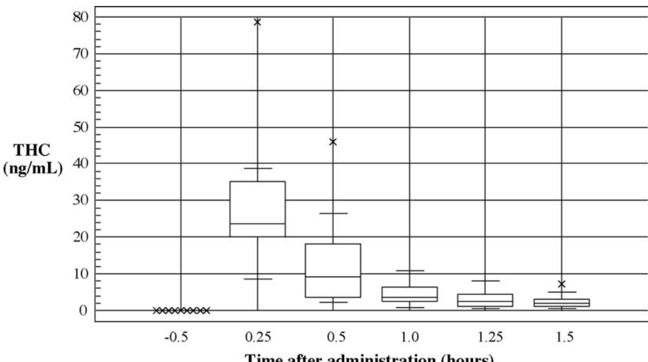

Fig. 4. Box- and whisker plots of THC levels in preserved oral fluid samples from nine healthy volunteers following smoking of a single marijuana cigarette. Oral fluid samples were taken 0.5 h prior to smoking and at 0.25, 0.5, 1, 1.25 and 1.5 h after smoking. Concentrations plotted on the Y-axis are expressed as ng/mL. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value, excluding "outside" (not present) and "far out" values (cross marker) which are displayed as separate points.

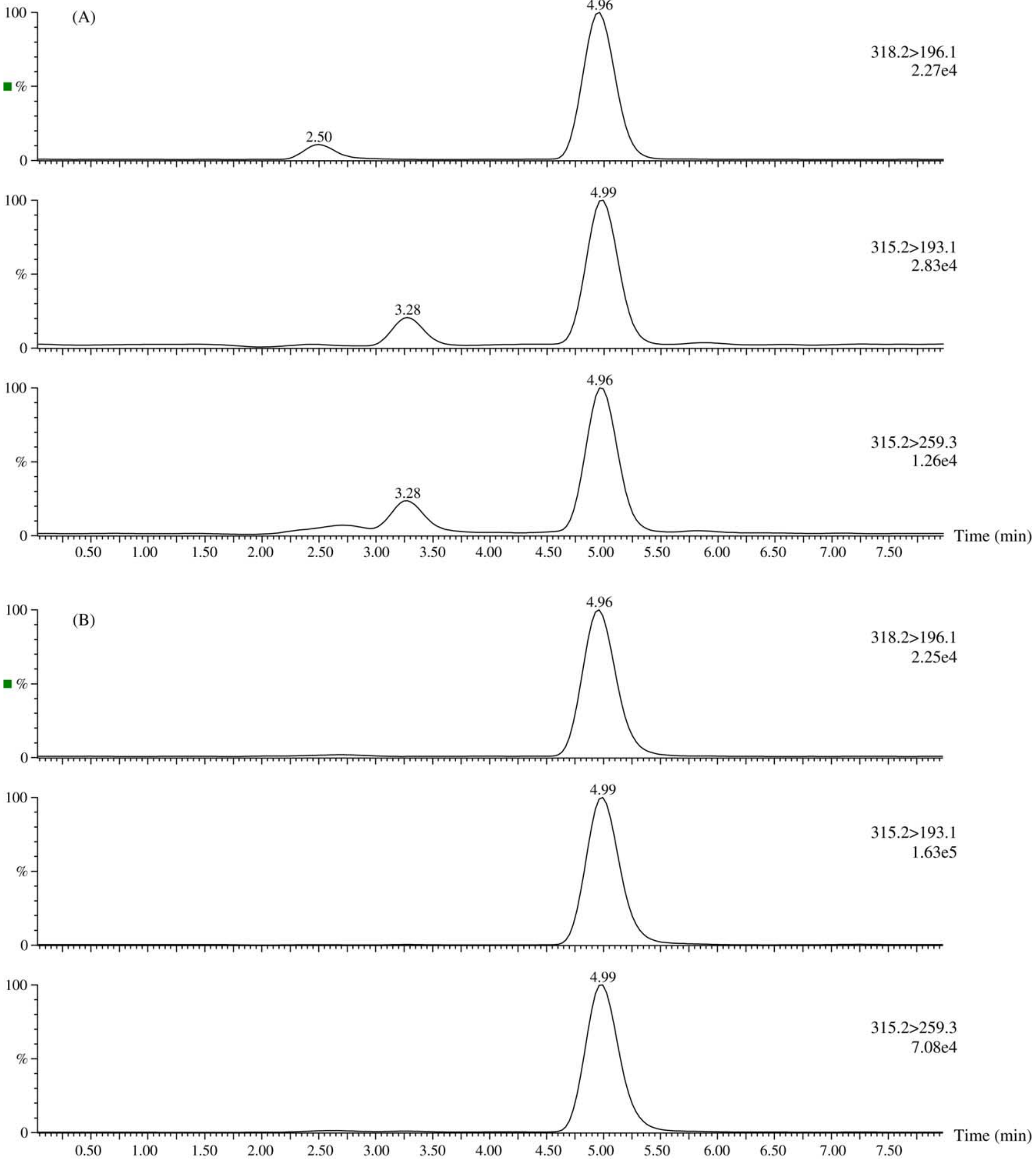

Fig. 5. Typical MRM chromatograms obtained following the analysis of two authentic preserved oral fluid specimens obtained from drivers in a roadside setting. Concentrations were 5.7 ng/mL (A) and 50.8 ng/mL (B). The figure shows the response for THC-d_3 (top trace) and for the two transitions of THC (quantifier and qualifier; middle and bottom traces respectively). Peak intensity is shown in the top right-hand corner of each trace.

be confirmed, thus 500 μL of oral fluid was used for the analysis. For samples where the response exceeded the upper limit of the standard curve, reanalysis of only 100 μL was performed. At −0.5 h all specimens were negative for THC, except for three subjects in which low concentrations were found (0.2, 0.4 and 2.2 ng/mL). However, it should be noted that in both the placebo and marijuana condition, THC could be detected, probably due to incomplete removal of THC for the preparation of the placebo cigarette. Mean peak (±1 SD) THC concentration in the marijuana condi-

Table 3
Results obtained applying the method to 48 preserved oral fluid samples collected by the police at the roadside

Sample identity	THC (ng/mL)	Sample identity	THC (ng/mL)
1	5.7	25	60.2
2	7.0	26	3.9
3	4.6	27	52.2
4	18.5	28	25.4
5	2.5	29	193.5
6	95.8	30	111.2
7	<LOQ	31	7.3
8	84.7	32	14.6
9	<LOQ	33	1.9
10	0.5	34	4.7
11	4.5	35	100.0
12	3.9	36	23.0
13	31.9	37	57.1
14	50.8	38	88.6
15	34.6	39	3.9
16	56.0	40	375.8
17	81.1	41	3.7
18	11.9	42	4.4
19	107.4	43	4.2
20	92.1	44	4.2
21	10.0	45	4.2
22	17.6	46	4.1
23	94.8	47	4.0
24	37.2	48	4.4

tion occurred at the first specimen collection (0.25 h) and was 30.6 ng/mL (±21.6 ng/mL). Thereafter, THC concentrations declined steadily to mean concentrations of 2.6 ng/mL (±2.3 ng/mL). Overall, concentrations were quite variable; this has also been reported by other authors [8] and may be due to the lack of exact volume measurement of the collection device. The Intercept device is a collection device on which the specimen is absorbed onto a matrix, leading to variable absorbed volumes.

The mean peak concentration is lower than the one reported by Niedbala et al. using the same collection device [8]. This could be due to the fact that the samples were only analysed several months after sampling. During this time the samples were conserved at −20 °C on the pad, i.e. without prior centrifugation. However, no stability studies on this aspect were available from the manufacturer.

During roadside controls for drugged driving, the police collected 48 authentic oral fluid samples for a confirmatory analysis in the laboratory. In these cases only 100 μL of preserved oral fluid was used due to limited sample volume. Fig. 5 shows typical MRM chromatograms of Intercept samples obtained from two marijuana users. In Fig. 5A, the presence of cannabidiol (at a retention time of 3.28 min) was also noted. A summary of the quantitative results for the positive samples is presented in Table 3. In these samples, the median THC concentration was 13.3 ng/mL with a range from 0.5 to 375.8 ng/mL. The measured THC concentrations varied considerably and some samples had to be reanalysed after dilution (one in five dilution with blank oral fluid).

4. Conclusions

A fully validated LC–MS-MS method for the determination of THC in preserved oral fluid, collected with the Intercept device, was developed. The method offers the combination of a very simple liquid–liquid extraction to avoid ion suppression, a high recovery and excellent precision and accuracy, when using either 100 or 500 μL of collected sample. The method was successfully applied to Intercept samples collected at the roadside and collected after a controlled study with cannabis.

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Quantitative analysis of multiple illicit drugs in preserved oral fluid by solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract

We present a validated method for the simultaneous analysis of basic drugs which comprises a sample clean-up step, using mixed-mode solid-phase extraction (SPE), followed by LC–MS/MS analysis. Deuterated analogues for all of the analytes of interest were used for quantitation. The applied HPLC gradient ensured the elution of all the drugs examined within 14 min and produced chromatographic peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, and two precursor-product ion transitions for the non-deuterated analogues. Oral fluid was collected with the Intercept®, a FDA approved sampling device that is used on a large scale in the US for workplace drug testing. However, this collection system contains some ingredients (stabilizers and preservatives) that can cause substantial interferences, e.g. ion suppression or enhancement during LC–MS/MS analysis, in the absence of suitable sample pre-treatment. The use of the SPE was demonstrated to be highly effective and led to significant decreases in the interferences. Extraction was found to be both reproducible and efficient with recoveries >76% for all of the analytes. Furthermore, the processed samples were demonstrated to be stable for 48 h, except for cocaine and benzoylecgonine, where a slight negative trend was observed, but did not compromise the quantitation. In all cases the method was linear over the range investigated (2–200 μg/L) with an excellent intra-assay and inter-assay precision (coefficients of variation <10% in most cases) for QC samples spiked at a concentration of 4, 12 and 100 μg/L. Limits of quantitation were estimated to be at 2 μg/L with limits of detection ranging from 0.2 to 0.5 μg/L, which meets the requirements of SAMHSA for oral fluid testing in the workplace. The method was subsequently applied to the analysis of Intercept® samples collected at the roadside by the police, and to determine MDMA and MDA levels in oral fluid samples from a controlled study.
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Keywords: LC–MS/MS; Oral fluid; SPE; Ion suppression

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1. Introduction

A variety of body specimens other than urine, such as saliva (oral fluid), sweat and hair have been used to document drug exposure for pre-employment screening, in forensic toxicology laboratories and in clinical applications [1–4]. The advantage of these samples over traditional matrices like urine and blood is that collection is almost non-invasive, relatively easy to perform, and may be achieved under close supervision to prevent adulteration or substitution of the sample. Tools for the detection of drugs in alternative specimens often utilise traditional technology, though some limitations are imposed which require special attention: the specimen volume or mass is often small, the target analytes are different from urine and the analyte concentration is lower than in urine [1–5].

Oral fluid can be extracted and analysed like other biological fluids such as blood. In general, there will be less interference from endogenous compounds than with blood or urine [1]. Laboratory immunoassay procedures (EIA) to screen for drugs of abuse in oral fluid have been validated for cocaine [6,7] opiates [8,9] and cannabinoids [10]. Many reports on oral fluid refer to the common GC–MS procedures for certain classes of drugs in blood using electron impact mode. These methods utilise the deuterated analogues for parent drugs, e.g. Δ^9-tetrahydrocannabinol (THC) and cocaine and also for relevant metabolites, e.g. 6-acetylmorphine (6-AM) [1,4,5]. Due to the small sample volume of oral fluid specimens, chromatographic procedures using tandem mass spectrometry, either GC or LC, have been developed for the detection of a single class of analytes [11,12] or for the analysis of multiple classes of illicit drugs simultaneously [13,14]. The high specificity and the increased signal-to-noise in combination with short chromatographic run times and a potential to reduce sample preparation because there is no need for derivatization, make LC–MS/MS the technique of choice for high-throughput confirmation of multiple illicit drugs in oral fluid samples.

One major issue that needs to be addressed is the choice of sampling method and the influence of the collected matrix on the LC–MS/MS analysis. Mortier et al. used liquid chromatography quadrupole-time-of-flight mass spectrometry with electrospray ionisation to successfully determine morphine, codeine, cocaine, benzoylecgonine and amphetamines in oral fluid samples obtained by spitting and subjected to solid-phase extraction (SPE). However, when authentic samples collected with a specific device were analysed, interferences were noticed compromising the quantitative analysis [13]. Dams et al. investigated the influence of oral fluid matrix components (samples collected with a neutral Salivette®) during post-column infusion of morphine, after different sample preparation steps [15].

The Intercept® is a FDA approved sampling device that is used on a large scale in the US for workplace drug testing [11]. It is also used to collect oral fluid samples for confirmation analyses in the joint roadside study between the European Union and the US to detect driving under the influence of drugs [16]. The collection system contains stabilizing salts, non-ionic surfactants for surface wetting and antibacterial agents, and guarantees a good stability for most illicit drugs and their metabolites during storage at 4 °C. However, these ingredients can also cause interferences, e.g. ion suppression during LC–MS/MS analysis in the absence of a suitable clean-up method.

We have validated a newly developed LC–ESI–MS/MS method combined with a routine SPE clean-up for the simultaneous quantitation of the major analytes which can be detected after consumption of basic illicit drugs; the oral fluid was collected using the Intercept® device. The method was applied to Intercept® samples collected at the roadside and after a controlled administration of MDMA to volunteers.

2. Materials and methods

2.1. Materials

Ammonium formate and ammonium hydrogen carbonate were purchased from Sigma–Aldrich (Steinheim, Germany). Tetrahydrofuran (chromatographic grade) and hydrochloric acid (fuming, 37%) were from the same supplier. Formic acid, ammonia solution (32%, extra pure), hydrochloric acid solution (0.1N) and all other solvents (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Solid-phase extraction (SPE) cartridges Oasis® MCX (30 mg, 1 cm^3) were from Waters (Milford, MA).

Individual stock solutions of the drugs and their deuterated analogues were purchased from LGC Promochem (Molsheim, France). Stock solutions of amphetamine-d_{11}, methamphetamine-d_5, MDMA-d_5, MDA-d_5, cocaine-d_3, benzoylecgonine-d_8, morphine-d_3, codeine-d_6 and 6-acetylmorphine-d_6 were obtained at concentrations of 0.1 g/L in methanol or acetonitrile. Amphetamine, methamphetamine, MDMA, MDA, cocaine, benzoylecgonine, morphine, codeine and 6-AM were certified at a concentration of 1 g/L in methanol or acetonitrile. Separate working solutions of the drugs, for tuning and selectivity experiments, were prepared in the laboratory at a concentration of 1 mg/L in methanol. A mixed working solution of non-deuterated compounds at 4 mg/L in methanol was used for the preparation of calibrators and QC samples within each run. A mixed internal standard working solution of 1 mg/L was prepared in methanol. Working solutions were stored at −20 °C, and were prepared monthly. To obtain the lower concentrations needed for internal standardization and validation of each experiment, further dilutions in water were prepared the same day.

2.2. Specimens

Oral fluid used for the preparation of blanks, calibrators and QC samples was obtained from healthy volunteers and

collected with the Intercept® collection device (OraSure Technologies, Bethlehem, PA) according to the manufacturer's instructions. Briefly, after gently wiping the collector pad between gum and cheek for approximately 2 min (as a kind of toothbrush), the device is placed in the supplied vial, which contains a stabilizing buffer solution, and sealed. After centrifugation, the recovered fluid was transferred in cryotubes and represents a mixture of the collected oral fluid and the buffer in a proportion of approximately 1:2. The device collects an average of 0.38 ± 0.19 mL of oral fluid and a dilution factor of 1 in 3 is arbitrarily accepted [11]. The tubes were sealed and stored at -20 °C prior to analysis.

Authentic oral fluid samples were collected by the police at the roadside during roadblocks to intercept drivers under the influence of drugs, using the same procedure as described for the blank samples.

A third series of oral fluid samples were obtained with a similar protocol from 18 volunteers who received either placebo or a high (100 mg) or a low (75 mg) dose of MDMA. Oral fluid samples were collected at 1.5 and 5.5 h after administration of the drug. The study protocol was approved by the ethics committee of the University Hospital of Maastricht in The Netherlands.

2.3. Sample preparation

Twenty-five microliters of concentrated hydrochloric acid, 50 μL of an internal standard working solution (at 0.2 mg/L) and 750 μL of water were added to 250 μL of oral fluid specimen collected with the Intercept® device. After conditioning with 1 mL of methanol and 1 mL of 0.1N hydrochloric acid, the diluted oral fluid samples were applied onto the SPE columns. Clean-up was accomplished with successive 1 mL washes of 0.1N HCl, tetrahydrofuran and a mixture of methanol and water (50:50, v/v). The cartridges were dried by applying full vacuum for 5 min before elution with 0.5 mL of 5% ammonia in methanol. After the extraction, the elution solution was treated according to a variety of different protocols: a simple dilution with 1 mL of water which had been previously proposed [17]; a fairly rapid and controllable concentration step to 50–100 μL, performed in a vacuum centrifuge (Jouan RC 10.22) at 40 °C, followed by addition of 950 μL of ammonium formate buffer (10 mM, with 0.01% formic acid, pH 4.2) before injection onto the LC system; complete evaporation of the elution solution performed with and without adding 50 μL of a 5% hydrochloric acid solution in methanol before taking to dryness. In the latter case the dry residue was then reconstituted in 1 mL of a mixture of the ammonium formate buffer pH 4.2 and methanol (95/5, v/v).

2.4. Chromatographic conditions

LC was performed using a Waters Alliance 2695 separation module. All aspects of system operation and data acquisition were controlled using MassLynx NT 3.5 software (Micromass UK Limited, UK). Analytes were separated on a XTerra MS C_{18} column (2.1 mm × 150 mm, 3.5 μm) (Waters) using a gradient elution with 10 mM ammonium bicarbonate (pH 10) (A) and methanol (B), at a flow rate of 0.25 mL/min. A gradient was carried out starting from 30% B at 3 min, B was then increased to 50% over the next 1 min. From 4 min to 12 min, B was linearly increased to 75%. At 12 min, B was increased to 90% in 1 min before returning to its initial conditions within 0.1 min and equilibrating for 6.9 min, which resulted in a total run time of 20 min. An injection volume of 20 μL was used.

2.5. Mass spectrometry

A Quattro *Ultima* tandem mass spectrometer (Micromass UK Limited, UK) fitted with a *Z-Spray* ion interface was used for all analyses. Ionisation was achieved using electrospray in the positive ionisation mode (ES+). The following conditions were found to be optimal for the analysis: capillary voltage, 1.0 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 800 L/h. The appropriate multiple reaction monitoring (MRM) conditions for the individual analytes and their respective deuterated analogues, were determined by direct infusion into the mass spectrometer. The cone voltage (CV) was adjusted to maximise the intensity of the protonated molecular species $[M + H]^+$ and collision induced dissociation of each protonated molecule was performed. Collision gas (argon) pressure was maintained at 2.7×10^{-3} mbar and the collision energy (eV) adjusted to optimise the signal for the most abundant product ions, which were subsequently used for MRM analysis.

2.6. Method validation

2.6.1. Selectivity, stability, recovery, assessment of matrix effects

The ability of the analytical method to differentiate and quantify the analyte in the presence of other components in the matrix and of other target analytes was assessed by including blank specimens from a different origin in every run, and by injection of single analyte solutions and evaluation of the MRM transition signal.

Analyte stability in the final extract was checked by repeated injections of an extracted calibrator at 80 μg/L over 48 h, and plotting of the absolute peak areas as a function of time.

Recoveries were estimated by comparing the responses of an 80 μg/L calibrator when the non-deuterated compounds were added before the extraction step with those obtained when the non-deuterated analytes were added after sample preparation.

To assess any potential suppression or enhancement of ionisation due to the sample matrix, two types of experiments were performed. In the first experiment, standards were added after the sample pre-treatment, i.e. just before

Table 1
MRM transitions and conditions for all compounds and their deuterated analogues

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (V)	Collision energy (eV)
Amphetamine	136.10	119.10, *91.00*	20	9, 17
Amphetamine-d_{11}	147.10	98.00	35	18
Methamphetamine	150.10	119.00, *91.00*	20	9, 20
Methamphetamine-d_5	155.10	92.00	20	20
MDA	180.05	*105.00*, 77.00	20	22, 30
MDA-d_5	185.00	168.10	20	10
MDMA	194.05	*163.05*, 105.10	40	12, 25
MDMA-d_5	199.10	165.10	40	13
Cocaine	304.15	*182.10*, 82.10	20	18, 28
Cocaine-d_3	307.15	185.10	22	20
Benzoylecgonine	290.15	*168.10*, 105.00	45	20, 30
Benzoylecgonine-d_8	298.10	171.00	45	20
Morphine	286.10	*165.10*, 152.00	75	40, 57
Morphine-d_3	289.00	165.10	75	47
6-AM	328.10	*165.00*, 152.00	80	40, 70
6-AM-d_6	334.20	165.10	75	40
Codeine	300.10	*165.10*, 128.10	70	43, 58
Codeine-d_6	306.20	165.10	70	45

Italicised transitions were used for quantitation.

injection and the peak responses were compared to those obtained from a methanolic standard diluted in the same volume of ammonium formate buffer. In addition, for an assessment of the effects of untreated samples, the diluted oral fluid samples that would normally be applied to the SPE cartridge were also directly injected into the LC. The second type of experiment involved a continuous post-column infusion of a mixture of the analytes of interest and their deuterated analogues (10 μg/L at a flow rate of 10 μL/min) to produce a constant elevated response in each MRM channel. The interference of this constant response was monitored following the injection of samples either prior to or after SPE clean-up.

2.6.2. Linearity, intra-assay and inter-assay precision, accuracy

Quantitation was performed by integration of the area under the specific MRM chromatograms in reference to the integrated area of its respective deuterated analogue which was added before the extraction procedure. Freshly prepared working solutions of 0.02, 0.1, 0.4 and 1 mg/L in water were used to prepare oral fluid calibrators at a concentration of 2, 10, 20, 40, 80, 120 and 200 μg/L. Standard curves were freshly prepared with each batch of QC and authentic samples. Standard curves were generated using a least-squares linear regression, with a $1/x$ weighting factor. Quality control samples (QC) were prepared for every run in blank oral fluid

Table 2
Stability of 6-AM and cocaine in oral fluid samples following a variety of post-SPE protocols

Post-SPE protocols		Results		
		pH	Stability over 48 h	
			6-AM	Cocaine
1	Dilution with water	11	Unstable	Unstable
2	Concentration to 50–100 μL and dilution with water	8–9	Stable	Unstable
3	Concentration to 50–100 μL and reconstitution in 0.95 mL ammmonium formate buffer 10 mM; 0.01% formic acid)[a]	4–5	Stable	Stable
4	Complete evaporation and reconstitution in 0.95 mL ammmonium formate buffer 10 mM:0.01% formic acid)[b]	4-5	Stable	Stable
5	Concentration to 100 μL, addition of 50 μL MeOH:HCl (95:5), complete evaporation and reconstitution in 0.95 mL ammmonium formate buffer 10 mM:0.01% formic acid)[c]	4–5	Stable	Stable

[a] Regression analysis showed a slight negative trend for cocaine and, to a lesser extent, for benzoylecgonine over a period of 48 h. The decrease in peak area was significantly different from zero (t-test, $p < 0.05$). For all other compounds, there was no significant change of the peak area.
[b] Loss of amphetamines has been reported in the evaporation process.
[c] This procedure extends the sample preparation considerably.

Table 3
Extraction recovery and matrix suppression

Compound	Percent recovery (mean ± 1S.D.)	Estimated effect after SPE (%)
Amphetamine	90.7 ± 4.4	−6.2
Methamphetamine	83.7 ± 4.1	−4.6
MDA	82.2 ± 2.9	−7.8
MDMA	76.6 ± 2.3	−1.9
Cocaine	93.1 ± 0.7	+5.1
Benzoylecgonine	93.9 ± 2.0	+12
Morphine	99.0 ± 0.9	−13
6-AM	91.8 ± 1.6	−9.8
Codeine	94.4 ± 2.0	+3.4

Data represent the mean of four experiments with an 80 μg/L calibrator.

at a concentration of 4, 12 and 100 μg/L. Intra-assay precision was evaluated by replicate ($n = 4$) analysis of the three QC samples in one run. Inter-assay precision was evaluated by replicate analysis of the QC samples in several experiments performed on four different days by two operators. Comparing the calculated concentrations of all calibrators and QC samples to their respective nominal values, provided data on the accuracy of the method.

2.6.3. Limit of detection and limit of quantitation

The limit of quantitation (LOQ) was defined in this study as the lowest calibrator with an acceptable relative uncertainty (coefficient of variation ≤20% and an accuracy of 100 ± 20%). The limit of detection (LOD) was estimated from extracted oral fluid samples, spiked with decreasing concentrations of the analytes, where the response of the quantitative ion was equal to 10 times the response of the blank extract.

3. Results and discussion

3.1. Method validation

The applied gradient ensured the elution of all the drugs examined within 14 min and produced chromatographic peaks of acceptable symmetry. Selectivity of the method was achieved by a combination of retention time, precursor and product ions.

With the exception of MDA, the most prominent precursor-product transitions were used for quantitation of the non-deuterated compounds and the next most abundant, used as qualifiers. For MDA an elevated background response was noted when using the MRM transition based on the most prominent product, i.e. m/z 180 > 163. Improved sensitivity (based on signal-to-noise) was achieved when the MRM transition utilised an alternative product ion (Table 1).

For the corresponding deuterated analogues, only one transition was monitored. Injection of single analyte solutions did not produce interference in the other MRM channels.

One of the limiting factors of LC–MS(-MS) applications is the potential presence of a matrix effect, leading to suppression or enhancement of the analyte response. This typically occurs as a result of insufficient clean-up of the matrix, and although partly overcome by the use of deuterated internal standards, it leads to variable sensitivities, and decreased precision and accuracy. Dams et al. [15] observed no signal suppression for morphine in oral fluid collected with a Salivette® after a simple dilution step, and a small ion suppression effect after SPE (maximum 10–15%) in the ESI mode. Protein precipitation resulted in suppression of 50–70% in some areas of the chromatogram. The Intercept® collector contains a variety of chemicals, i.e. sodium chloride, sodium benzoate, potassium sorbate, bovine gelatin, tween 20, chlorhexidine digluconate and a blue dye, some of which can interfere with the LC–MS/MS detection signal. After direct injection of diluted spiked oral fluid samples collected from seven individuals and analysed without SPE clean-up, relatively small variations in the responses for cocaine and benzoylecgonine (ranging from 12% suppression to 24% enhancement) were observed. In all cases there was an enhancement in the responses for MDMA and methamphetamine, which was very variable ranging from 2 to 62%. The most dramatic effect was the suppression of the analyte signal for morphine and 6-AM (68–87%), and to a lesser extent for codeine, amphetamine and MDA (33–67%). The Oasis® MCX (30 mg, 1 cm^3) SPE cartridges utilise mixed-mode (cation-exchange) sorbents, which pro-

Table 4
Equation of a typical calibration curve with coefficient of determination (r^2), and the estimated limits of quantitation (LOQ) of the method

Compound	Equation	r^2	LOQ (μg/L)
Amphetamine	$y = 1.0306x + 0.1952$	0.999735	2.0
Methamphetamine	$y = 1.8859x + 0.0032$	0.999279	2.0
MDA	$y = 0.4206x - 0.0843$	0.999731	2.0
MDMA	$y = 1.1554x + 0.0752$	0.999598	2.0
Cocaine	$y = 1.0043x + 0.8846$	0.999952	2.0
Benzoylecgonine	$y = 1.4007x + 0.2487$	0.999781	2.0
Morphine	$y = 1.4769x + 0.0991$	0.999467	2.0
6-AM	$y = 0.9387x + 0.1621$	0.999717	2.0
Codeine	$y = 1.0836x + 0.0802$	0.999288	2.0

vide effective sample clean-up for basic drugs. The proposed SPE procedure takes approximately 35 min for a batch of 20 samples and uses three washing steps for selective clean-up of the different components. The 0.1N HCl solution locks the analytes and matrix onto the sorbent using ion-exchange mechanisms and removes any salts and weakly retained species. Tetrahydrofuran was selected because of its water miscibility and because it removes a significant fraction of surfactant material. The third wash with methanol/water (50:50) removes any remaining surfactants. The elution solution was chosen to elute the analytes while retaining the strong organic bases (antibacterial) from the preservative solution. After the extraction, the elution solution was treated according to a variety of different protocols summarised in Table 2. Since pH is a crucial factor in the stability of cocaine and 6-AM, efforts were made to lower the pH of the injected solutions. Stability of analytes was monitored by means of repeated injections of extracted samples over 48 h, and plotting absolute peak areas as a function of time. Evaporation of the eluate to approximately 50–100 μL and reconstitution with 0.95 mL of an ammonium formate buffer (10 mM:0.01% formic acid) was considered the most suitable (Table 2).

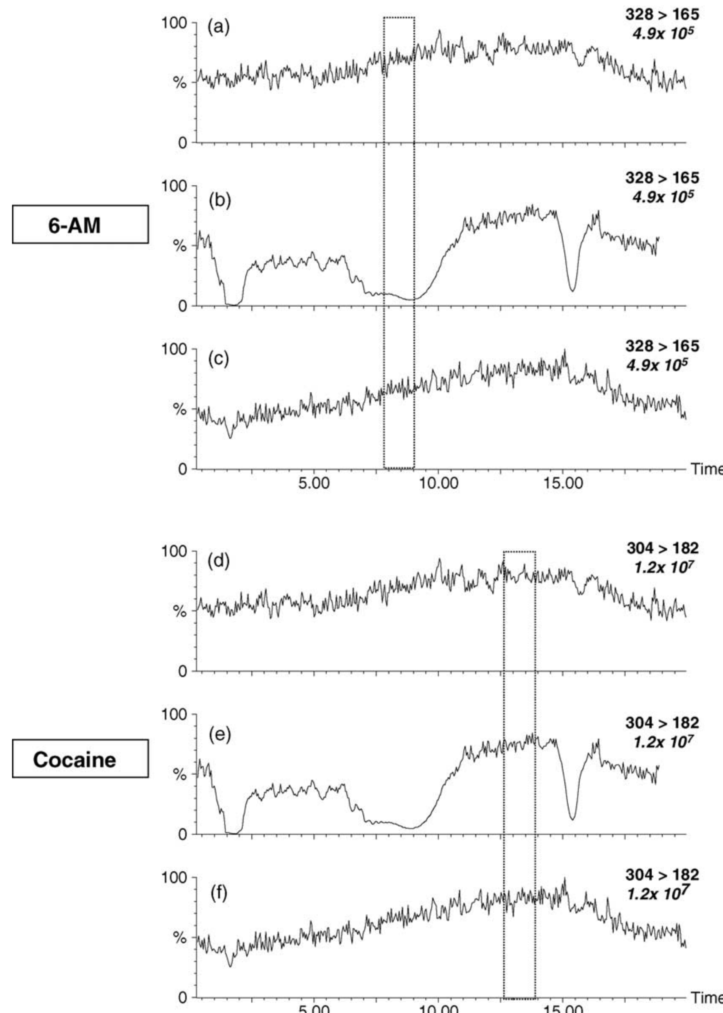

Fig. 1. Evaluation of the effect of the matrix on 6-AM and cocaine response by post-column infusion following an injection of a water-only control (a and d) and a blank sample prior to SPE (b and e) and the same sample post SPE clean-up (c and f). The dotted areas indicate the elution position for 6-AM and cocaine, respectively. Peak intensity is shown on the right-hand corner of each trace.

Table 5
Intra-assay and inter-assay precision (expressed as variation coefficient) and accuracy (expressed as percent deviation) of the QC samples spiked at a concentration of 4.0, 12.0 and 100.0 μg/L

Compound	Intra-assay precision ($n = 4$)			Inter-assay precision ($n = 8$)			Accuracy		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Amphetamine	1.9	1.0	3.1	5.4	5.3	8.6	−3.1	−0.8	−1.8
Methamphetamine	3.3	1.9	1.1	2.2	0.5	1.7	−4.2	−2.0	−1.4
MDA	3.9	3.0	0.8	1.1	1.5	2.6	−3.4	0.1	1.7
MDMA	1.8	1.3	4.5	5.7	5.0	4.9	−5.7	−1.0	−0.6
Cocaine	1.9	1.2	1.2	4.3	5.3	1.8	−5.1	−1.8	−0.6
Benzoylecgonine	1.0	1.2	0.9	5.0	12.6	2.3	−1.4	−1.3	0.1
Morphine	1.0	1.5	1.2	3.3	2.2	3.9	−1.4	−2.2	−1.4
6-AM	2.3	2.6	0.3	4.2	3.2	3.1	−1.5	1.0	1.6
Codeine	7.7	1.4	2.2	1.2	1.3	2.9	−1.1	−0.1	0.5

Table 6
SAMHSA proposed threshold concentrations for oral fluid testing laboratories

Drug classes	Analytes	Threshold concentrations (μg/L)	
		Screening test	Confirmatory test
Amphetamines	d-Methamphetamine[a]	160[(1)]/50[(2)]	160[(1)]/50[(2)]
	d-Amphetamine		160[(1)]/50[(2)]
Cocaine	Benzoylecgonine[a]	20	8
Opiates	Morphine[a]	40	40
	6-AM		4
Marijuana	Δ^3-Tetrahydrocannabinol[a]	4	2[(1)]/4[(2)]

(1) Substance Abuse and Mental Health Service Administration. Mandatory guidelines for federal workplace drug testing programs. Draft #3. December 2000. (2) Substance Abuse and Mental Health Service Administration. Mandatory guidelines for federal workplace drug testing programs. Draft #4. September 2001.
[a] Target analyte for initial screen.

Table 7
Summary of oral fluid concentrations (μg/L) of cocaine (COC), benzoylecgonine (BE), 6-AM, morphine (MOR), codeine (COD), amphetamine (AMP), MDMA and MDA in authentic samples from potentially intoxicated drivers

ID	COC	BE	6-AM	MOR	COD	AMP	MDMA	MDA
1	>2000	>2000	238	725	107	–	–	–
2	26	146	–	–	–	–	–	–
3	915	46	–	–	–	–	–	–
4	–	–	–	–	–	802	–	–
5	–	–	–	–	–	1329	–	–
6	1726	1019	291	1063	132	–	3	–
7	–	–	–	–	–	–	1198	15
8	22	< 2	–	–	–	–	11	–
9	–	–	–	–	–	425	2	–
10	115	199	–	–	–	13	2	–
11	–	–	–	–	–	1797	151	15
12	12	63	–	–	–	75	1066	8
13	–	–	–	–	–	358	< 2	–
14	–	–	–	–	–	488	>2000	123
15	–	–	–	–	–	325	13	<2
16	>2000	>2000	–	–	–	–	–	–
17	191	124	–	–	–	30	330	41
18	555	186	–	–	–	–	–	–
19	72	140	–	–	–	41	598	33
20	–	–	–	–	–	745	–	–

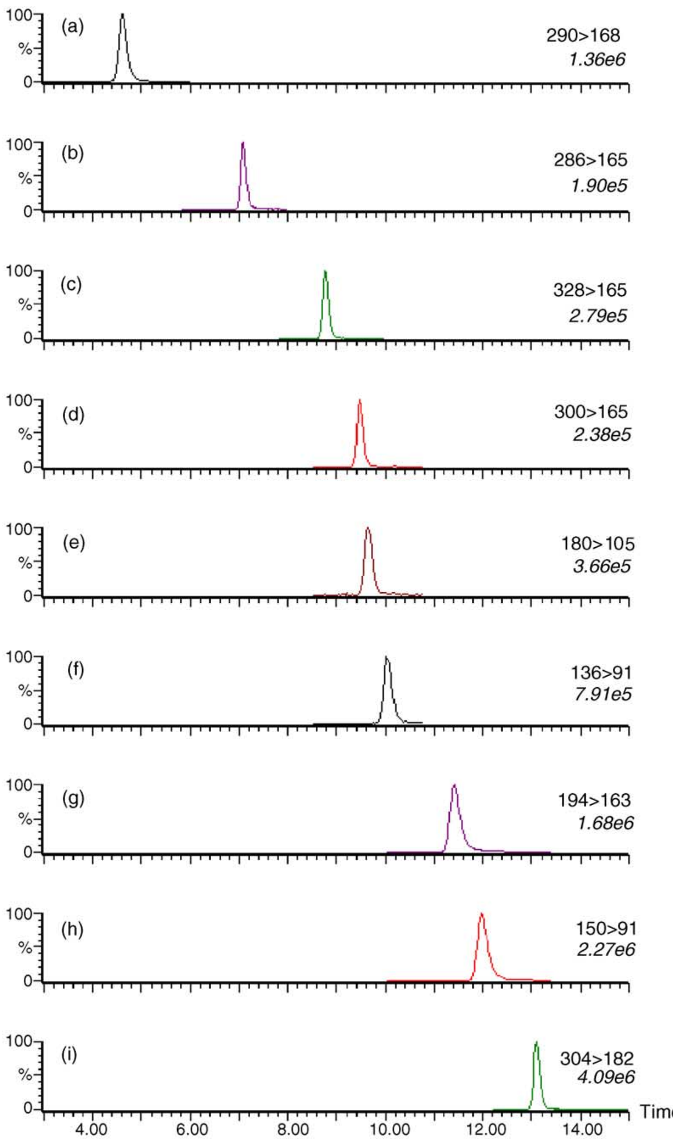

Fig. 2. The precursor-product transitions used for quantitation of (a) benzoylecgonine; (b) morphine; (c) 6-AM; (d) codeine; (e) MDA; (f) amphetamine; (g) MDMA; (h) methamphetamine; and (i) cocaine in the 12 μg/L calibrator prepared by the routine SPE method. Peak intensity is shown on the right-hand corner of each trace.

The results of the extraction recovery study are presented in Table 3. Very high and reproducible recoveries were obtained with this SPE procedure for all analytes.

No clear interferences were detected during the analysis of blank samples. Application of the SPE procedure to selectively spiked samples (one compound and its deuterated analogue), showed no detectable signal (>LOD) in other MRM channels, except when 6-AM was extracted. There was a clear response for morphine and its deuterated analogue after extraction of 6-AM only samples. The estimated concentration of the morphine detected was on average $4.2 \pm 0.05\%$ of the 6-AM concentration in the sample and remained unchanged for a 48-h injection time. As expected, the ratio of the 6-AM

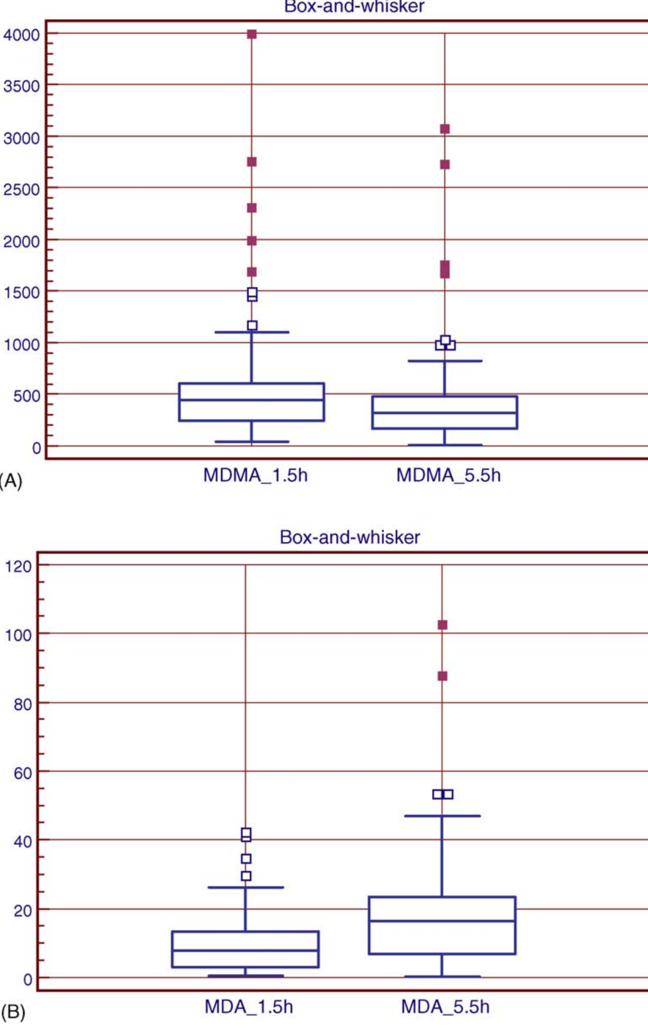

Fig. 3. Box-and whisker plots of MDMA (A) and MDA (B) levels in oral fluid samples collected with Intercept® after a controlled administration of a single dose of MDMA. Concentrations plotted on the *Y*-axis are expressed as μg/L. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line represents the median. The horizontal line extends from the minimum to the maximum value, excluding ''outside'' (square marker) and ''far out'' values (filled square marker) which are displayed as separate points.

response to its deuterated analogue remained identical to a non-extracted standard.

An assessment of the matrix effect after SPE clean-up is presented as a percentage of ion suppression or enhancement in comparison to a standard. The results are shown in Table 3 and clearly indicate that there is only a minimum effect on ionisation due to the presence of the matrix. The post-column infusion experiments were performed to assess the effect of the matrix throughout the course of the *whole* chromatographic run and not just at the elution time for the analytes. The effect on the response for one of the most severely affected compounds, i.e. 6-AM, and one of the least affected compounds, i.e. cocaine, are given in Fig. 1. Fig. 1a and d show the responses obtained in these channels following an injection of a water-only control. The slight changes observed in baseline correspond to the changes in the LC gradient. The responses following the injection of a sample prior to SPE and the same sample following SPE, are also given. The results confirm the usefulness of the SPE method as a sample clean-up before chromatography to obtain

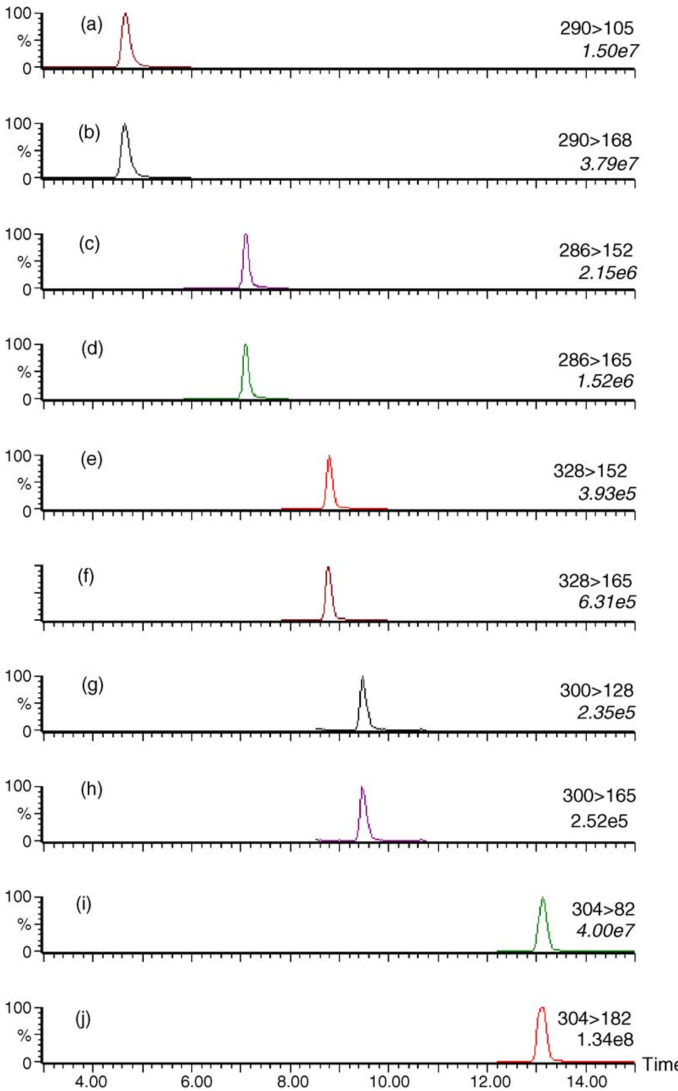

Fig. 4. MRM chromatograms obtained following the analysis of an authentic oral fluid specimen obtained from a driver in a roadside setting. The figure shows the response for the two transitions (qualifier and quantifier) of benzoylecgonine (a, b), morphine (c, d), 6-AM (e, f), codeine (g, h) and cocaine (i, j). Peak intensity is shown on the right-hand corner of each trace.

reproducible and reliable quantitative results for all compounds without major interferences of matrix components.

Calibration curves were made for each compound by plotting the peak-area ratios (compound/internal standards) against the concentration. A weighted (1/*x*) linear regression line was applied for each compound. Linear responses were obtained for all the compounds over the range investigated (2–200 μg/L). Linearity, intra-assay and inter-assay precision, and limits of quantitation are summarised in Tables 4 and 5. Coefficients of variation at the low (4 μg/L), medium (12 μg/L) and high (100 μg/L) QC level were consistently below 10%, except in one case. Fig. 2 shows an MRM chromatogram after extraction of a medium QC sample. The limit of quantitation for all analytes (Table 4) was sufficiently low to meet the requirements of SAMHSA for oral fluid testing in the workplace (Table 6). Limits of detection were estimated to be between 0.2 and 0.5 μg/L.

For each compound two MRM transitions were monitored to provide additional confidence in identification. The ratio of these ions (qualifier ion to quantitation ion) was calculated. For all of the compounds investigated, ion ratios were found to be very reproducible with variation (as %CV) less than 8%.

3.2. Samples

One hundred and eight oral fluid samples were collected with Intercept® from volunteers who received a single representative dose of MDMA (75 or 100 mg), or placebo. The quantitative results are presented as box-plots in Fig. 3. Samples with a concentration above the linear range of the calibration curve were diluted appropriately and re-analysed. Concentrations of MDMA were quite variable and generally high, though not as elevated as in previous studies where the same dose of MDMA was administered and samples were collected by spitting [18,19]. The corresponding MDA concentrations remained quite low, even at 5.5 h. To our knowledge, this is the first study to report on typical concentrations of MDMA and MDA in oral fluid samples collected with Intercept® after a controlled administration and using a validated method of analysis.

During roadside controls for drugged driving, the police collected fifty-five authentic oral fluid samples for a confirmatory analysis in the laboratory. Fig. 4 shows the typical MRM transitions of an Intercept® sample of a mixed cocaine/heroin user. The presence of 6-AM is an important feature of oral fluid analysis in comparison to blood, and has been demonstrated with this collection device in a previously published paper [20]. In Table 7 a summary of the quantitative results for the positive samples is presented. The measured concentrations varied considerably and several samples had to be re-analysed after a 1 in 10 dilution with blank oral fluid and with water. Identical results were obtained which indicated that the method was also applicable to a smaller sample size. The choice of sample volume will largely depend on the application and the requirements for sensitivity. For instance, Dams et al. [21] analysed oral fluid samples collected from pregnant methadone maintenance treatment participants using the Salivette® device. In contrast to the Intercept® specimens, Salivette® samples are not further diluted, e.g. with stabilizing buffer prior to analysis, however, nevertheless, in this population low median concentrations were reported, i.e. 7.5 μg/L morphine, 5.2 μg/L 6-AM, 3.4 μg/L benzoylecgonine and 6.4 μg/L cocaine.

4. Conclusions

We have validated a method for the simultaneous analysis of the most prevalent basic illicit drugs and their metabolites in oral fluid collected with Intercept®. The method combined SPE with LC–MS/MS and provided a thorough clean-up of the matrix to avoid ion suppression, in combination with a high recovery, excellent precision and accuracy in the linear range from 2 to 200 μg/L, using 250 μL of sample. The method was successfully applied to Intercept® samples collected at the roadside and after a controlled administration of MDMA to volunteers.

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