

Nouveaux produits de synthèse : analyse, consommation et métabolisme ; Applications cliniques et médicolégales

Islam Amine Larabi

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Nouveaux produits de synthèse : Analyse, consommation et métabolisme ; Applications cliniques et médicolégales

New psychoactive substances: Analysis, prevalence and metabolism; Clinical and forensic applications

Thèse de doctorat de l'université Paris-Saclay

Ecole doctorale n°577 structure et dynamique des systèmes vivants (SDSV)

Spécialité de doctorat : Sciences de la vie et de la santé

Unité de recherche : Université Paris-Saclay, UVSQ, Inserm, Infection et inflammation, 78180, Montigny-Le-Bretonneux, France

Référent : Université de Versailles-Saint-Quentin-en-Yvelines

Thèse présentée et soutenue à Paris Saclay, le 26/10/2020, par

Islam Amine LARABI

Composition du Jury

Lamiae GRIMALDI-BENSOUDA Professeur des universités, Université Paris Saclay (INSERM U1181)	Présidente
Bruno MEGARBANE Professeur, Université Paris Descartes (INSERM U1144)	Rapporteur & Examinateur
Anne-Laure PELISSIER Professeur, Université Aix-Marseille (INSERM INMED)	Rapportrice & examinatrice
Pascal KINTZ Professeur, Université de Strasbourg	Examinateur

Direction de la thèse

Jean-Claude ALVAREZ Professeur, Université Paris Saclay (INSERM U1173)	Directeur de thèse

Thèse de doctorat

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Liste des abréviations

3-FF : 3-fluorofentanyl

3-HO-PCE : 3-Hydroxy-PCE

3-HO-PCP : 3-Hydroxyphencyclidine

3-MeO-PCP : 3-methoxyphencyclidine

3-MMC : 3-methylmethcathinone

4-HO-MET : 4-hydroxy-N-methyl-N-ethyltryptamine

4-MEC : 4-methylethcathinone

4-MeO-PCP : 4-methoxyphencyclidine

4-MMC : 4-methylmethcathinone

ANRS : Agence nationale de recherche contre le SIDA

BAT : Biochip array technology

CPG : Chromatographie en phase gazeuse

CS : Cannabinoïdes de synthèse

CYP450 : Cytochrome P450

DA : Dopamine

DASH : Depressants-Analgesics-Stimulants-Hallucinogenics

DAT : Dopamine transporter

DMT : Dimethyltryptamine

DXM : Dextrométhorphane

ECG : Electrocardiogramme

EMA : Agence européenne des médicaments

EWS : Early Warning System

FMO : Flavin-containing Monooxygenase

FN : Faux négatif

FP : Faux positif

Fu-F : Furanylfentanyl

GABA : Acide γ-aminobutyrique

GC-MS : Gas chromatography coupled to mass spectrometry

GC-MS/MS : Gas chromatography tandem mass spectrometry

HLM : Human liver microsomes

HPLC : High performance liquid chromatography

HRMS : High resolution mass spectrometry

HSH : Hommes ayant des rapports sexuels avec d'autres hommes

LC-HRMS : Liquid chromatography coupled to high resolution mass spectrometry

LC-MS : Liquid chromatography coupled to mass spectrometry

LC-MS/MS : Liquid chromatography tandem mass spectrometry

MDMA : 3, 4-méthylènedioxyméthamphétamine

MD-PHP: Méthylènedioxy-α-pyrrolidinohexanophénone

MDPV : Méthylènedioxypyrovalérone

MeO-AF : methoxyacetylfentanyl

NA : Noradrénaline

NMDA : N-méthyl-D-aspartate

NOS : Nouveaux opioïdes de synthèse

NPS : Nouveaux Produits de Synthèse (*français*) ou New Psychoactive Substances (*anglais*)

OEDT: Observatoire Européen des Drogues et des Toxicomanies

OFDT : Observatoire français des drogues et des toxicomanies

O-PCE : 2'-Oxo-PCE

PCP : Phencyclidine

PMA : Paramethoxyamphetamine

PrEP : Prophylaxie Pré-Exposition ou Pre-Exposure Prohylaxis

RC : Research chemicals

RdR : Réduction des Risques

RMN : Résonance magnétique nucléaire

SER : Sérotonine

SERT : Sérotonine transporter

SIDA : Syndrome d'immunodéficience acquise

SINTES : Système d'identification national des toxiques et substances

SOHT : Society of hair testing

SPA : Substances psychoactives

THC : Tétrahydrocannabinol

TREND : Tendances récentes et nouvelles drogues

UE : Union européenne

UNODC : United Nations Office on Drugs and Crime

α-PVP : Alpha-pyrrolidinovalérophénone

Liste des articles

1. **Islam Amine Larabi**, Nicolas Fabresse, Isabelle Etting, Emuri Abe and Jean Claude Alvarez. Rapid and simultaneous screening of new psychoactive substances and conventional drugs of abuse. A comparative study of Biochip Array Technology versus LC-MS/MS in whole blood and urine. **TIAFT Bulletin 2019, 49(2)**
2. Nicolas Fabresse, **Islam Amine Larabi**, Tim Stratton, Robert Mistrik, Gregory Pfau, Geoffroy Lorin de la Grandmaison, Isabelle Etting, Stanislas Grassin Delyle, Jean-Claude Alvarez. Development of a sensitive untargeted liquid chromatography–high resolution mass spectrometry screening devoted to hair analysis through a shared MS2 spectra database: A step toward early detection of new psychoactive substances. **Drug Test Anal. 2019; 11(5): 697-708.**
3. **Islam Amine Larabi,** Mohammed Riffi, Nicolas Fabresse, Isabelle Etting, Jean-Claude Alvarez Validation of an UPLC-MS/MS method for the determination of sixteen synthetic cannabinoids in human hair. Application to document chronic use of JWH-122 following a non-fatal overdose. **Toxicol. Anal. Clin 2019;31:283–292**
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7. Julie Chas, MD, Rebecca Bauer, **Islam Amine Larabi,** PharmD, Gilles Peytavin, MD, Perrine Roux, PhD, Eric Cua, MD, Laurent Cotte, MD, Armelle Pasquet, MD, Catherine Capitant, MSc, Laurence Meyer, MD, PhD, Francois Raffi, MD, PhD, Bruno Spire, MD, PhD, Gilles Pialoux, MD, PhD, Jean-Michel Molina, MD, PhD, Jean-Claude Alvarez, MD, PhD. Evaluation of drug abuse by hair analysis and self-reported use among MSM under PrEP: Results from a sub-study of the ANRS-IPERGAY trial. **Soumis dans: Addiction (Aout, 2020)**
8. **Islam Amine Larabi,** Marie Martin, Nicolas Fabresse, Isabelle Etting, Yve Edel, Gregory Pfau, Jean Claude Alvarez Hair testing for 3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl, carfentanil, acetylfentanyl and fentanyl by LC–MS/MS after unintentional overdose. **Forensic Toxicol 38, 277–286 (2020)**
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PREMIERE PARTIE
REVUE BIBLIOGRAPHIQUE

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1. Introduction

Les nouveaux produits de synthèse (NPS) ou Nouvelles Substances Psychoactives (NSP) se définissent par référence à la définition de l'Office des Nations Unies contre la Drogue et le Crime (UNODC), comme les substances naturelles ou synthétiques, consommés sous forme pure ou de préparation pour leurs effets psychoactifs, non contrôlés par la convention des Nations Unies de 1961 sur les stupéfiants, ni celle de 1971 sur les psychotropes, mais qui peuvent constituer une menace pour la santé publique [1].

Le terme NPS privilégié par l'Observatoire Français des Drogues et des Toxicomanies (OFDT) correspond à l'acronyme anglais NPS (New Psychoactive Substances).

Les NPS sont obtenus par des modifications chimiques sur les structures de base des stupéfiants classiques (dits conventionnels) tels que l'amphétamine, la 3,4-méthylène-dioxy-méthamphétamine (MDMA ou ecstasy), le fentanyl, la kétamine ou d'autres drogues, afin d'accentuer leurs effets, et d'échapper à la législation sur les stupéfiants; les nouvelles substances ayant des structures différentes des drogues classiques dont elles sont issues, elles ne sont donc ni listées comme stupéfiants, ni facilement identifiables par les techniques d'analyse usuelles. Ces produits sont vendus via des sites Internet ou dans des magasins spécialisés sous des appellations ambiguës comme «produits chimiques de recherche, Research Chemicals RC», « euphorisants légaux » (legal highs), « euphorisants végétaux » (herbal highs), «sels de bain», « engrais pour plantes », « encens », et sont étiquetés comme impropre à la consommation humaine « Not for Human consumption » afin de dissimuler leur vraie nature [2].

2. Données épidémiologiques

Le marché des NPS est un marché mondial complexe et très dynamique car des substances peuvent apparaître puis disparaître rapidement, mais leur nombre en circulation reste relativement élevé. 120 pays ont déjà signalé à l'UNODC un ou plusieurs NPS sur leur territoire. Jusqu'en décembre 2019, plus de 950 substances ont été signalées par les gouvernements, les laboratoires et les organisations partenaires (**figure 1**).

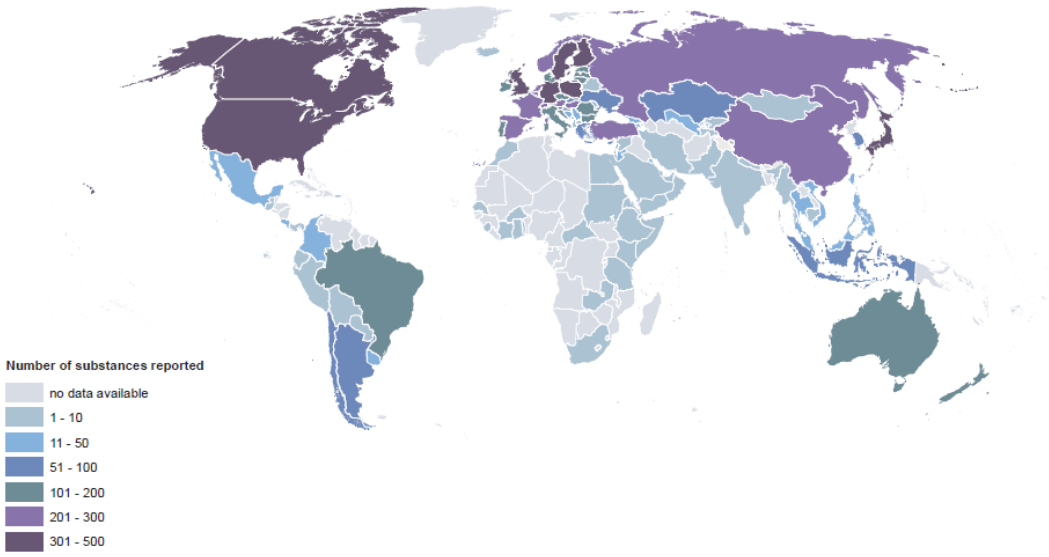


Figure 1 : Émergence mondiale des nouvelles substances psychoactives jusqu'en décembre 2019 (Source : https://www.unodc.org/LSS/Page/NPS)

A la fin de l'année 2018, l'Observatoire Européen des Drogues et des Toxicomanies (OEDT) surveillait plus de 731 NPS, parmi lesquels 55 avaient été détectés cette année-là pour la première fois, et 390 substances ont été détectées l'année précédente (2017) (dont 51 pour la première fois), soit environ la moitié des NPS surveillés par le système d'alerte précoce (EWS : Early Warning System) de l'Union Européenne (UE) [3].

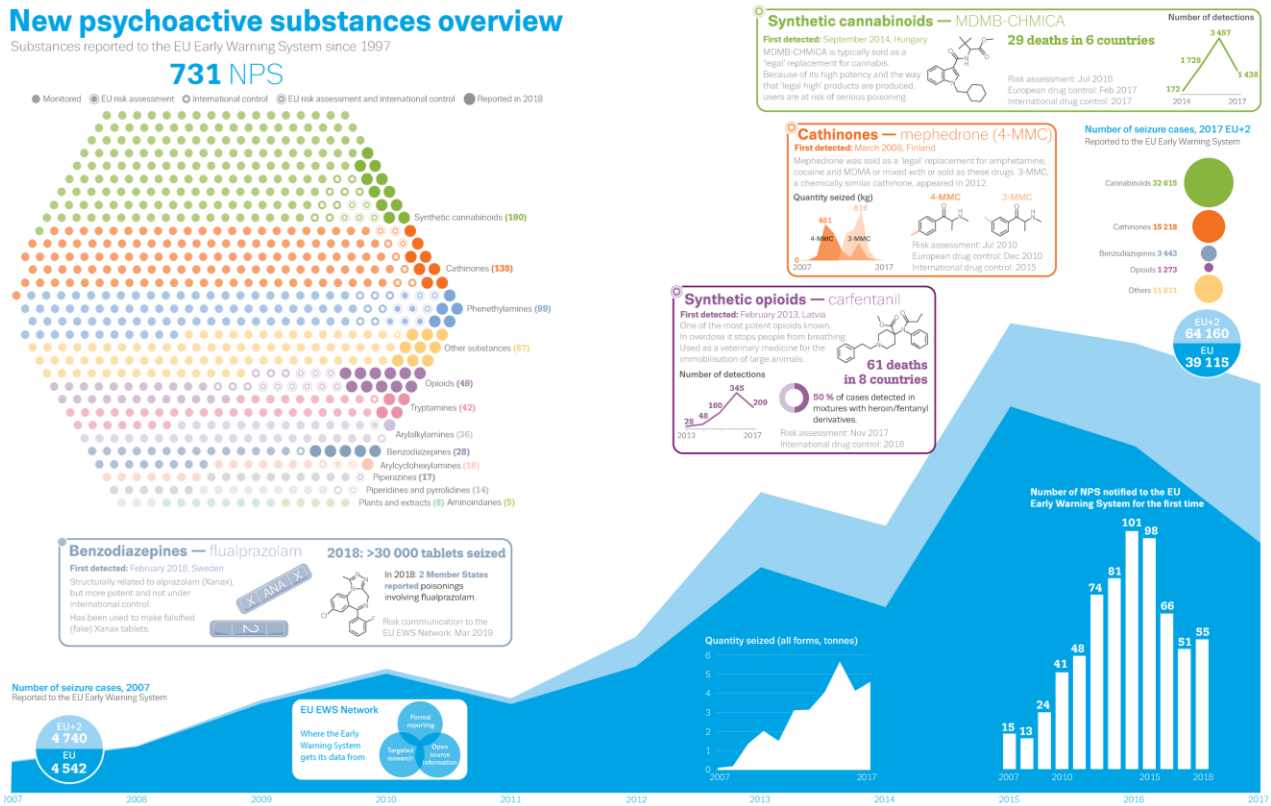


Figure 2 : Aperçu de l'évolution du nombre de NPS par classe chimique signalés au système d'alerte précoce de l'union européenne depuis 1997.
(Source : *https://www.emcdda.europa.eu/media-library/infographic-new-psychoactive-substances-nps-overview-%E2%80%94%C2%A0substances-reported-eu-early-warning-system-1997_en*)

En France, entre 2007 et 2014, le nombre d'identifications de NPS était en constante augmentation. Depuis 2015, ce nombre recule mais reste tout de même élevé. Au total, 222 nouvelles substances ayant circulé au moins une fois en France ont été recensées depuis l'apparition des premières molécules jusqu'à 2018 (**figure 3**).

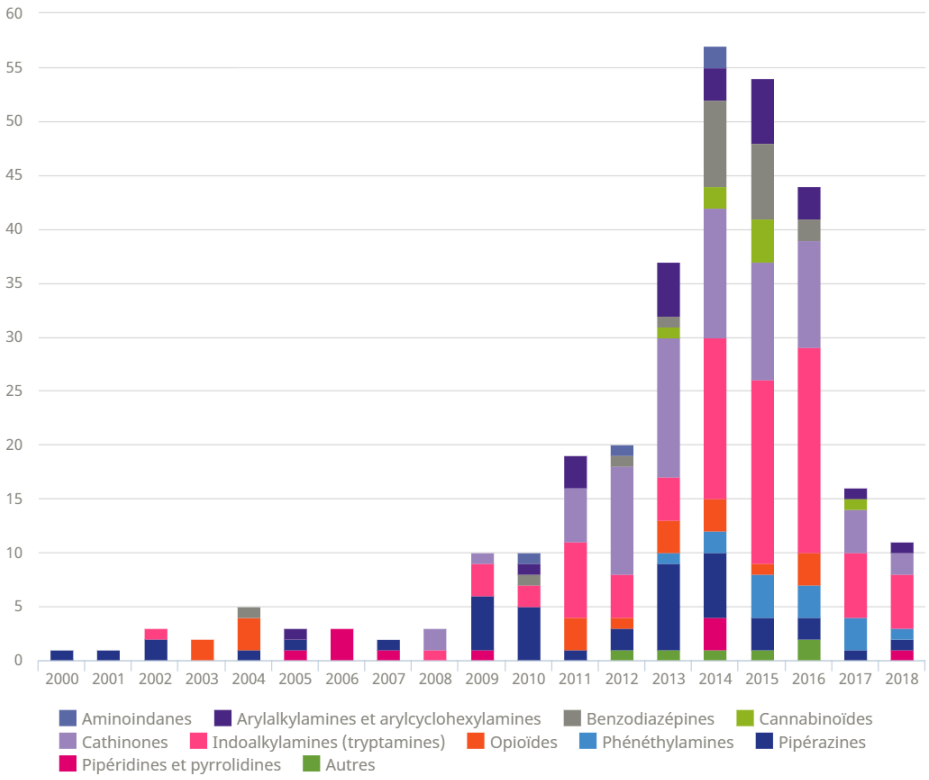


Figure 3: Nombre d'identification de NPS en France par classe chimique. (Source : https://www.ofdt.fr/produits-et-addictions/de-z/nouveaux-produits-de-synthese/#:~:text=L'abr%C3%A9viation%20NPS%20est%20g%C3%A9n%C3%A9ralement,%2C%20cannabis%2C%20etc.).

3. Statut juridique des NPS

Le problème du contrôle de la prolifération de ces substances réside dans leur variété, leur facilité de synthèse par les laboratoires clandestins (souvent implantés en Chine ou en Inde), leur faible coût (prix moyen dépassant rarement 20 euro/g contre 70 euro/g pour se procurer la même quantité de cocaïne), et le fait qu'ils soient indétectables par les analyses toxicologiques usuelles [4].

Néanmoins, afin de limiter leur propagation, des mesures d'interdiction et de contrôle ont été mis en place à l'échelle nationale et internationale, même si le statut juridique de plusieurs NPS reste considérablement différent d'un pays à l'autre. Jusqu'en 2019, plus de 60 pays ont mis en œuvre des réponses juridiques pour contrôler les NPS, de nombreux pays ayant utilisé ou modifié la législation existante et d'autres ayant utilisé des instruments juridiques innovants. Plusieurs pays dont la France ont adopté des contrôles sur des groupes entiers de NPS en utilisant une approche dite générique, qui invoque le principe de «similitude chimique» entre une substance déjà contrôlée et une autre non explicitement mentionnée dans la législation [1]. Malgré tous ces efforts réalisés, beaucoup reste à faire dans ce jeux « du chat et de la souris » entre les laboratoires clandestins fabriquant d'une façon frénétique ces substances et les gouvernements s'appuyant sur les laboratoires d'analyse souvent dépassés, et qui tentent d'identifier rapidement ces produits pour permettre leur contrôle une fois des preuves suffisantes de leurs effets néfastes établies (**figure 4**)

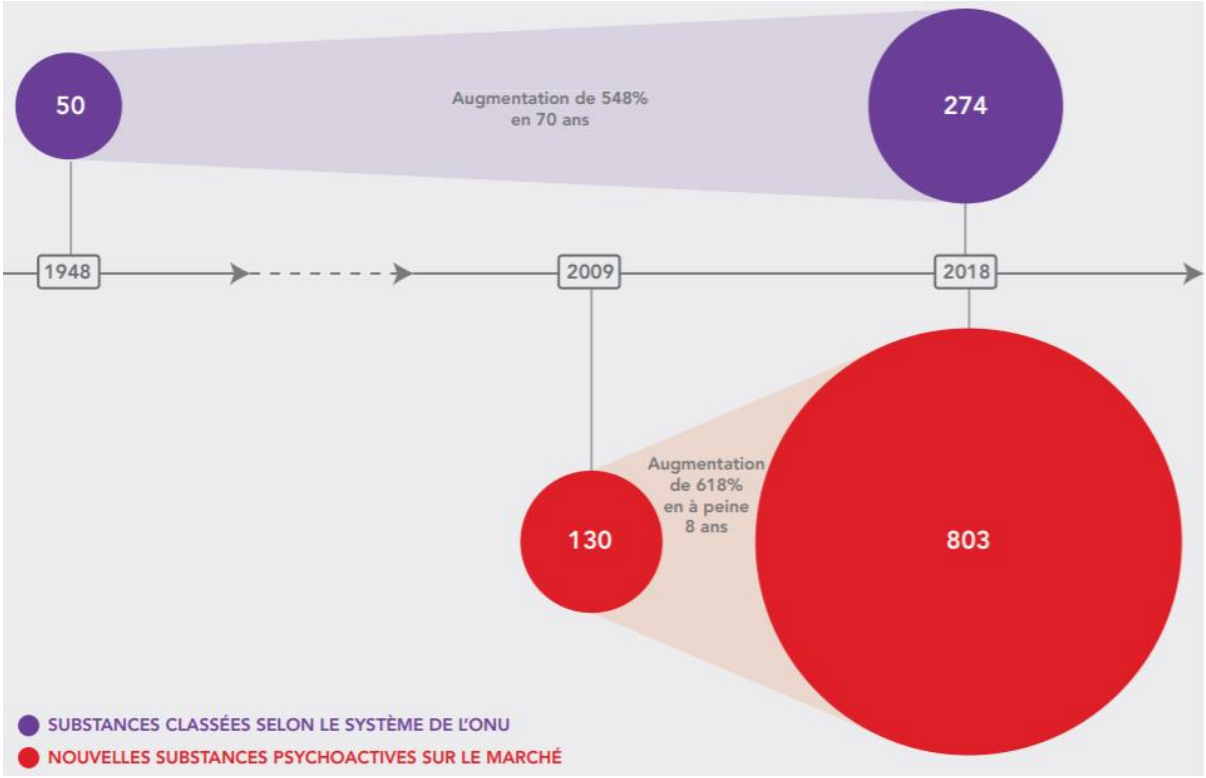


Figure 4 : Défis du contrôle international des nouveaux produits de synthèse montrant le décalage entre le nombre de NPS disponibles sur le marché (en rouge) et ceux classés sous contrôle international (en violet)
(Source : https://www.globalcommissionondrugs.org/reports/classification-psychoactive-substances)

4. Profil des consommateurs de NPS

La mise en place de plusieurs dispositifs de suivi des consommateurs de NPS (exemple en France : TREND, SINTES…etc.) par les institutions et les associations de lutte contre les addictions a largement contribué à une meilleure connaissance des profils, des motivations et des pratiques des usagers de NPS et a permis de proposer des solutions de soins et d'accompagnement pour la réduction des risques (RdR) inhérents à la consommation de ces drogues [5]. Plusieurs profils peuvent ainsi être distingués [6]:

- Les « ***connaisseurs***», appelés communément « **psychonautes** » qui se proclament comme des experts de l'expérimentation de NPS. Leur but est de réaliser de nouvelles expériences de consommation à la recherche d'un effet nouveau ou supérieur sans pour autant courir le risque d'une overdose ou de dépendance. Ils sont réunis en communautés à travers le net et partagent leurs expériences à travers des forums sous forme de récits de voyage ou « trip report ».
- Les « ***habitués*** » ou usagers traditionnels de drogues classiques qui expérimentent les NPS pour des raisons de prix pour les plus modestes (ex : milieu carcéral) ou pour relancer leur carrière de consommateur pour les plus insérés d'entre eux (ex : anciens usagers d'opiacés, sous traitement de substitution) [6].
- Les « ***novices*** ». Ce groupe est constitué d'usagers occasionnels, souvent jeunes, masculins et socialement insérés. Ils se servent d'Internet pour réaliser leurs achats et sont donc peu visibles par les systèmes de soins ou de RdR. Ils sont considérés comme des sujets « à risque » de développement de dépendance ou de survenue de surdosages. Leur expérience avec les NPS se fait souvent à leur insu de par les disparités qui existent entre les produits commandés (souvent des drogues classiques) et ceux réellement livrés (souvent des NPS ou contenant des NPS).
- Les usagers du ***milieu festif gay***, déjà consommateurs de drogues classiques et à la recherche de nouvelles sensations et d'effets en contexte sexuel (appelé **Chemsex**). Ils sont très familiers d'Internet qui leur permet de faire aussi des rencontres. Les NPS sont souvent utilisés pour augmenter la stimulation et le rendement sexuel et faciliter la sociabilité. Une pratique très répandue au sein de cette communité connue sous le nom « Slam » consiste à s'injecter les produits. Parmi les NPS les plus utilisés, on note la méphédrone et 4-methylethcathinone (4-MEC).

- Les jeunes adultes fréquentant ***l'espace festif alternatif techno***, et déjà usagers de drogues conventionnelles. L'effet recherché est la sociabilité et la stimulation pour l'amusement et la fête. Certains usagers de ce groupe ont pu expérimenter les NPS à leur insu lorsque ces derniers sont vendus sous des noms de substances classiques, particulièrement de la MDMA (ecstasy) ou la kétamine.

5. Classification des NPS

La classification chimique est l'une des façons choisies pour représenter les différentes catégories de NPS. La liste des classes chimiques de NPS peut cependant différer en fonction des organismes émetteurs (**tableau 1**).

Tableau 1 : Liste des classes chimiques de NPS selon l'UNODC et l'OEDT
(Source : https://www.npsproject.eu/nps/#1464280658218-93fffffc-133c)

Classes chimiques de NPS selon l'UNODC	Classes chimiques de NPS selon l'OEDT
Aminoindanes	Pipérazines
Cannabinoïdes de synthèse	Benzodiazépines
Cathinones de synthèse	Arylamines
Kétamine et dérivés de la phencyclidine (PCP)	Tryptamines
Autres substances	Opioids
Phenethylamines	Phenethylamines
Pipérazines	Autres substances
Dérivés de plantes	Cannabinoïdes de synthèse
Tryptamines	Cathinones de synthèse

La classification pharmacologique des substances psychoactives (SPA) a aussi été bouleversée par l'arrivée des NPS et a considérablement évolué. Initialement, les SPA étaient classées en « **sédatifs** », « **stimulants** » et « **hallucinogène**s ». La classe des sédatifs a été ensuite divisée en deux sous-groupe « **dépresseurs** » et « **analgésiques** » à la fin du 20e siècle selon le modèle DASH (Depressants, Analgesics, Stimulants, Hallucinogenics).

En 2011, un nouveau modèle dit « The Drugs wheel » (roue de drogues) de Mark Adley [7] a encore élargi le spectre des SPA pour comprendre les **cannabinoïdes, dissociatifs** et **entactogènes** (ou empathogènes). Basé sur ce modèle, le centre d'addictovigilance de Paris a proposé une représentation distinguant les drogues classiques (ou conventionnelles) des NPS afin de simplifier la présentation et permettre de comprendre la complexité des NPS et leur diversité (**figure 5**).

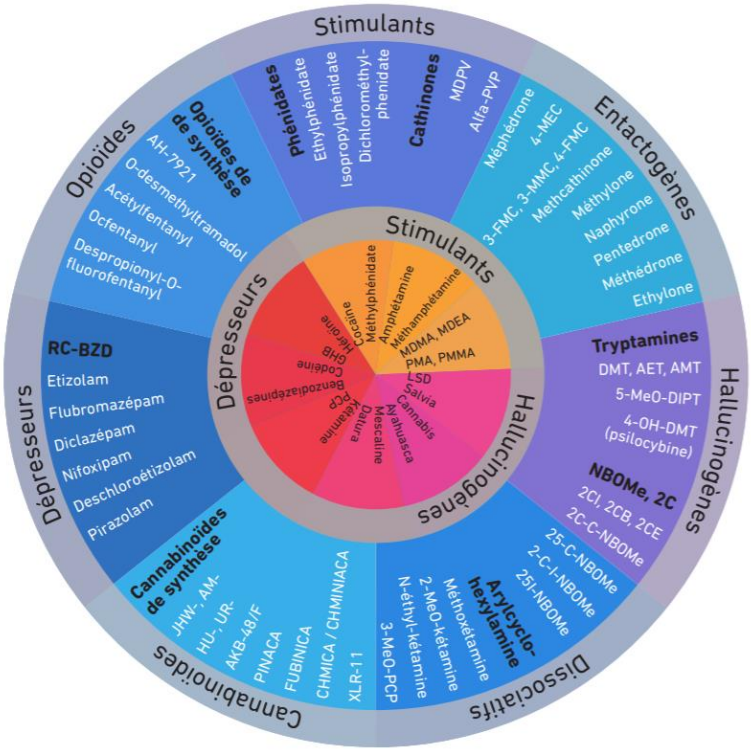


Figure 5 : Roue de drogues (Drugs Wheel) selon le centre d'addictovigilance de Paris. Cercle intérieur : substances psychoactive classiques, Cercle extérieur : nouveaux produits de synthèse. (Source : http://addictovigilance.aphp.fr/2015/12/03/drugs-wheel-nouvelle-ere-des-drogues/).

A l'horizon 2020, la majorité des NPS rapportés à l'UNODC sont des stimulants (cathinones, phenethylamines...etc.), des cannabinoïdes de synthèse et des hallucinogènes classiques avec une augmentation notable des nouveaux opioïdes et benzodiazépines de synthèse ces dernières années (**figure 6**).

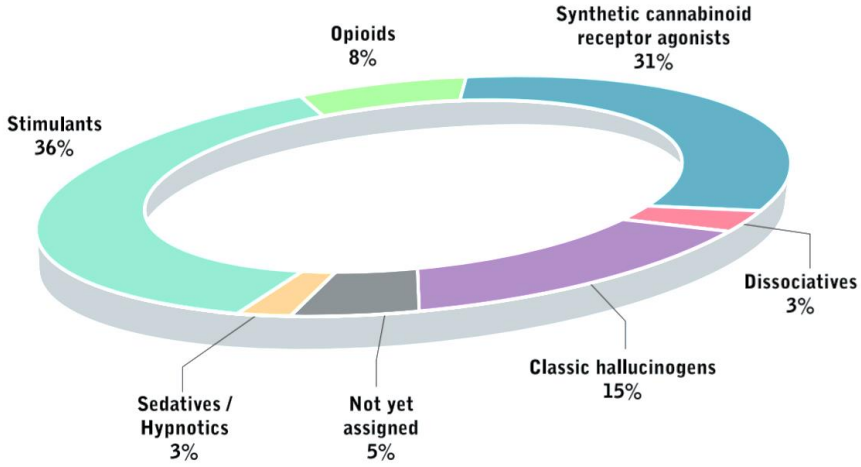


Figure 6: NPS par groupe d'effets, signalées à l'ONUDC jusqu'en décembre 2019. (Source : https://www.unodc.org/LSS/Page/NPS)

6. Mécanisme d'action

Les NPS interagissent avec diverses cibles monoaminergiques. En règle générale, les ***stimulants*** inhibent le transport de la dopamine (DA) et de la noradrénaline (NA) (ex : cathinones de type pyrovalérone) ou induisent la libération de ces monoamines dans l'espace présynaptique (ex : phenethylamines, cathinones dites amphétamine et méthamphétamine-like), les ***entactogènes*** augmentent la libération de sérotonine (SER) de façon similaire à la MDMA (ecstasy) (ex : phénylpipérazines, aminoindanes, amphétamines para-substituées et cathinones dites MDMA-like). L'activité sérotoninergique versus dopaminergique déterminée in vitro par le ratio d'inhibition du transporteur de dopamine (DAT) sur celui de la sérotonine (SERT) (DAT/SERT) peut être utile pour prédire les effets psychotropes (pharmacologiques et toxiques) des NPS et permettre de les classer en substances **méthamphétamine-like** possédant plus de composante stimulante et addictogène (ex : cathinone, methcathinone, ethcathinone, flephedrone…etc.) ou **MDMA-like** avec une composante entactogène accentuée, mais moins addictogène (ex : paramethoxyamphetamine PMA, methedrone…etc.) (**figure 7**) [8].

Un groupe intermédiaire appelé **cocaïne-MDMA-like** (ex : méphédrone, 4-MEC, methylone, ethylone, butylone) comprend des NPS avec une activité inhibitrice de DAT et SERT similaire à la cocaïne et libèrent la sérotonine au même titre que la MDMA. La consommation des produits appartenant à ce groupe présente un risque élevé de pharmacodépendance.

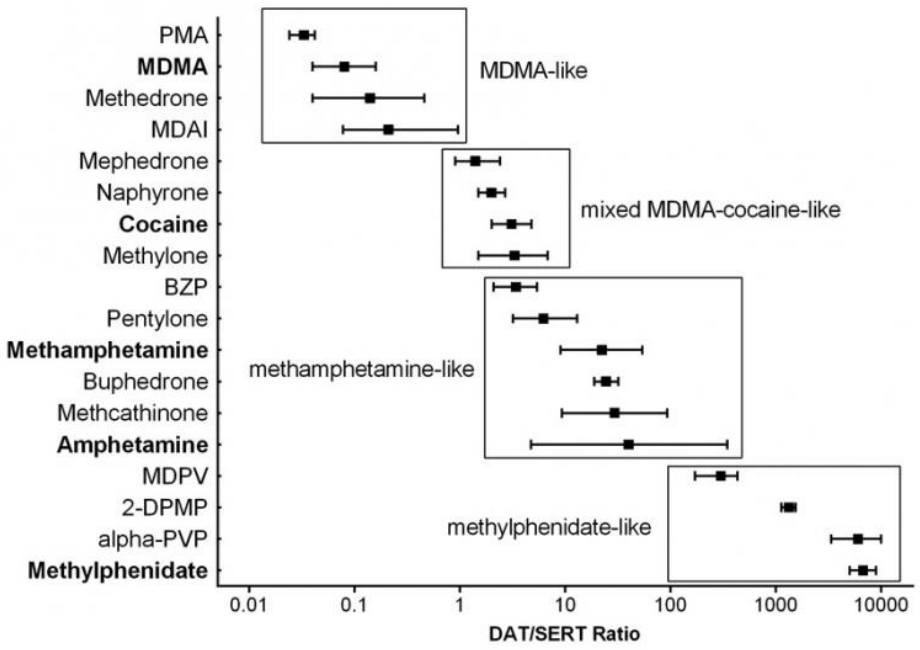


Figure 7 : Classification fonctionnelle des NPS stimulants et entactogènes en fonction du ratio d'inhibition du transporteur de la dopamine (DAT) sur celui de la sérotonine (SERT).

Un faible rapport d'inhibition DAT/SERT (<0,1) indique une activité sérotoninergique dix fois supérieure à celle de la MDMA. Un rapport d'inhibition DAT/SERT élevé (> 10) indique une activité stimulante supérieure à la celle de la méthamphétamine avec un potentiel addictogène élevé. (Source: https://smw.ch/article/doi/smw.2015.14043) [8].

Le groupe des ***hallucinogènes*** se compose de deux catégories de composés : *1) les* ***hallucinogènes*** dits ***classiques*** ou ***psychédéliques*** (ex : LSD, dimethyltryptamine DMT, phénéthylamines) qui produisent une perception altérée de la réalité connue sous le nom de « synesthésie » en agissant comme agonistes des récepteurs sérotoninergiques 5-HT2A ; *2)* les ***anesthésiques dissociatifs*** (ex: kétamine et ses dérivés, phencyclidine, méthoxétamine… etc.) qui provoquent une sensation de détachement et de dissociation de soi et de l'environnement par modulation des récepteurs cérébraux de type N-méthyl-D-aspartate (NMDA). Ce deuxième groupe possède aussi des propriétés stimulantes par inhibition de la recapture des monoamines (DA et NA) [9].

Les ***cannabinoïdes de synthèse (CS)*** miment l'effet du tétrahydrocannabinol (THC) en agissant comme agonistes complets du récepteur cannabinoïde CB1 qui fait partie du système endocannabinoïde responsable de la régulation du comportement, l'humeur, le sommeil, l'appétit et l'immunité. De par leurs structures très variées et souvent différentes du THC, les CS sont des hallucinogènes plus puissants que le THC, et certains ont aussi un effet stimulant [10,11].

Les ***nouveaux opioïdes de synthèse (NOS) (ex** : carfentanil, furanylfentanyl, U-47700, MT-45…etc.**)*** agissent sur les récepteurs opioïdes (souvent de type μ) centraux et parasympathiques dont les effets sont similaires à ceux de l'opium (morphine), alors que les ***benzodiazépines de synthèse*** sont des modulateurs positifs du système GABA-ergique (GABA-A) et agissent de la même façon que les benzodiazépines de prescription. Les deux classes de NPS font partie des dépresseurs du système nerveux central.

7. Effets des NPS sur la santé

Les risques de complications liés à l'usage de NPS sont d'autant plus importants que leurs effets pharmacologiques sont souvent plus prononcés que ceux des congénères dont ils dérivent. De plus, les disparités entre le contenu réel des produits proposés à la vente et le contenu annoncé, en termes de quantité, qualité, ou composition sont à l'origine de plusieurs cas d'intoxications accidentelles, voire de décès [2].

Ces complications vont des convulsions à l'agitation, l'agressivité, la psychose (délire et paranoïa) aiguë ainsi qu'au développement potentiel de la dépendance [1]. Les NPS sérotoninergiques (entactogènes et hallucinogènes) sont associés au syndrome sérotoninergique aigu, à l'hyperthermie, aux convulsions et à l'hyponatrémie. Les médicaments dopaminergiques créent une forte dépendance et la toxicité aiguë comprend une stimulation prolongée, une insomnie, une agitation et une psychose.

D'autres symptômes inquiétants peuvent être rencontrés avec les cathinones de synthèse : tachycardie, douleurs thoraciques, hypertension artérielle, anomalies à l'ECG, mydriase, bruxisme, douleurs abdominales, vomissements, céphalées, œdème cérébral, tremblements, parkinsonisme, rhabdomyolyse, insuffisance rénale, vasoconstriction des extrémités [12]. Plusieurs décès imputés aux dérivés de cathinones sont rapportés dans la littérature [13–17].

Les surdosages aux CS sont dominés par l'agitation, l'anxiété, la paranoïa, les rêves vifs et l'hallucination. Sur le plan somatique, sont observés une tachycardie, une hypertension, des convulsions, une perte de conscience, une dépression respiratoire, une rhabdomyolyse, une insuffisance rénale, et plus rarement un infarctus du myocarde [8]. Plusieurs cas de décès ont été attribués aux CS seuls ou en association avec d'autres NPS [18–21].

Les NOS provoquent un toxidrome pathognomonique des opiacés associant un trouble de conscience avec coma calme hypotonique, myosis bilatéral serré, bradypnée (fréquence respiratoire 12/min), bradycardie, hypotension, vomissements et constipation.

Leurs effets peuvent être jusqu'à 10000 fois plus puissants (ex : carfentanil) que ceux des opiacés classiques. Le risque de décès par dépression respiratoire sévère est majeur [22].

Les surdosages aux benzodiazépines de synthèse sont caractérisés par des troubles de la conscience et de la coordination, coma calme hypotonique, des vertiges, une hypotension, et une amnésie antérograde. Le risque d'une dépression respiratoire sévère peut être majoré en cas d'association avec les opioïdes. Cependant, les décès attribués à cette classe de NPS demeurent rares [23].

8. Traitement des intoxications aux NPS

Les intoxications par les stimulants et/ou entactogènes sont souvent modérées en dehors des cas graves liés à un syndrome sérotoninergique avec hyperthermie, des convulsions et un œdème cérébral dû à une hyponatrémie [24]. Le traitement est principalement symptomatique. La fréquence cardiaque, la pression artérielle et la température corporelle doivent être surveillées. Un bilan biologique comprenant les électrolytes, la créatine kinase, les enzymes

hépatiques et cardiaques est recommandé [8]. L'analyse toxicologique permet de confirmer l'origine de l'intoxication et corréler les concentrations obtenues aux effets observés.

Le traitement d'un toxidrome sympathomimétique (hypertension, tachycardie, mydriase, agitation et hypersudation) comprend principalement l'administration de benzodiazépines et un remplissage hydrique pour contrôler l'agitation, la stimulation cardiovasculaire et l'hyperthermie. L'utilisation d'halopéridol sans benzodiazépines n'est généralement pas recommandée car les seuils de convulsions et de dysrythmie sont abaissés et les effets psychologiques négatifs induits par le médicament, y compris l'anxiété, peuvent augmenter [25]. L'hypertension doit être traitée principalement avec des nitrates. Les bêtabloquants doivent être évités en raison d'une stimulation α-adrénergique sans opposition entraînant une augmentation supplémentaire de la pression artérielle [26]. Un refroidissement physique et une relaxation peuvent être nécessaires en cas d'hyperthermie sévère, mais les antipyrétiques ne sont d'aucune utilité [8].

Les intoxications aux NOS sont souvent graves voire mortelles par dépression respiratoire et arrêt cardiaque, en absence de prise en charge rapide et d'administrations de naloxone, un antagoniste des récepteurs μ qui permet de reverser l'intoxication en quelques minutes après administration parentérale [22]. Un spray nasal est désormais disponible et des programmes de distribution de la naloxone pour une administration en ambulatoire par une personne non médicale se multiplient afin de prévenir la survenue de surdosages [27].

Les intoxications aux benzodiazépines sont traitées par administration de flumazenil, un antagoniste spécifique compétitif du récepteur GABA-A à délai d'action très rapide (environ 1 minutes avec une durée d'effet qui varie de 1 à 2h). Ce traitement reste efficace dans les surdosages aux benzodiazépines de synthèse [28].

9. Analyse des NPS

La diversité des NPS et leur spectaculaire diffusion depuis leur réapparition sur le marché de drogues dans les années 2000 avait incité le développement de nombreuses méthodes analytiques sensibles et fiables pour leur identification et quantification dans différentes matrices biologiques (sang, urine, salive, cheveux…etc.) ou non biologiques (poudres, liquides, plantes...etc.) dont le but est de permettre leur contrôle et limiter leurs dégâts.

Classiquement, un dépistage par immunoanalyse est souvent réalisé dans des laboratoires de biochimie ou de toxicologie non spécialisés sur des automates dotés d'une sensibilité et d'une rapidité largement suffisantes pour répondre à des situations d'urgence (ex : intoxications

aigues). Cependant, ces techniques sont limitées au dépistage de drogues classiques (cocaïne, amphétamines, THC, opiacés) et certains médicaments (ex : benzodiazépines, barbituriques, antidépresseurs) et ne permettent donc pas de détecter les NPS. De plus, elles ont l'inconvénient majeur de générer des résultats faussement positifs (FP) à cause des réactions croisées entre l'anticorps employé et des composés endogènes ou exogènes présentant une similitude structurelle avec les composés d'intérêt, ce qui rend obligatoire la confirmation de tout résultat positif par une technique plus spécifique.

Néanmoins, l'intérêt que représente le marché des NPS et les demandes des laboratoires ont poussé les quelques rares firmes pionnières dans le domaine de l'immunoanalyse telles que Randox® à développer des kits de dépistage dédiés à la détection des NPS [29–31]. Malheureusement, ces techniques bien que très utiles, notamment en toxicologie d'urgence ne sont pas déployées dans tous les laboratoires. Nous aurons l'occasion d'exposer plus loin (partie I des travaux personnels) l'évaluation de l'un des kits de criblage des NPS par immunoanalyse dans différentes matrices biologiques en toxicologie clinique et médicolégale.

L'avènement des techniques séparatives telles que la chromatographie liquide (HPLC : chromatographie liquide haute performance) ou gazeuse (CPG : chromatographie en phase gazeuse) et leur couplage à la spectrométrie de masse simple (LC-MS, GC-MS) ou en tandem (LC-MS/MS, GC-MS/MS), ainsi que leur démocratisation ont permis de s'affranchir de certains problèmes liés aux techniques précédentes.

Les spectromètres de masse sont considérés comme des balances de très haute précision pouvant identifier et doser une quantité de matière aussi infime que le yoctogramme (1 yoktogramme = 10^{-27} kg) par mesure des rapports de masse sur charge (ou m/z) des molécules d'intérêt (ex : NPS) soumises à un champ magnétique. Ces techniques ont révolutionné le champ de la toxicologie et le domaine de l'analyse de traces en général, et ont permis d'analyser des matrices autrefois considérées comme trop complexes, telles que les cheveux.

Le spectre de masse correspond à l'empreinte spécifique d'une molécule et présente plusieurs fragments générés après la cassure de cette dernière et l'analyse ciblée des fragments les plus caractéristiques (d'où l'appellation criblage ou screening ciblé). Cette empreinte spectrale ne peut être partagée avec d'autres substances (à quelques exceptions près, comprenant les isomères de position), ce qui élimine donc la possibilité de tout résultat FP et confère une grande spécificité à ce type d'approche.

Cependant, cette analyse dite quadripôlaire (du nom de l'analyseur le plus employé) appelée aussi spectrométrie de masse de basse résolution nécessite l'établissement préalable d'une bibliothèque de spectres des molécules d'intérêt à l'aide d'étalons de référence achetés auprès de l'industrie chimique. Le nombre de molécules recherchées par chaque laboratoire dépend donc de sa capacité à se procurer ces étalons de référence, notamment ceux des NPS qui présentent la limite du cout (très élevé) et de la disponibilité.

On comprend maintenant pourquoi les différentes études évaluant la prévalence de NPS par cette approche analytique ciblée présentent une certaine hétérogénéité des résultats obtenus, car elles sont directement conditionnées par le nombre et le type de molécules recherchées. Par conséquence, une sous-estimation du nombre de cas de NPS pouvant être identifiés doit être assumée de par la limite imposée par le nombre de composés contenus dans ces bibliothèques de spectres.

Plus récemment, une nouvelle approche de la spectrométrie de masse, dite spectrométrie de masse haute résolution ou HRMS (High Resolution Mass Spectrometry) basée sur le calcul de la masse exacte d'un nombre considérable de composés d'intérêt en toxicologie a permis de s'affranchir de l'emploi des étalons de référence. Des milliers de spectres de masse de haute résolution générés et conservés dans des bibliothèques peuvent être partagées en ligne (ex : bibliothèque mzCloud de ThermoFisher®) et être exploités gratuitement par tous les laboratoires disposant de cette technologie.

Actuellement, la littérature scientifique déborde de centaines de revue techniques, d'articles originaux, ou de case-report faisant mention d'analyse de NPS par LC-MS/MS [32–38] ou par LC-HRMS [39–47].

- ***Place de l'analyse des cheveux dans l'évaluation des expositions aux NPS***

Le sang et l'urine sont les matrices biologiques les plus couramment analysées en toxicologie car elles fournissent des informations très utiles sur la consommation récente de drogues (durée de détection de quelques heures dans le sang à quelques jours dans les urines) et sont faciles à collecter. De plus, le sang est la seule matrice permettant de corréler les effets pharmacologiques ou toxiques observés à la concentration mesurée de drogue. Cependant, à l'exception des intoxications aigues aux NPS où les patients sont admis rapidement dans les services des urgences ou de réanimation qui réalisent des prélèvements précoces de sang ou d'urine, ces matrices présentent peu d'intérêt quand il s'agit des usagers qui sont vus dans les

structures de suivi en addictologie ou en RdR à distance de leur dernière consommation, car souvent, les analyses toxicologiques sont négatives. De plus, plusieurs NPS ont des demi-vies d'élimination courtes ne permettant pas de les déceler dans ce type de matrices.

Les cheveux sont une matrice alternative très utile quand il s'agit d'évaluer des expositions uniques ou répétées moins récentes aux drogues [48–51]. De par l'intégration de la plupart des drogues dans la kératine du cheveu, et de la vitesse de pousse évaluée à 1 cm/mois chez l'adulte, la matrice capillaire offre une large fenêtre de détection, pouvant aller jusqu'à des mois avant le prélèvement en fonction de la longueur de la mèche analysée. De plus, les cheveux sont impérissables et peuvent être conservés à température ambiante. Enfin, il est admis que les composés parents s'intègrent beaucoup mieux que leurs métabolites dans les cheveux et sont donc recherchés en priorité, ce qui évite certaines problématiques liées à l'absence des étalons de référence des métabolites de NPS [52]. Comme évoqué plus haut, l'utilisation des cheveux a accompagné l'évolution fulgurante des techniques d'analyse par spectrométrie de masse qui ont permis d'évaluer des expositions à des concentrations très faibles de l'ordre de picogramme par milligramme de cheveux, et en ont fait aujourd'hui la matrice de prédilection dans un grand nombre d'expertises toxicologiques.

10. Modèles d'études du métabolisme des NPS

Comme tout xénobiotique, les NPS sont métabolisés majoritairement au niveau du foie, bien que d'autres tissus et organes peuvent être impliqués [53]. L'étude des différentes voies métaboliques permet la mise en évidence de nouveaux métabolites considérés comme marqueurs d'expositions plus pertinents que la molécule mère, de par leurs abondances ou leurs demi-vies plus longues qui offrent une fenêtre de détection plus large. Cette étude explore également les interactions métaboliques entre les NPS et d'autres drogues ou médicaments, à l'origine de certains des effets pharmacologiques ou toxiques rapportés.

Pour des raisons d'éthique, les études de ces molécules sur les humains ne peuvent être réalisées. Les études in-vivo sur animal constituent une première alternative aux essais cliniques, mais requièrent des installations spécifiques, et une connaissance plus approfondie de la physiopathologie animale. C'est pourquoi les modèles in-vitro sont devenus l'alternative idéale pour les études de métabolisme des NPS, notamment dans les laboratoires dotés d'équipements de spectrométrie de masse et/ou de résonance magnétique nucléaire (RMN).

Différents modèles in vitro sont utilisés tels que les cultures d'hépatocytes ou les fractions S9, mais les microsomes du foie humain (HLM) sont les mieux établis et les plus fréquemment

utilisés en toxicologie de par leur faible cout, leur bonne conservation (-80°C pendant des années) et leur utilisation simple [54].

Même si ce modèle ne permet pas d'explorer toutes les voies métaboliques car ne disposant que des enzymes de phase I (Cytochrome CYP450, oxydoreductase, Flavin-containing Monooxygenase FMO) et certaines enzymes de phase II (ex : UDP Glucuronyl transferase), son utilisation dans les études du métabolisme des NPS ne cesse d'augmenter et les publications à ce sujets en sont le meilleur reflet [55–59].

11. Conclusion et perspectives

Le phénomène des nouveaux produits de synthèse constitue un réel problème de santé publique auquel les gouvernements doivent faire face. Des mesures juridiques et médicales ont été mises en place ces dernières années afin de protéger les populations et limiter les risques liés à la consommation de ces produits dangereux. L'évolution rapide du marché et la diversité des produits en circulation nécessitent la mise en place efficace et rapide d'un ensemble d'outils à la fois législatifs et analytiques pour contrer ce fléau.

L'avènement des techniques d'analyse de pointe telles que le criblage non ciblé par immunoanalyse sur biopuces et la spectrométrie de masse haute résolution ou le criblage ciblé par spectrométrie de masse en tandem a permis d'objectiver la présence ou l'exposition à ces substances dans différentes situations cliniques ou médico-judiciaires, mais beaucoup reste à faire afin de mieux appréhender la problématique liée à leur usage.

Il en est de même pour les populations d'usagers de NPS dont les profils, les pratiques et les motivations doivent être cernés et étudiés afin que des solutions et des mesures préventives et curatives efficaces et à grande échelle (population générale) puissent être proposées.

Enfin, les données scientifiques relatives à la pharmacologie et la toxicologie de ces nouveaux produits restent insuffisantes et des efforts sont à mener pour étudier leur mécanisme d'action, leur métabolisme et leur toxicité afin d'améliorer les connaissances dans ce domaine et élaborer un plan d'action global de prévention.

DEUXIEME PARTIE
TRAVAUX PERSONNELS

PARTIE I : Aspects analytiques des nouveaux produits de synthèse

1. Rationnel de l'étude

L'évolution constante des NPS sur le marché de drogues et leurs diversités chimiques et fonctionnelles nécessitent le développement continu de techniques performantes d'identification et de dosage afin d'objectiver leur présence chez des populations d'usagers, ou dans des échantillons non biologiques issus des saisis, et ce, pour permettre une prise en charge médicale adéquate dans le 1er cas, ou une décision quant à leur contrôle dans le second.

La démocratisation de la spectrométrie de masse qui offre plusieurs avantages en termes de rapidité, de robustesse et de fiabilité des résultats a poussé d'une façon paradoxale plusieurs laboratoires à s'affranchir de l'utilisation des techniques de dépistage rapide par immunoanalyse qui présentent comme nous l'avons évoqué plusieurs inconvénients liés aux réactions croisées nécessitant une confirmation des résultats obtenus, ou pour la plupart de ces techniques à leur incapacité à détecter les NPS.

Néanmoins, ces techniques restent très utiles notamment en toxicologie d'urgence où un résultat préliminaire rapide peut orienter le diagnostic et permettre au clinicien une mise en place rapide d'un traitement adéquat (ex : naloxone pour les intoxications aux opioïdes). De plus, comme les anticorps employés sont souvent dirigés contre un nombre considérable de molécules appartenant à une même classe chimique, ces techniques constituent un bon complément des techniques de criblage ciblé par spectrométrie de masse où le nombre de molécule recherché est limité par le nombre de spectres préalablement introduits dans les bibliothèques. Seule l'utilisation de la spectrométrie de masse haute résolution (HRMS) qui, parallèlement à l'immunoanalyse, est une autre technique de criblage non ciblé, peut complètement remplacer cette dernière. Cependant, ce type de technologie n'est pas disponible dans tous les laboratoires (coût élevé), et l'exhaustivité de la recherche réalisée (incluant des milliers de spectres) engendre une certaine faiblesse quant à la sensibilité de la méthode (sa limite de détection), et peut limiter ainsi ses applications.

Cette première partie des travaux a pour objectif de développer une stratégie globale incluant deux approches analytiques d'identification et de dosage des NPS dans le sang, l'urine ou les cheveux issus des prélèvements du vivant ou en post-mortem :

- La première approche est basée sur le criblage non ciblé par ***1)*** chimiluminescence sur biopuces où nous aurons l'occasion d'évaluer l'un des rares kits permettant actuellement le dépistage rapide de NPS (***publication n°1***), ou par ***2)*** chromatographie liquide couplée à la spectrométrie de masse haute résolution (LC-HRMS) où nous mettrons en place et validerons une technique dédiée à l'analyse des NPS dans les cheveux (***publication n° 2***).

- La deuxième approche correspond à un criblage ciblé par spectrométrie de masse en tandem (LC-MS/MS) qui a pour but de mettre à jour la liste des NPS recherchés au laboratoire par l'ajout d'autres familles comme les cannabinoïdes de synthèse (***publication n° 3***) et les nouveaux opioïdes de synthèse (***publication n°4***)

Publication n° 1

Rapid and simultaneous screening of new psychoactive substances and conventional drugs of abuse. A comparative study of Biochip Array Technology versus LC-MS/MS in whole blood and urine

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RAPID AND SIMULTANEOUS SCREENING OF NEW PSYCHOACTIVE SUBSTANCES AND CONVENTIONAL DRUGS OF ABUSE

A comparative study of Biochip Array Technology versus LC-MS/MS in whole blood and urine

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Introduction

New psychoactive substances (NPS) are derived from conventional drugs of abuse (DOA) like amphetamines, cocaine, ketamine, fentanyl or benzodiazepines by slight chemical modification in order to obtain new products. They are ambiguously labeled "not for human consumption" and sold as legal highs online or in head shops in different and imaginative packages to avoid regulation [1]. By the end of 2018, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) was monitoring more than 730 new psychoactive substances with about one additional NPS being reported every week in Europe, illustrating how complex this market has become. These substances make up a broad range of drugs, such as synthetic cannabinoids (SC), synthetic cathinones, new synthetic opioids (NSO) and recently, synthetic benzodiazepines [2]. The molecules belonging to each class mimic the effects of the narcotics from which they derive. For example, SC exert the effects on CB1 and/or CB2 endocannabinoid receptors as tetrahydrocannabinol (THC), the active ingredient of cannabis, cathinones mimic the effects of MDMA (ecstasy) and / or cocaine, NSO mimic those of morphine, and synthetic benzodiazepines those of prescribed benzodiazepines. Their particularity lies in their high potency, often greater than those of parent molecules, especially for NSO. Part of the problem in controlling the proliferation of designer drugs lies in their ease of synthesis and purchase, low cost, and the fact that most are undetectable by standard toxicology screening [3]. Cases of NPS-related intoxications or deaths continue to increase, hence the need to detect them, particularly in the presence of any unexplained symptoms associated with negative standard screening.

The identification of NPS in biological samples requires reliable analytical techniques based both on liquid or gas chromatography coupled with mass spectrometry, which requires expensive reference standards (RS) that are often unavailable or difficult to obtain. Consequently, due to the limited number of NPS targeted by laboratories screening, it is likely that the number of reported cases is underestimated. The use of high-resolution mass spectrometry methods (HRMS) based on a shared MS spectra database has allowed to screen for several designer drugs without a need for RS. However, such approaches remain rare and require the use of sophisticated software as well as expensive equipment [4–8]. Despite the unprecedented spread of NPS, there are still no government mandated screening and confirmation cutoffs in any biological matrix [9]. Thus, high-throughput and cost-effective immunoassay screening has emerged as an alternative and complementary techniques to GC-MS and/or LC-MS/MS. Some of these immunoassays have been developed by Randox® as a part of their novel biochip array technology (BAT) [9–11].

The aim of the present study is to evaluate the performance of the new DOA blood and urine assays for the screening of SC, pyrovalerones, fentanyl analogs and benzodiazepines-type NPS as well as conventional DOA using the Evidence® MultiSTAT in authentic whole blood and urine samples from forensic and clinical casework.

Material and methods

Chemicals and reagents

BAT assay

DOA blood and urine assays were provided in a separate kit from Randox toxicology ltd (UK). Each kit contains test cartridges, blood or urine cut-off (thresholds of the concentrations considered positive), positive control, blood diluent and reconstitution buffer. MultiSTAT Tip cartridge were provided in a third kit.

Each cartridge contains a biochip (solid substrate containing immobilized antibody discrete test regions DTRs), assay diluent, conjugate (horseradish peroxidase - labelled drug derivatives), wash buffer, and signal reagent (luminol and peroxide, 1:1).

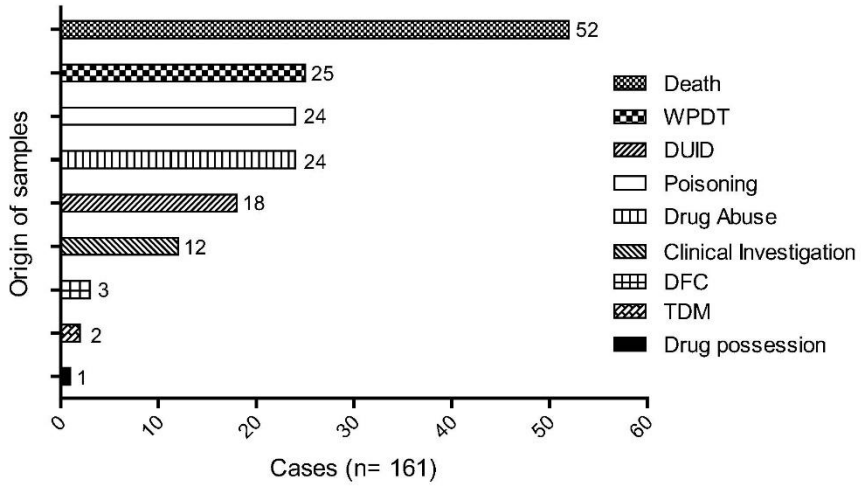


FIGURE 1. CIRCUMSTANCES OF ANALYZED CASES.

ABBREVIATIONS: WPDT: WORKPLACE DRUG TESTING; DUID: DRIVING UNDER THE INFLUENCE OF DRUGS; DFC: DRUG- FACILITATED CRIMES; TDM: THERAPEUTIC DRUG MONITORING

MultiSTAT reconstitution buffer and sample diluent are stable up to the expiry date when stored at +2 to +8°C protected from light.

LC-MS/MS assay

Reference and certified standards were purchased from 3 suppliers: LGC Standards (Molsheim, France), Sigma–Aldrich (Paris, France) and Lipomed AG (Arlesheim, Switzerland) in 1 mg/mL vials or as a powder to be reconstituted with methanol. Acetonitrile, dichloromethane, hexane, ethyl acetate, formic acid and methanol were supplied by Sigma–Aldrich (Paris, France) in mass spectrometry or high-performance liquid chromatography (HPLC) grade. Sodium carbonate and sodium hydrogenocarbonate were from Prolabo (Paris, France). Ultra-pure water (18 MΩ) was obtained by ultrafiltration using a Direct-Q UV3 apparatus (Millipore Corp., Molsheim, France). Formate buffer (containing 2 mM ammonium formate in 0.1% formic acid) was prepared in ultra-pure water and stored after each analysis at +4°C away from light for a maximum of one week. Calibration standards (CS) containing a mixture of all screened compounds at 1, 5, 10, 50, 100 and 500 ng/mL were prepared by spiking stock solutions of reference standards with appropriate volume into a 200 µL blank blood or urine sample. Quality controls (QC) were prepared by the same way at concentrations of 25, 75 and 375 ng/mL. Free-drug blood and urine were obtained respectively from the French blood department (Versailles, France) and volunteers who did not undertake any treatment.

Method

BAT screening on the Evidence® MultiSTAT

The Biochip Array System is a solid-state device made of an array of DTRs containing immobilized antibodies specific to different DOA classes. An enzyme-linked immunosorbent assay (ELISA) is thus performed by chemiluminescence with direct competition between the drug in the specimen (e.g. JWH-018) and the same drug labelled with horseradish peroxidase (HRP) for the antibody binding sites. Signal reagent, containing a 1:1 mixture of luminol and peroxide solutions, is automatically added to the biochip and produces a chemiluminescent signal when HRP-labeled analyte binds to the antibody sites. This signal generated from each of the DTRs is inversely proportional to the analyte concentration and is detected using a digital imaging camera called a charged coupled device (CCD) and compared to that from a cut-off material.

Whole blood or urines samples were centrifuged at 13,000 rpm (11,000 G) for 10 minutes. Two hundred µL of the supernatant of urine, or WB previously diluted with sample diluent (1:4) were loaded into the cartridge and analyzed. A 17 minute single run allows the detection of 20 panels (each with a specific antibody) including a large number of structurally similar molecules. The DOA urine array has an additional panel to test for creatinine and determine whether the sample has been adulterated by dilution or not.

AB-CHMINACA derivatives, PCP and pregabalin were screened only in blood while JWH-018, UR-144 and benzodiazepine II (mainly for lorazepam and clonazepam detection) were screened only in urine. The α-PVP panel does not cross-react with mephedrone-like molecules, thus, these designer drugs were not screened by this BAT assay. AB-CHMINACA confirmation by LC-MS/MS was not performed as the reference standard was not available at the time of the study. All NPS cross-reactivity profiles are available in the product insert.

LC-MS/MS confirmation

The extraction procedure for NPS and conventional DOA screening except THC is derived from previously published methods and adapted to 200 µL of blood or urine [12,13]. Briefly, chromatography was performed on Accela liquid chromatography pump (Thermofisher, Les Ulis, France) coupled with a CTC auto sampler (Thermofisher), using a Hypersil GOLD PFP column (100x2.1 mm, 1,9 µm) preheated at 30°C. The mobile phase is a gradient of acetonitrile and 2mM formate buffer in 0.1% formic acid starting from 20% of acetonitrile to 90% in 10 min at a flow rate of 300 µL/min. The run lasts 12 min. Compounds were detected by a TSQ Vantage triple-

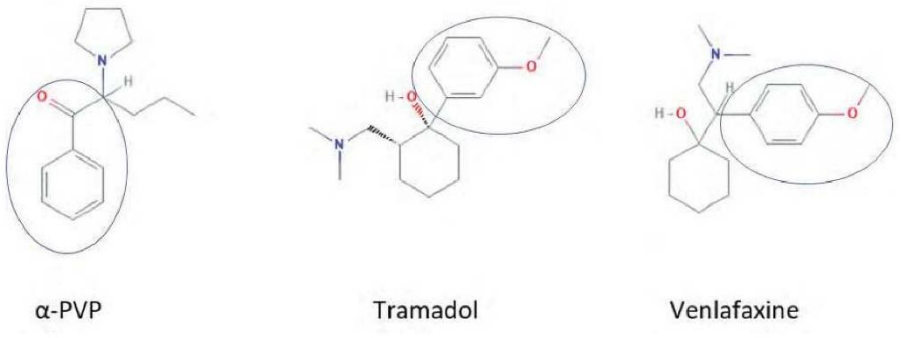


FIGURE 2. HYPOTHESIS OF STRUCTURAL SIMILARITY BEHIND THE CROSS-REACTIONS BETWEEN α-PVP, TRAMADOL AND VENLAFAXINE AT α-PVP ANTIBODY BINDING SITES.

quadrupole mass spectrometer (Thermofisher) equipped with an electrospray ionization (ESI) source set in a positive mode. Data were collected in selected reaction monitoring (SRM) mode, with two m/z transitions per analyte. The method is fully validated. Positive BAT assay and negative LC-MSMS results were confirmed by a LC-HRMS method previously described [5]. THC and its two main metabolites (11-OH-THC and THC-COOH) were screened in blood by a previously published LC-MS/MS method [14]. Only THC-COOH was screened in urine. Ethylglucuronide (EtG) results were correlated to ethanol quantitation by a GC-FID accredited method (LOD = 60 ng/mL). Only discordant results were confirmed by the determination of EtG by LC-MS/MS. Barbiturates were confirmed by GC-MS.

Study design

One hundred and sixty-one whole blood (80) and urine (81) specimens were sampled in different circumstances and analyzed on the Evidence® MultiStat (figure 1). The confirmation was performed using LC-MS/MS and/or LC-HRMS as described above. The evaluation included the following panels: α-PVP, JWH-018, UR-144, AB-PINACA and fentanyl derivatives, PCP, benzoylecgonine (BZE), amphetamine (AMP)/MDA, methamphetamine (MAMP)/MDMA, THC, opiates, oxycodone, 6-monoacetylmorphine (6-MAM), methadone, buprenorphine, benzodiazepines, tricyclic antidepressants (TCA), barbiturates, tramadol, pregabalin, and EtG.

BAT sensitivity (Sn), specificity (Sp) and efficiency were evaluated based on LC-MS/MS cut-offs. Samples were considered true positive (TP) if the BAT assay and LC-MS/MS were positive and true negative (TN) if both results were negative. A negative BAT assay and LC-MS/MS positive was considered false negative (FN). Positive BAT assay and negative LC-MS/MS were false positive (FP). Sensitivity was calculated as TP/(TP+FN) ×100, specificity as TN/(TN+FP) ×100, and efficiency as (TP+TN)/total number of specimens×100. Statistics and illustrations were performed using GraphPad Prism 5.01 software.

Results & Discussion

Overall, 38 toxicological panels were evaluated in 161 blood and urine samples. The comparison of BAT versus chromatographic methods showed more than 90% agreement in 34 out of 38 panels among which 19 panels (50%) showed 100% agreement. Two hundred and sixty-four true positives (blood = 169, urine = 95) were recorded out of 2402 acquired results (11%): 51 for benzodiazepines, 47 for opiates, 42 for THC, 30 for cocaine, 23 for tramadol, 16 for EtG, 10 for methadone, 8 for buprenorphine, 7 for amphetamine, 6 for methamphetamine, 5 for tricyclic antidepressants, 4 for α-PVP, 4 for fentanyl and fentanyl analogs, 4 for oxycodone, 3 for pregabalin, 2 for 6-MAM and 2 for barbiturates. No sample tested positive for synthetic cannabinoids (n=143) and PCP (n=77).

Ninety-one discordant results (57 blood; 34 urine) (3.8%) were observed with the Evidence® MultiSTAT detection compared to chromatographic methods, with 58 FP: (30 blood and 28 urine) (2.4%) and 33 FN (21 blood and 11 urine) (1.4%) (table I).

The specificity was excellent (≥ 94%) for fentanyl analogs, SC, THC, BZE, opiates, 6-MAM, methadone, buprenorphine, PCP, barbiturates, benzodiazepines and EtG in both blood and urine, α-PVP, tramadol, oxycodone and pregabalin in blood, and amphetamine, methamphetamine and tricyclic antidepressants in urine, It remains acceptable (81-88%) for the remaining panels: amphetamine, methamphetamine and tricyclic antidepressants in blood, and α-PVP, tramadol and oxycodone in urine.

The sensitivity when applicable was also excellent (≥ 94%) for fentanyl analogs, BZE, 6-MAM, oxycodone, tramadol, methadone and benzodiazepines both in blood and urine, barbiturates and pregabalin in blood, and α-PVP, THC, opiates, buprenorphine and EtG in urine. It remains suitable for THC, opiates and tricyclic antidepressant in blood (79, 81 and 83%, respectively). However, the sensitivity was insufficient (≤ 67%) for amphetamine, methamphetamine, buprenorphine and EtG in blood (table I).

In previous studies, Ellefsen et al. and Castaneto et al. evaluated the performance of the first three biochip immunoassays on the Evidence® EV 180-120 analyzer (Randox LTD, UK) to screen 20,017 authentic urine for 28 synthetic cathinones (DOA-V), phenylpiperazines (PNP I and II) and benzylpiperazines (BZP) derivatives, as well as 22 SC and 37 metabolites. Sensitivity, specificity and efficiency increased with optimized cut-offs except a slight drop in sensitivity (97.5 to 93.8%) with piperazine derivatives. However, results demonstrated a large negative bias for mephedrone (bath salt I), MDPV (bath salt II) and SC [9–11]. Even if these studies

Table 1.1. Efficiency, sensitivity and specificity of BAT on the Evidence® MultiSTAT according to LC-MSMS cut-off.

Matrix	Blood					Urine					Cut-off ng/mL
Panel	LC-MSMS (+)	LC-MSMS (-)	Efficiency (%)	Sn (%)	Sp (%)	LC-MSMS (+)	LCMSMS (-)	Efficiency (%)	Sn (%)	Sp (%)	
FENTANYL											
MultiSTAT+	1	0	100	100	100	3	0	100	100	100	0.1
MultiSTAT-	0	52				0	56				
AB-PINACA											
MultiSTAT+	0	0	100	N/A	100	0	0	100	N/A	100	1
MultiSTAT-	0	79				0	64				
JWH18											
MultiSTAT+	N/A	N/A	N/A	N/A	N/A	0	0	100	N/A	100	1
MultiSTAT-	N/A	N/A				0	64				
UR 144											
MultiSTAT+	N/A	N/A	N/A	N/A	N/A	0	0	100	N/A	100	5
MultiSTAT-	N/A	N/A				0	64				
α-PVP											
MultiSTAT+	0	0	100	N/A	100	4	8	85	100	85	5
MultiSTAT-	0	73				0	46				
AMP/MDA											
MultiSTAT+	3	13	79	50	81	4	3	92	67	94	10
MultiSTAT-	2	54				3	52				
MAMP/MDMA											
MultiSTAT+	3	1	78	60	81	3	0	97	60	100	10
MultiSTAT-	3	67				2	56				
THC											
MultiSTAT+	15	0	95	79	100	27	0	100	100	100	0.1
MultiSTAT-	4	57				0	39				
BZE											
MultiSTAT+	15	0	100	100	100	15	0	98	94	100	5
MultiSTAT-	0	58				1	44				
Opiates											10
MultiSTAT+	25	0	92	81	100	22	0	100	100	100	
MultiSTAT-	6	42				0	40				

described a high throughput screening that requires only a small urine volume, the negative bias of some tests could be a drag on their use. Moreover, fentanyl analogs, and some SC have not been investigated.

In the present study, five newly marketed NPS were screened positive for α-PVP in 4 urines: N-ethylpentylone (48 µg/mL), α-PHP, MDPHP, pentedrone and hexedrone. However, no blood sample tested positive for α-PVP derivatives (n= 73). The test exhibited a good sensitivity (100%) but specificity in urine dropped to 85% due to the interference of tramadol (n=5) or venlafaxine (±norvenlafaxine) (n=3) when concentrations exceeded 2000 ng/mL. The similarity in chemical structures of the 3 compounds could explain these false positives (FP) (figure 2), but further investigations should be conducted to confirm it as this interference has never been reported so far. Furthermore, mephedrone (2500 ng/mL), 3-MMC (154 and 13020 ng/mL) and 4-MEC (3075 ng/mL) were not detected in 4 urines as these substances do not cross-react with α-PVP antibody. Although pyrovalerone-type NPS are increasingly encountered in drug market, old type cathinones like mephedrone or 4-MEC remain highly abused [15]. Thus, the integration of a specific antibody recognizing these designer drugs should be considered in this screening.

Three fentanyls were screened positive (case 1: 3-fluorofentanyl <1 ng/mL and fentanyl at 3.3 ng/mL; case 2: ocfentanyl at 284 ng/mL; case 3: fentanyl at 4.2 ng/mL). This is the first time 3-fluorofentanyl is identified in Europe in urine, as well as in recovered powder (purity 86%) and hair from a drug addict [16]. Fentanyl was also detected in one blood sample at 14 ng/mL. Good sensitivity, specificity and efficiency were achieved (100%) in both matrices. According to the National Center for Health Statistics, among the 70,200 drug overdose

TABLE 1.2 (CONTINUED). EFFICIENCY, SENSITIVITY AND SPECIFICITY OF BAT ON THE EVIDENCE® MULTISTAT ACCORDING TO LC-MSMS CUT-OFF.

Matrix	Blood					Urine					Cut-off ng/mL
Panel	LC-MSMS (+)	LC-MSMS (-)	Efficiency (%)	Sn (%)	Sp (%)	LC-MSMS (+)	LCMSMS (-)	Efficiency (%)	Sn (%)	Sp (%)	
6-MAM											
MultiSTAT+	0	0	100	N/A	100	2	0	100	100	100	10
MultiSTAT-	0	73				0	59				
Oxycodone											
MultiSTAT+	1	4	94	100	94	3	7	89	100	88	10
MultiSTAT-	0	68				0	52				
Tramadol											
MultiSTAT+	13	0	100	100	100	10	6	90	100	88	5
MultiSTAT-	0	50				0	45				
Methadone											
MultiSTAT+	5	0	100	100	100	5	0	100	100	100	5
MultiSTAT-	0	59				0	61				
Buprenorphine											
MultiSTAT+	2	0	97	50	100	6	0	100	100	100	0.1
MultiSTAT-	2	55				0	55				
Phencyclidine (PCP)											
MultiSTAT+	0	0	100	N/A	100	N/A	N/A	N/A	N/A	N/A	5
MultiSTAT-	0	77				N/A	N/A				
Barbiturates											
MultiSTAT+	2	0	100	100	100	0	1	97	N/A	97	10
MultiSTAT-	0	55				0	36				
Benzodiazepines											
MultiSTAT+	30	2	97	100	93	21	0	98	95	100	1
MultiSTAT-	0	26				1	40				
TCA											
MultiSTAT+	5	7	87	83	88	0	3	94	N/A	94	10
MultiSTAT-	1	50				0	48				
Pregabalin											
MultiSTAT+	3	1	98	100	98	N/A	N/A	N/A	N/A	N/A	100
MultiSTAT-	0	54				N/A	N/A				
EtG						GC-FID +	GC-FID -				
MultiSTAT+	13	2	93	59	96	3	0	100	100	100	5
MultiSTAT-	9	45				0	33				

deaths estimated in 2017 in the US, the sharpest increase occurred among deaths related to fentanyl and fentanyl analogs with more than 28,400 overdose deaths. Their screening should be performed routinely [17].

Unlike cathinones and fentanyl analogs, SC screened negative for the 143 analyzed samples both by BAT and LC-MS/MS. Even if SC remain the largest group of NPS seized and monitored in Europe [2], they are rarely detected in biological samples in comparison to NSO or cathinones [15]. This could be explained by their rapid metabolism, high potency and thus their use at very low doses, all contributing to a reduction in their detectability. Their low reporting could also show the effectiveness of regulation that slows down their spread.

Etizolam, a synthetic benzodiazepine was detected in one urine sample at 125 ng/mL. Its similar structure to prescribed benzodiazepines has allowed its detection by the antibody targeting oxazepam (benzodiazepine I) which is very convenient allowing the detection of these new drugs in a simple way.

For conventional DOA, THC detected well in both matrices with over 95% agreement with LC-MS/MS (n=142). THC at 0.1, 0.3 and 0.9 ng/mL and THC-COOH ≤ 7 ng/mL (n=4) reported negative in blood (sensitivity: 79%). These very low concentrations suggest either an exposure to cannabis probably for more than 24 hours [14] or a passive exposure to cannabis smoke [18].

BZE was detected in both blood (n=15) and urine (n=15) with an efficiency ≥ 98%. One FP in urine reported BZE concentration at 18 ng/mL (sensitivity: 94%). This concentration probably reflects an exposure for more than 48 h since the last consumption, or a potential contamination [19]. In a controlled study, the detection times for BZE ranged from 31 to 106 h. BZE is often used to identify cocaine use in the workplace, treatment, and judicial programs. However, cocaine can be converted to BZE in urine when the pH is basic, allowing the possibility of a positive test due to external contamination. Some officials have suggested testing for EME, another metabolite of cocaine, to circumvent this problem, but other studies demonstrated that cocaine can also be converted to EME when urine pH > 7 [20]. Thus, concentrations below official cut-off (e.g. SAMHSA: 100 ng/mL) should be interpreted with caution.

Opiates were detected in 47 samples (25 blood and 22 urines), 6 FN were reported in blood with concentrations of codeine at 5 ng/mL (alone) and 12 ng/mL associated with morphine (31 ng/mL). Morphine was detected in 4 further samples at concentrations ranging from 11 to 44 ng/mL (sensitivity 81%; specificity: 100%). The interpretation of opiates results are still tricky as the presence of both codeine and/or morphine has four likely explanations: 1) the subject took codeine only; 2) the subject took codeine and morphine; 3), the subject used heroin and finally, 4) the subject took morphine only. Other possibilities might exist (codeine and/or morphine intake in combination with heroin, or consumption of several grams of poppy seeds) but these scenarios are less likely to occur [21,22]. To confirm heroin intake, the BAT on the MultiSTAT integrates a specific antibody of 6-MAM, the main metabolite of heroin. 6-MAM was detected in 2 urines samples (≤ 244 ng/mL) and no cross-reactivity occurred with other opiates present in the samples (specificity: 100%, n= 134). The BAT also includes an antibody for oxycodone screening, a very abused opioid. This drug was screened positive in 4 cases (1 blood and 3 urines) at concentrations ranging from 9 up to 2000 ng/mL. However, several FP have occurred (n= 11) in the presence of other opiates (codeine, morphine, hydrocodone, hydromorphone, ethyl morphine, and dihydrocodeine). The lowest concentrations that read positive for oxycodone in urine was 129 ng/mL of codeine associated with 14 ng/mL of morphine. Therefore, positive results should be confirmed by LC-MS/MS to distinguish between oxycodone or another opiate intake.

MDMA can be detected with the antibody directed against methamphetamine with an approximate cross-reactivity of 66%. Thus, the two compounds can be screened simultaneously. Methamphetamine was detected in one urine specimen (> 2000 ng/mL) and MDMA in 3 blood (560, 588 and 864 ng/mL) and 2 urine samples (400 et > 2000 ng/mL). However, 2 blood samples screened negative for MDMA at concentrations of 69 and 72 ng/mL, and 2 urine for methamphetamine (80 ng/mL) and MDMA (160 ng/mL). The blood cut-off set at 50 ng/mL seems low as the performance of the test was not improved with regard to LC-MS/MS cut-off. A higher cut-off of blood test at 100 ng/mL seems to be more adequate, but should be validated in a larger set of samples, especially with concentrations around the cut-off.

The amphetamine immunoassay is known to show broad antibody cross-reactivity with structurally similar compounds (e.g., pseudoephedrine, ranitidine, phenylephrine, trazodone, m-CPP, fenofibrate, bupropion, labetalol, propranolol, dimethylamylamine, phentermine) [23]. Indeed, in the present study, 16 FP were noticed, 13 from a post-mortem blood, probably related to the presence of some putrefactive amine (e.g. putrescine, cadaverine) or the presence of propranolol in one case (1197 ng/mL) as reported above. The same type of interference could be suspected in the 3 FP from living patients, since biogenic amines (e.g. dopamine, adrenaline, histamine, etc.) could interfere with the detection of amphetamines [24,25]. Moreover, 3 FN were recorded in blood with the presence of amphetamine at 59 ng/mL, pseudoephedrine at 101 ng/mL, and MDA at 37 ng/mL (associated with MDMA at 72 ng/mL). Two further FN in urine were observed (amphetamine at 55 ng/mL and MDA at 90 ng/mL).

The low sensitivity of buprenorphine assay is due to the low number of positive cases and very low concentrations of buprenorphine (0.3 and 1.8 ng/mL) and norbuprenorphine (0.9 and 1.3 ng/mL) found in the samples.

As the quantitation of EtG was not performed in all samples due to the extensive workload during the study, a correlation of EtG with EtOH concentrations was conducted first where 11 bloods reported discordant results out of 105 analyzed samples (efficiency: 93%). The quantification of EtG by LC-MS/MS (LOD: 100 ng/mL) in discordant samples confirms BAT findings. Thus, the sensitivity was improved from 59 to 100%.

Finally, the screening of other psychotropic drugs by BAT was concordant with LC-MS/MS findings in over 87%. False positives with the tricyclic antidepressants immunoassay were often due to the presence of phenothiazine type compounds (alimemazine: n=4; cyamemazine: n=2; chlorpromazine n= 1; levomepromazine: n= 1) or some antipsychotics (quetiapine n=1; zuclopenthixol n=2) [26].

Conclusion

Biochip array technology on the Evidence® MultiSTAT allows rapid detection of a wide panel of designer and conventional drugs of abuse, especially in complex matrices like post-mortem blood with a good agreement regarding LC-MS/MS results. The inclusion of fentanyl analogs and some new SC (e.g. AB-CHMINACA) in this screening will allow better monitoring of these NPS, that are increasingly encountered in drug related intoxications or deaths. The interference of tramadol or venlafaxine with α-PVP antibody has never been described before and should be extensively investigated. As for the previously described immunoassay methods, the results obtained for amphetamines remain insufficient. Amphetamine FP in post-mortem samples should probably be addressed with regard to the presence of some putrefactive bases. Also, the screening of mephedrone-like derivatives is recommended in future development as these molecules remain highly abused and do not cross-react with α-PVP panel. Finally, BAT offers informative preliminary results, especially in urgent circumstances, that should be confirmed by a reference method like LC-MS/MS or GC-MS.

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Publication n° 2

Development of a sensitive untargeted liquid chromatography–high resolution mass spectrometry screening devoted to hair analysis through a shared MS2 spectra database: A step toward early detection of new psychoactive substances

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RESEARCH ARTICLE

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Development of a sensitive untargeted liquid chromatography–high resolution mass spectrometry screening devoted to hair analysis through a shared MS2 spectra database: A step toward early detection of new psychoactive substances

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Abstract

Untargeted liquid chromatography–high resolution mass spectrometry (LC–HRMS) techniques have become indispensable tools for systematic toxicological analysis. They allow the research of an almost unlimited number of drugs within a single analytical cycle, but shared mass spectra libraries are still missing to identify newly marketed compounds, along with defined analytical procedures. This article describes the optimization, validation, and application of an untargeted screening method devoted to hair analysis using data-dependent analysis (DDA) and a shared HRMS database. This method used an ultra-high performance liquid chromatography coupled to a benchtop Orbitrap. Raw MS data were processed with Compound Discoverer software coupled to the mzCloud™ library. Optimizations were performed on blank hair spiked with 19 analytes having different physical and chemical properties. To validate the effectiveness of a shared spectra database, 20 compounds spectra were added and then retrospectively screened. Sensitivity and reliability were evaluated on 317 compounds of interest in toxicology. The method was then applied to 11 hair samples. The matrix effect range by ion suppression/enhancement was 40%–110%. The method allows the detection of 284 among the 317 screened compounds, including 72 new psychoactive substances (NPS). Lower limit of identification (LLOI) and lower limit of detection (LLOD) were 1 to 1000 pg/mg and 1 to 500 pg/mg, respectively. The method was successfully applied to 11 clinical cases and 144 compounds were identified including 24 NPS including AKB48-5F for the first time in hair. We developed and validated an LC–HRMS untargeted screening of 284 compounds and successfully applied it to 11 real hair samples.

KEYWORDS

hair, HRMS, NPS, toxicology

1 | INTRODUCTION

In recent years, the diversity and availability of new designer drugs have increased tremendously. According to the European Monitoring Center For Drugs and Drug Addiction (EMCDDA), two new substances were detected every week over the last five years. By the end of 2016, the EU Early Warning System was monitoring more than 620 new psychoactive substances (NPS), including 66 substances reported for the first

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time. These observations are correlated to an increase number of acute poisonings and deaths.[1-3] The illicit drug market is continuously evolving to circumvent legislation, making it difficult for regulators and forensic toxicologists to monitor these substances.[4]

Screening tools rely mainly on mass spectrometry with homemade spectra databases, which may represent up to a few hundred compounds. New compounds are basically added one by one depending on the laboratory's own cases, the literature, the law, and the availability of certified reference materials (CRMs). This approach is cumbersome and may miss newly marketed drugs. This underlines the increasing need to share information and knowledge regarding designer drugs between laboratories. On the other hand, high-resolution mass spectrometers (benchtop Orbitrap and TOF instruments) allow the research of an almost unlimited number of drugs within a single analytical cycle, but shared spectra databases are still missing, along with defined analytical procedures.

Liquid chromatography–high resolution mass spectrometry (LC–HRMS) untargeted screenings are commonly performed through two kinds of experiments: Data independent analysis (DIA) or data dependent analysis (DDA). DDA includes a full-scan analysis within a defined range of m/z, with a fragmentation of ions having intensity over a specified threshold. This strategy produces specific MS2 spectra allowing an accurate identification, but it will miss a lot of compounds having poor intensities. DIA also includes a full scan analysis, but it is followed by a fragmentation of all ions in a defined m/z range. This approach enables the fragmentation of all ions including those presenting a low intensity. However, it produces complex MS2 spectra and requires data processing software equipped with deconvolution algorithms to reveal MS2 spectra for each precursor.[5]

Drug testing in biological matrices relies on blood, urine, oral fluid, and hair but also tissue analysis in post-mortem cases. The exploration time of drug exposure in blood and oral fluid is usually 24 hours and in urine up to three days. Tissues are devoted to post-mortem analyses. On the contrary, hair is an easy sample to collect in both clinical and forensic cases, which allows the study of a wider exposure window, ranging from weeks to months or even years according to shaft's length. Hair remains of particular interest for screening NPS when the sampling is far from the drugs' intake.[6] For example, synthetic cannabinoids present a short half-life and are mainly excreted as metabolites that are not always known,[7] while hair analysis allows preferably the detection of parent compounds, whose identification is easier.

The aims of this study were (a) to develop an untargeted screening method devoted to hair analysis using DDA and a shared HRMS spectra database, (b) to evaluate the specificity and sensitivity of the method for 284 analytes, and (c) to apply this method to real forensic and clinical cases.

2 | MATERIALS AND METHOD

2.1 | Chemicals and reagents

Water, formic acid, methanol (MeOH), acetonitrile (ACN), heptane, dichloromethane, and isopropanol were MS grade and provided by Merck (Darmstadt, Germany). All other chemicals were analytical grade and provided by Sigma Aldrich (Saint-Louis, USA). Methanolic solutions of each CRM were prepared from pure powders or directly obtained in methanolic solution at a concentration of 1 mg/mL and stored at −20°C. A 1 µg/mL mixture solution, containing all studied CRMs was then prepared by dilution of the 1 mg/mL solutions in MeOH, and stored at −20°C.

2.2 | Sample preparation

Sample preparation including hair extraction with phosphate buffer and liquid–liquid extraction (LLE) was previously published.[8] Briefly, hair samples were decontaminated twice using dichloromethane and washed with warm water. Two weights were evaluated: 20 mg and 50 mg. Each sample was then incubated for 10 minutes at 95°C in 1 mL of phosphate buffer at pH 5.0. LLE was performed by 4 mL of a mixture of heptane/ethyl acetate (v/v: 90/10) after addition of 1 mL of carbonate buffer at pH 9.7. After 15 minutes of agitation and 5 minutes of centrifugation, a second extraction was performed by dichloromethane/isopropanol (v/v: 80/20). Organic phases were conserved and then evaporated to dryness. The residue was reconstituted in 80 µL of mobile phase A (water, ammonium formate 2 mmol/L, 0.1% formic acid). Two injection volumes were tested: 10 µL and 20 µL.

Optimizations (sample preparation, LC conditions, MS-Orbitrap conditions, and data processing) were performed on blank hair with 19 analytes having different physical and chemical properties and representing the different toxicological classes of interest. Each hair sample was spiked with standard solutions to have successive concentrations of 1, 5, 10, 50, 100, 500, and 1000 pg/mg. Chemical characteristics (formula, exact mass, Log P, pKa) of these compounds and hair concentrations found in the current literature after chronic administration are presented in Table 1. Sensitivity was evaluated with 317 toxicologically relevant substances including 72 NPS (Table S1 in the Supporting Information).

2.3 | Instrument

2.3.1 | LC conditions

Chromatography was performed using an Ultimate 3000 pump (Thermo Fisher, Les Ulis, France). Two reverse phase columns were evaluated: Accucore™ phenyl-hexyl (PH, 100x2.1 mm, 2.6 µm, Thermo, Waltham, USA) and Accucore™ pentafluoro-phenyl (PFP, 100x2.1 mm, 2.6 µm, Thermo, Waltham, USA). They were maintained at 40°C. The flow rate was set at 500 µL/min. Elution was achieved as follow: 99% A for 1 minute, linear gradient to 99% B (ACN/MeOH, v:v 50:50, 1% water, 0.1% formic acid), held for 1.5 minutes. Two runs were evaluated: 10 and 20 minutes. The column re-equilibration was performed with a linear gradient to 99% A in 0.2 minutes, held for 3.0 minutes.

2.3.2 | MS-Orbitrap conditions

Compounds were detected using an Orbitrap mass spectrometer (Q-Exactive, Thermo, Waltham, USA) equipped with a heated electrospray ionization (HESI) probe operating in positive ionization mode. Four tune configurations were tested (Table 2), including the

TABLE 1 Compounds selected for method development: Exact mass, Log P, Pka, and hair levels found in literature

N°	Compound	Molecular Formula	*m/z*	Log P	Pka	Hair levels Found in the Literature (pg/mg)	References
1	5-MeO-DALT	$C_{17}H_{22}N_2O$	271.1805	2.8	-	-	-
2	Amphetamine	$C_9H_{13}N$	136.1121	1.8	10.1	1,620 (*n* = 484)	Burgueno et al.[9]
3	Atenolol	$C_{14}H_{22}N_2O_3$	267.1703	0.2	9.6	140–270 (*n* = 6)	Deveaux et al.[10]
4	Benzoylecgonine	$C_{16}H_{19}NO_4$	290.1387	−0.3	3.2/9.5	30–2,980 (*n* = 23)	Alves et al.[11]
5	Bromazepam	$C_{14}H_{10}BrN_3O$	316.0080	1.7	2.7/12.2	880 (*n* = 155)	Unpublished data
6	Buprenorphine	$C_{29}H_{41}NO_4$	468.3108	4.5	7.5/12.5	57 (*n* = 36)	Belivanis et al.[12]
7	*meta*-chlorophenylpiperazine (*m*-CPP)	$C_{10}H_{13}N_2Cl$	197.0840	2.07	8.9	-	-
8	Chlorpromazine	$C_{17}H_{19}ClN_2S$	319.1030	5.4	9.3	2,900–68,200 (*n* = 16)	Shen et al.[13]
9	3-Fluorofentanyl	$C_{22}H_{27}N_2OF$	355.2180	4.12	8.7	60–150 (n = 3)	Larabi et al.[14]
10	Haloperidol	$C_{21}H_{23}ClFNO$	376.1474	3.7	8.7	12,200; 20,100	Shen et al.[13], Weinmann et al.[14]
11	JWH-018	$C_{24}H_{23}NO$	242.1852	6.43	-	0.8–70,5 (*n* = 9)	Salomone et al.[16]
12	Ketamine	$C_{13}H_{16}ClNO$	238.0993	3.35	7.5	100–99,300 (*n* = 887)	Leung et al.[17]
13	MDPV	$C_{16}H_{21}NO_3$	276.1594	3.97	8.4	120; 1000	Alvarez et al.[8], Salomone et al.[18]
14	Mephedrone	$C_{15}H_{15}NO$	178.1226	2.39	8.05	59; 50	Salomone et al.[18]
15	Methiopropamine	$C_8H_{13}NS$	156.0842	1.9	-	-	-
16	Methoxetamine	$C_{15}H_{21}NO_2$	248.1645	0.8	9.3	7.7; 28; 27	Salomone et al.[18]
17	Morphine	$C_{17}H_{19}NO_3$	286.1438	0.89	8.2	280; 1240	Gouveia et al.[19]
18	Paroxetine	$C_{19}H_{20}FNO_3$	330.1500	3.1	9.8	805 ± 710 (n = 4)	Ferrari et al.[20]
19	Zopiclone	$C_{17}H_{17}ClN_6O_3$	389.1123	0.97	6.9/13.0	37; 66	Villain et al.[21]

TABLE 2 HESI probe parameters evaluated

Tune	Spray Voltage (Kv)	Capillary Temperature (°C)	Probe Heater Temperature (°C)	RF Lens
Low	1500	250	100	50
Medium	3000	250	200	75
High	5000	250	300	100
Default	3000	320	320	60

configuration already used for routine analysis (default tune). Max spray current was fixed at 100 AU (arbitrary unit). Nitrogen (Model N2–45, nitrogen generator, Parker, Haverhill, MA, USA) was employed as sheath and auxiliary gas at a pressure of 60 and 10 arbitrary units, respectively. Data were acquired in DDA mode. Precursors' masses and their related fragments ions were measured with a resolution of 70 000 and 17 500 FWHM at m/z 200, respectively. Two analytical mass ranges were evaluated: 125–650 m/z and 125–1000 m/z. Automatic gain control (AGC) target values were $1e^6$ for full MS scan experiment and $1e^5$ for DDA. The minimum value set for precursor ions was $8e^2$ with an intensity threshold set at $1.3e^4$, if the mass peak of interest reaches this minimum AGC target value within the maximum injection time (MIT), a data dependent scan will be initiated. Optimal settings for MIT, dynamic exclusion time (DET) and mass isolation window (MIW) were evaluated using LogViewer™ software.[22] MIT was set at 800 ms for MS and MS2 experiments in order to evaluate the influence of DET and MIW. The evaluation was performed for several parameters: MS MIT, MS2 MIT, repeats (m/z analyzed twice or more), MS and MS2 events. The following DET and MIW values were respectively tested: 3, 6, and 9 seconds, and 0.4, 1 and 4 m/z. The maximum number of precursors per MS cycle was set to 5, and priority was executed according to abundance only (no inclusion list). The influence of normalized collision energy (NCE) was evaluated at three levels: 35%, 45%, and 55%.

Two exclusion lists were built and evaluated. The first list (EL1) was created from three blank hair (=free of drugs with a negative screening) collected from three different donors. Features (m/z, RT) were extracted using Compound Discoverer 2.0 software (Thermo, Waltham, USA) and duplicates were deleted. Among the 19 selected compounds for optimization bromazepam was the analyte providing the lower signal at a concentration of 50 pg/mg. Consequently, bromazepam intensity at 50 pg/mg ($1e^6$) was chosen as the cut-off between a background noise and a significant signal. The exclusion lists was built from features presenting areas greater than $1e^6$, with effective exclusion at m/z retention times (RT) ± 0.5 minutes. The final list contained 2,870 m/z. The second exclusion list (EL2) was created from raw files containing solely the mobile phase processed with the developed method. In this case, features were excluded all along the analysis, generating 464 m/z.

The mass spectrometer was calibrated every day with an MRFA solution (L-methionyl-arginyl-phenylalanyl-alanine acetate) at 1 µg/mL, caffeine at 2 µg/mL and Ultramark® 1621 0.001% (a commercially available mixture of fluorinated phosphazenes) over a mass range 50–2000 m/z.

Chromatographic data acquisition was performed using Xcalibur software (v.4.0, Thermo, Waltham, USA). Exact masses and retention times were determined by injection of a methanolic mixture containing all compounds into the LC–HRMS system.

2.4 | Data processing

Raw data were processed with Compound Discoverer 2.0 software (Thermo, Waltham, USA). Identification was performed by comparison of raw data with the mzCloud™ data, (https://www.mzcloud.org), and relies on exact mass, elemental composition, isotopic pattern and MS2 spectra. Four algorithms are proposed with the Compound Discoverer software (HighChem HighRes, HighChem DP, Cosine, and NIST). The four were evaluated for the 19 compounds at different concentrations; the matching threshold was set at 60%. The algorithm providing lower limits of identification (LOIs) was conserved for the final method. The mzCloud™ database contains 17 compound classes, screenings were performed through six of them: therapeutics/prescription drugs, drugs of abuse/illegal drugs, sports doping drugs, natural products/medicines, natural toxins, and counterfeit drugs (therapeutic).

2.5 | Validation procedure

2.5.1 | Specificity

Specificity was assessed by analyzing drug-free hair from donors after they signed an informed consent. Matrices included brown (M1, n = 1), blond (M2, n = 1), red (M3, n = 1), African (M4, n = 1), and Japanese hair (M5, n = 1).

2.5.2 | Matrix effect

Ion suppression or enhancement (%) was determined by measuring the substance mixture at 100 pg/mg without and in the presence of the extract from six hair samples and calculated as the ratio between peak area in the presence and without a matrix.

2.5.3 | Sensitivity

Sensitivity was evaluated by injecting three different hair matrices spiked with a mixture of 317 substances at different concentrations (1, 5, 10, 50, 100, 500, and 1000 pg/mg). LOI was defined by the lowest concentration of analyte that could be correctly identified by the screening software. Limit of detection (LOD) was defined as the lower concentration exhibiting a signal at least 3-fold the background noise.

2.5.4 | Identification reliability

Reliability was assessed by analyzing three quality controls (QC) A, B, and C, provided by the Society of Hair Testing (SoHT) containing known and unknown substances for inter-laboratory proficiency evaluation (Proficiency Test 2017–1). QC results were then compared with other laboratories' results.

2.5.5 | Library incrementing and retrospective analysis

HRMS experiments allow the analysis of a previously acquired raw file to look for newly added compounds that were absent from the mass spectra database during the first analysis. MzCloud™ is a shared MS2 database continuously incremented with new high resolution MS and MS2 spectra. To highlight these two advantages, compounds from our own laboratory and missing from the database were added to the library: 1-propionyl-LSD, 2-oxo-3-hydroxy-LSD, agomelatine, diacetolol, etizolam, fenethylline, HU-210, hydroxytriazolam, lercanidipine, manidipine, nalmefene, nefopam, niaprazine, oxomemazine, procaine, prozapine, tertatolol, tetracaine, tiagabine, and zaleplon. Compounds were analyzed by flow injection analysis. A 10 µg/mL solution was prepared in water, acetonitrile, and methanol (2:1:1), and was injected. No chromatographic column was used, the syringe was directly connected to the mass spectrometer. The flow rate was 50 µL/min. Data were acquired at the higher resolution (140 000 FWHM) and analyses were performed in parallel reaction monitoring (PRM) mode with an isolation mass window of 1 m/z. Fragmentation was performed with collision energies from 10% to 200% NCE with 10% increments; each scan was acquired three times. Spectra, structures, and metadata were rigorously manually and electronically evaluated for errors, checked for consistency and correctness, filtered, recalibrated and compared with external databases. Once added, raw files presenting unidentified peaks were processed a second time.

2.6 | Application

The method was applied to 11 routine cases with a history of proven or suspected drug use. Eight patients followed were in addiction clinics and three patients were hospitalized in intensive care units. For each case, a hair strand was sampled to explore their history of consumption.

3 | RESULTS

3.1 | Optimization of LC–HRMS parameters

Results are summarized in Table 3. The comparison between the two LC columns shows non-significant differences between peak areas. However, slightly smaller peak widths (n = 3 injections) were observed with PH column (mean: PH: 0.18 minutes vs PFP: 0.19 minutes). Tune parameters exhibiting higher peaks areas were those already used in routine screening (Figure 1). Results of the influence of MIW and DET are presented in Table 4. A DET set at 9 seconds makes it possible to obtain the lowest number of repeated analyses. Lower LOIs were observed with EL2. Average MS and MS2 MITs observed with the final method were respectively 3.3 ms and 80 ms (Table 4), to allow an optimal analysis they were adjusted to 10 ms and 100 ms, respectively. Regarding the four identification algorithms, some compounds remained not identified even at 1000 pg/mg (amphetamine/cosine, paroxetine/HighChem DP). The algorithm providing the lower LOIs along with an accurate identification for all the compounds was

TABLE 3 Final optimization LC, MS, and MS2 parameters

Hair sample	Amount (mg)	50
LC	Column	PH
	Gradient (min)	20
MS	Resolution MS	70,000
	AGC target MS	1^e6
	MIT MS (ms)	10
	Scan range MS (m/z)	125–650
	Resolution MS2	17,500
	AGC target MS2	1^e5
	MIT MS2 (ms)	100
	Isolation mass window (m/z)	1
	NCE (%)	55
	Exclusion list	EL2
	Dynamic exclusion time (sec)	9

HighChem HighRes. LOIs and LODs observed for the 19 compounds selected for optimization are presented in Table 5.

3.2 | Validation

3.2.1 | Specificity

Several compounds were found in matrices presumed to be free of drugs, M1: theobromine and piperine; M2: solanidine, quinidine, cinchonine and chlorpheniramine; M3: methylphenidate; M4: salbutamol, ketoconazole and pholcodine; M5: quinine. Among these molecules, the presence of cinchonine, chlorpheniramine, methylphenidate,

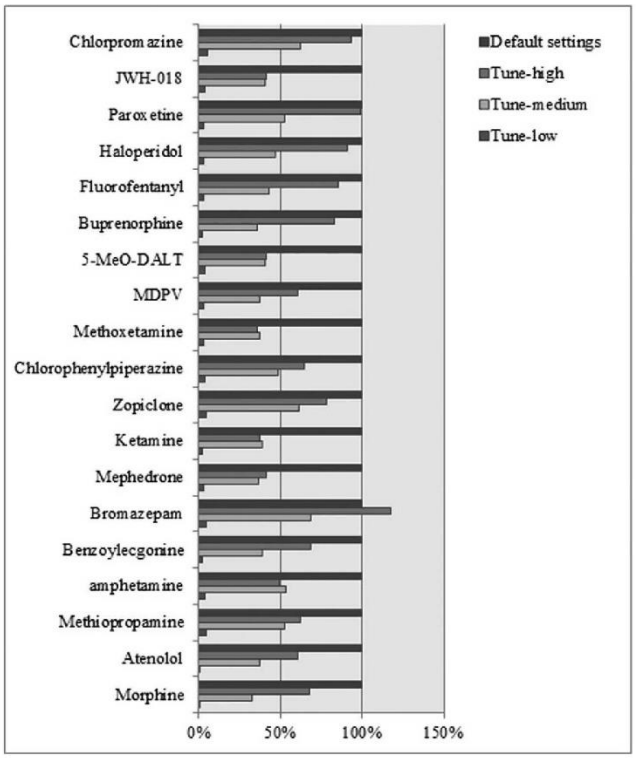


FIGURE 1 Average areas observed with the four different tunes (expressed as a percentage of the default settings area)

TABLE 4 MS and MS2 MIT, events and repeats observed with different mass isolation windows, DET and with the final parameters

	Mass Isolation Window (MIW) (m/z)			Dynamic Exclusion Time (DET) (sec)			Final Parameters
	0.4	1	4	3	6	9	
MS MIT (mean, ms)	2.2	3.1	3.2	3.1	3.2	2.9	3.3
MS2 MIT (mean, ms)	55	49	21	49	67	70	80
Repeats (%)	80	80	80	80	40	30	50
MS events (*n*)	1003	1041	1159	1041	947	938	1694
MS2 events (*n*)	5015	5205	5792	5015	4731	4688	8460

TABLE 5 LOI, LOD, and matrix effect obtained for the 19 compounds selected for optimization

Molecules	LOI (pg/mg)	LOD (pg/mg)	Matrix Effect 100 pg/mg (CV%)
5-MeO-DALT	50	1	66% (4%)
Amphetamine	50	50	89% (3%)
Atenolol	5	1	97% (2%)
Benzoylecgonine (BZE)	50	5	95% (11%)
Bromazepam	100	10	110% (10%)
Buprenorphine	10	5	58% (7%)
meta-Chlorophenylpiperazine (*m*-CPP)	500	5	67% (6%)
Chlorpromazine	100	5	70% (15%)
Fluorofentanyl	5	1	48% (13%)
Haloperidol	5	5	40% (8%)
JWH-018	50	5	100% (14%)
Ketamine	50	5	88% (3%)
Methylenedioxypyrovalerone (MDPV)	5	1	80% (3%)
Mephedrone	50	5	83% (11%)
Methiopropamine	50	50	103% (10%)
Methoxétamine	5	5	88% (8%)
Morphine	5	1	90% (4%)
Paroxetine	100	10	47% (17%)
Zopiclone	50	5	89% (1%)

salbutamol, ketoconazole and pholcodine was confirmed after a comparison against CRMs.

3.2.2 | Matrix effect

The matrix effect by ion suppression/enhancement was 40%–110%, with a CV < 17%. Details are given in Table 5.

3.2.3 | Sensitivity

ACE inhibitors (enalapril, lisinopril, moexipril, quinapril), angiotensin II receptor antagonists (candesartan, eprosartan, olmesartan, valsartan), antibiotic (sulfamethoxazole), anticonvulsants (gabapentine, pregabaline and vigabatrin), antidepressant (fluoxetine), calcium channel blockers (lacidipine, nitrendipine), hallucinogens (ibogaine, mescaline, muscarine, psilocybin, salvinorine A, salvinorine B), parasympathomimetics (nicotine), phosphodiesterase inhibitor (tadalafil), rodenticide (crimidine), and vitamin K antagonists (acenocoumarol, bromadiolone, chlorophacinone, difenacoum, diphacinone, fluindione, phenindione, tioclomarol) presented a lack of sensitivity. Mass resolution, LOIs and LODs for the 284 compounds are presented in Supporting Information S1. Among them, 20 were absent from the database and 16 were not identified due to a lack of sensitivity or interferences (2C-I, 4-fluoroamphetamine, clobazam, N-desmethyl clobazam, desmethylclomipramine, desmethylsertraline, duloxetine, estazolam, hydroxy-alprazolam, hydroxy-bromazepam, irbesartan, lorazepam, oxcarbazepine, psilocin, QUCHIC). This problem was solved for 11/16 compounds by injecting hair samples spiked only with these compounds. Mean LOI was 228 pg/mg (median = 50 pg/mg, min = 1 pg/mg, max = 1000 pg/mg) and mean LOD was 21 pg/mg (median = 5 pg/mg, min = 1 pg/mg, max = 500 pg/mg).

3.2.4 | Identification reliability

Drugs found in SoHT QC A and B with the developed method and by other laboratories are presented in Table 6. Eighteen additional compounds were found and confirmed with CRMs in sample A: Dihydromorphine, 6-acetylcodeine, hydrocodone, anhydroecgonine methyl ester, ecgonine methyl ester, trimethoprime, metoclopramide, doxylamine, diphenhydramine, diltiazem, citalopram, papaverine, noscapine, chloroquine, phenacetine, phenazone, lidocaine, and levamisole. Nineteen additional compounds were found in sample B: 6-acetylcodeine, dihydrocodeine, dextromethorphane, anhydroecgonine methyl ester, ecgonine methyl ester, venlafaxine, desmethylvenlafaxine, trimipramine, trimethoprime, paracetamol, diltiazem, citalopram, desmethylcitalopram, amisulpride, papaverine, noscapine, phenacetine, lidocaine, and levamisole. Five compounds were not found in samples A and B: nordiazepam, THC, THC-COOH, cannabinol, and cannabidiol. Diazepam was not found in sample A and norcocaine was not found in sample B. In sample C, we found trimethoprim and caffeine that had not been reported by other laboratories. There were no false positives; in QC A, QC B, and QC C, false negative rates were 16%, 15%, and 0%, respectively.

3.2.5 | Retrospective analysis

Among the 20 compounds successfully added to the database, 19 were correctly identified, retrospectively. HU-210 was detected (LOD = 500 pg/mg) but not identified probably because the LOI was over the concentration range evaluated by this method (1–1000 pg/mg). The LOI ranged from 5 to 1000 pg/mg and the LOD from 1 to 500 pg/mg. Details are given in Supporting Information S1.

3.3 | Application

Characteristics of patients and hair samples are given in Table 7. The results are presented in Table 8. Samples were analyzed with LC–HRMS for untargeted screening and with liquid chromatography–tandem mass spectrometry (LC–MS/MS) for targeted screening for confirmation and quantification when the provided sample was sufficient. Each compound detected with the untargeted screening was confirmed with CRM. Compounds detected with LC–HRMS and LC–MS/MS are presented in Table 8 column D (D = detected), compounds detected solely with LC–MS/MS are presented in Table 8 column FN (FN = false negative). Nineteen compounds were not detected. The corresponding hair levels have been bolded and underlined. AKB48-5F was detected in case n°10, a summary of the identification procedure is given in Figure 2. Each monoisotopic mass along with the corresponding MS2 spectra is compared to the mzCLoud™ library. Here, the feature m/z = 384.2431 generates an NCE spectra matching with AKB48-5F, with a similarity score provided by the algorithm at 83.7%.

TABLE 6 Drugs identified (+), unidentified (−) and average concentrations found by other laboratories (NA: not applicable; ND: not detected)

			SoHT QC A		SoHT QC B	
Classes	**Molecules**	**LOI (pg/mg)**	**Identification**	**Concentrations (pg/mg)**	**Identification**	**Concentrations (pg/mg)**
Amphetamines	Methamphetamine	100	(−)	ND	(+)	4,920
	Amphetamine	50	(−)	ND	(+)	4,480
	MDMA	1000	(−)	ND	(+)	1,960
Cannabinoids	THC	NA	(−)	30	(−)	90
	THC-COOH	NA	(−)	100	(−)	360
	Cannabinol	NA	(−)	70	(−)	120
	Cannabidiol	NA	(−)	50	(−)	60
Cocainics	Cocaine	5	(+)	8,560	(+)	1,200
	Benzoylecgonine	50	(+)	12,320	(+)	1,360
	Norcocaine	NA	(+)	170	(−)	20
	Cocaethylene	5	(+)	130	(+)	20
Opioids	Morphine	5	(+)	580	(+)	250
	6-monoacetylmorphine	10	(+)	1,050	(+)	700
	Codeine	5	(+)	140	(+)	90
	Dihydrocodeine	5	(+)	1,240	(−)	ND
	Tramadol	50	(+)	950	(+)	140
	Methadone	50	(+)	1,160	(+)	290
Pharmaceuticals	Diazepam	100	(−)	60	(+)	130
	Nordiazepam	100	(−)	50	(−)	19

TABLE 7 Characteristics of patients and hair samples

Case n°	Gender	Age (Years)	Department	Hair			
				Color	Length (cm)	Segment (cm)	Mass (mg)
1	M	41	Addictology	Black	3	1	20
2	M	43	Addictology	Brown	5	2	20
3	M	42	Addictology	Brown	6	2	20
4	M	50	ICU	Black	6	2	21
5	F	39	Addictology	Black	6	2	21
6	M	43	Addictology	Black	4	2	21
7	F	60	ICU	Blond	6	3	7
8	M	49	Addictology	Brown	6	1	20
9	F	28	ICU	Black	25	1	15
10	M	44	Addictology	Grey	1.5	1.5	42
11	M	30	Addictology	Black	2	2	45

4 | DISCUSSION

Performance of MS-based analysis is highly dependent on the sample itself, and even when optimally prepared, the analysis failed if the chosen LC and MS instrument parameters are not optimized. Several studies attempting to improve full MS and DDA experiments were conducted in proteomics and metabolomics fields, but to our knowledge, none in toxicology.[23,24]

Sample amount is an important parameter in hair analysis; most of the time, samples are just enough to perform one single analysis. In the developed method, 50 mg of hair was necessary to achieve lower LOIs. This amount appears to be high when compared to targeted quantitative methods using 20 to 30 mg.[8,25] Few HRMS methods devoted to wide drug screening in hair have been published, but they all used greater sample amounts (150–200 mg), or do not indicate the mass of the sample.[13,26,15,29]

Chromatographic gradient and duration are both important to allow a good separation of isobaric compounds (eg, ethylphenidate/meperidine/methoxetamine, amitriptyline/EDDP/maprotiline). A gradient duration set at 20 minutes was necessary to achieve a fine chromatographic resolution. Isobaric compounds were well separated except amitriptyline and maprotiline (m/z 278.1903 and RT: 11.3 minutes) generating a co-elution/co-isolation/co-fragmentation. RT of amitriptyline is slightly shorter than maprotiline, MS2 spectrum was identified as amitriptyline, and the 9-second DET prevents a second fragmentation, allowing maprotiline identification. This example highlights one of the main biases of DDA. When we spiked hair with maprotiline, good identification was achieved.

With the orbitrap technology, an appropriate MIT is necessary to acquire a fully informative mass spectrum. When MIW decreases, few ions are able to accumulate in the C-trap and MIT increases. By the same way, when DET increases, ions' intensities selected for DDA experiments decreased, which required a higher MIT to reach the AGC target value. These observations are well illustrated in Table 5 and consequently, appropriate MIT were selected. DET is a key parameter to avoid repeated analyses and to fragment molecules present at low concentration. Previous screening methods based on DDA without DET or with short DET are described.[24,28] In our method,

TABLE 8 Compounds detected with LC–HRMS and LC–MS/MS (D); compounds detected solely with LC–MS/MS (false negatives, FN); quantification (not quantified, NQ). Nineteen compounds were not identified, the corresponding hair levels have been bolded and underlined

Compounds	D (n)	FN (n)	Hair Levels (pg/mg)	Compounds	D (n)	FN (n)	Hair Levels (pg/mg)
• Amphetamines				Alimemazine	2		230; 1,550
Amphetamine	3		130–2,400	Alprazolam	2	1	**15**; 50
Ethylphenidate	1		1,210	Amiodarone	1		NQ
Methamphetamine	4	1	**100**–5,325	Amitriptyline	2		2; 4,488
N,N-DMA	1		NQ	Amoxapine	2		<1; 1,300
N-Ethylamphetamine	1		NQ	Aripiprazole	1		26
N-Formylamphetamine	3		NQ	Articaine	1		NQ
• Cathinones				Benazepril	1		NQ
3,4-Methylenedioxy-α-Pyrrolidinohexanophenone	1		NQ	Cetirizine	2	2	**12**; **30**
3,4-Methylenedioxy-N-Propylamphetamine	1		NQ	Citalopram	2	1	**9**; 36
3-Methylcathionone	1		NQ	Clobazam	2	1	**15**; 615
4-Methylethylcathinone	2		240; 625	Clozapine	1		NQ
α-Pyrrolidinohexanophenone	1		6,250	Cyamemazine	2		45; 300
α-Pyrrolidinovalerophenone	2		32; 140	Cyclobenzaprine	1		NQ
Cathine	1		NQ	Deacetyldiltiazem	1		NQ
3,4-Methylenedioxy-α-Pyrrolidinovalerophenone	2	1	**3**–310	Desmethylcarbodenafil	1		80
Mephedrone	5		18–52,160	Desmethylmirtazapine	1		21
Methcathinone	1		NQ	Desmethylnefopam	4		NQ
Methylone	1		50	Diazepam	3		53–1,800
Mexedrone	1	1	**33**	Diltiazem	1		NQ
N-Ethylpentylone	2	1	**15**–1,000	Diphenhydramine	1		560
Normephedrone	2	1	**60**–28,910	Doxepine	1		NQ
Pentylone	1		9	Doxylamine	2	1	**5**; 8,180
• Cocainics				Duloxetine	1		NQ
Anhydroecgonine methyl ester	5		45–2,400	Hydroxylidocaine	1		NQ
Benzoylecgonine	6		570–13,900	Hydroxymetronidazole	1		NQ
Cocaethylene	4		13–21,340	Hydroxymidazolam	1		165
Cocaine	7		70–77,570	Hydroxyzine	8		3.2; 2,850
Ecgonine methyl ester	5		27–1,100	Ketamine	4		130–2,320
Norcocaine	5		20–3,350	Ketoconazole	1		NQ
• Fentanyls				Lacosamide	1		NQ
3-Fluorofentanyl	1		15	Lamotrigine	1		NQ
Fentanyl	1		3	Laudanausine	1		NQ
Furanylfentanyl	1		60	Levamisole	7		28–34,840
• Dissociatives				Levetiracetam	3		4725
Deschloro-N-ethyl-ketamine	1		NQ	Lidocaine	8	1	**20**–4,140
Methoxetamine	1		NQ	Lidocaine-N-oxide	1		NQ
N-methyl-2AI	1		NQ	Loxapine	3		860
Dimethyltryptamine	1		NQ	Mepivacaine	1		NQ
• Opioids				Metoclopramide	1		7
6-Acetylcodeine	1		NQ	Metronidazole	1		NQ
6-Acetylmorphine	2		8; 550	Midazolam	1		210
Codeine	3		13–90	Minoxidil	1		NQ
Detromethorphan	1		10	Mirtazapine	1		60
Dextrorphan	1		NQ	Nalmefene	1	1	**96**
Dihydrocodeine	3		NQ	N-demethylclobazam	1		2,435
Heroine	1		40	N-desmethyltramadol	1		NQ
Morphine	2		10; 80	N-desmethylvenalfaxine	1		3,725
Norcodeine	1		NQ	Nefopam	5	1	**8**–1,250

(Continues)

TABLE 8 (Continued)

Compounds	D (n)	FN (n)	Hair Levels (pg/mg)	Compounds	D (n)	FN (n)	Hair Levels (pg/mg)
Normeperidine	1		NQ	Niflumic acid	1		NQ
Noscapine	2		NQ	Nordiazepam	3	1	**14**–1,670
O-Desmethyltramadol	5		NQ	Norketamine	3		10–114
Oxycodone	1		25	Norlidocaine	1		NQ
Papaverine	2		NQ	Nortriptyline	1		24,506
Pholcodine	1		300	O-desmethylvenlafaxine	1		NQ
Thebaine	1		NQ	Ofloxacine	2		NQ
Tramadol	9	2	**10**; **11**–12,240	Oxazepam	2	2	**19**; **45**
Tramadol N-oxide	1		NQ	Oxomemazine	1		NQ
• Phenethylamines				Paracetamol	3		2,000; 14,000
2-AI	1		NQ	Phenacetin	5		NQ
2C-E	1		70	Pheniramine	1		260
5-MAPB	1		140	Phloroglucinol	1		NQ
Ephedrine	1		1280	Prazepam	3	3	**5**; **7**; **42**
MDA	3	2	**6**; **90**–720	Prilocaine	3		18–240
MDMA	6	2	**6**; **15**–12,690	Quetiapine	2		110; 215
• Synthetic cannabinoids				Quinine	2		NQ
AKB48-5F (5F-APINACA)	1		65	Salbutamol	1		NQ
• Naturals				Salicylic acid	1		NQ
Anatabine	1		NQ	Sertraline	1		397
Caffeine	6		NQ	Sildenafil	4		60–320
Cotinine	2		NQ	Temazepam	1		37
Nicotine	1		NQ	Trimethoprime	1		312
• Pharmaceuticals				Tropatepine	1		4,275
1-(4-chlorophenyl)-phenylmethylpiperazine	2		NQ	Venlafaxine	1		10,35
3-hydroxycyclobenzaprine (cyclobenzaprine metabolite)	1		NQ	Venlafaxine N-oxide	1		NQ
3-hydroxylidocaine	1		NQ	Zolpidem	4		60–3,272
Alfuzosin	1		NQ	Zuclopenthixol	1		65

three DETs were evaluated, and 9 seconds generated the fewest repeated analyses. This result is coherent with an observed peak width of 0.18 minutes (10.8 seconds). MIW has a little impact on repeated analyses, MS, and MS2 events; the value of 1 m/z providing the best sensitivity was selected.

The optimal collision energy to obtain informative fragment spectra depends on molecular mass, which increases at higher mass. NCE allows an adaptation of the collision energy according the mass of the ion. Stepped NCE is often used in MS2 experiments to provide an appropriate fragmentation regardless molecule's mass.[26,30] However, this approach is time consuming (at least two MS2 cycles per m/z) and may miss numerous compounds presenting a weak intensity. Here, a single NCE was selected (55%) to avoid this matter while generating informative MS2 spectra.

A major problem with DDA is fragmentation of contaminants present in mobile phase all along the chromatographic analysis. To our knowledge, no toxicological screening relying on DDA used an exclusion list to avoid a fragmentation of these contaminants. EL1 was built from hair matrix contaminants; this exclusion list provided high LOI. This observation is due to the exclusion of masses (m/z) close to that of spiked compounds, preventing their fragmentation. EL2 was built only on mobile phase contaminants (mainly those released from tubings, analytical columns, and chemicals used for analysis), this exclusion list led to lower LOIs and was included in the final method. This underlines the importance of subtracting background noise when possible. A description of common contaminants encountered with mass spectrometry was done by Keller et al.[31] An inclusion list could be built to privilege the fragmentation of the ions of interest, but the aim of this study was to evaluate untargeted screening. For this reason and to avoid biases, no inclusion list was added.

Regarding sensitivity for the 19 compounds selected for optimization (Table 5), LOIs are satisfactory as they are below hair levels found in the current literature except for three compounds: zopiclone, bromazepam, and JWH-018 (Table 1). Hair levels for zopiclone and bromazepam correspond to a single intake found in drug-facilitated sexual assault cases. Regarding JWH-018, we observed a wide range of concentrations (0.8–70.5 pg/mg), the LOI is 50 pg/mg so some cases may be missed. Table 5 highlights a variable difference between LOI and LOD (LOI = 1- to 100-fold LOD). There is no rule to predict the LOI of a compound from its LOD; this parameter will depend on the signal generated by the compound and its ability to generate a DDA as well as an identification algorithm. Matrix effect remains

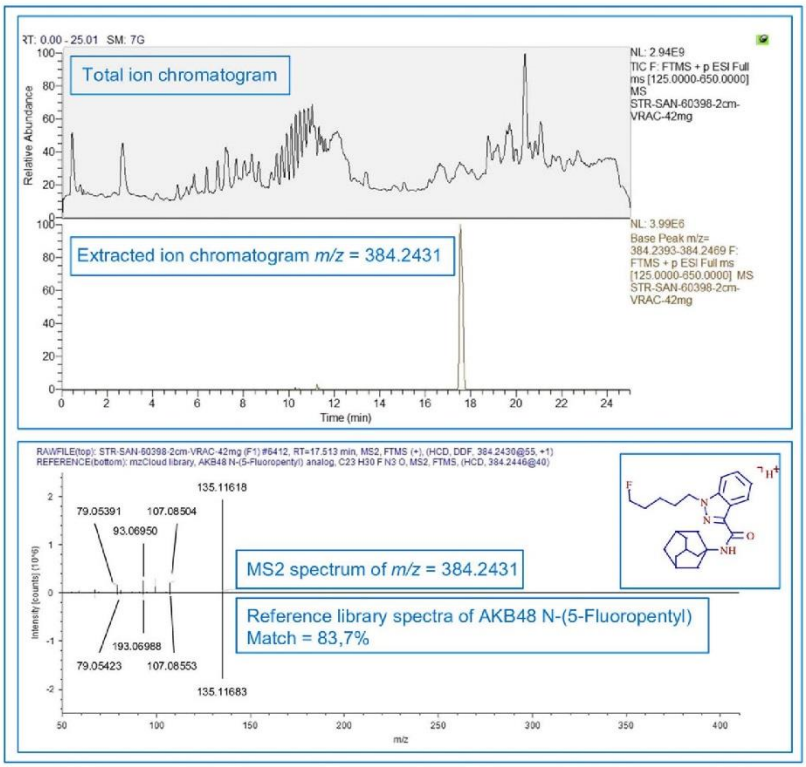


FIGURE 2 Identification procedure of AKB48-5F applied in case 1: Total ion chromatogram (125–650 m/z), extracted ion chromatogram of m/z = 384.2431, MS2, and reference library spectra [Colour figure can be viewed at wileyonlinelibrary.com]

acceptable (40%–110%) with little variability (2%–17%), the higher ion extinction is observed for haloperidol (40%) without alteration of sensitivity (LOI = 5 pg/mg).

Several compounds were found in blank hair. Some were natural, deriving from food consumption (piperine/pepper, theobromine/chocolate, solanidine/potatoes, quinine and cinchonine/sodas). Regarding pharmaceuticals, salbutamol was admitted to be taken as treatment of asthma, ketoconazole for hair dermatophytosis, pholcodine for cough treatment, and chlorphenirамine for allergic rhinitis. A volunteer suffering from attention-deficit hyperactivity disorder admitted being treated by Ritalin® (Methylphenidate, Novartis, Basel, Switzerland). These drugs were not false positives, confirming a good specificity of the developed method.

The hair extraction by acidic buffer as well as the subsequent LLE at pH 9.7 are selective for basic substances and exclude to a high extent acidic drugs (eg, some analgesics like ibuprofen) and lipophilic neutral drugs (eg, THC). Strongly hydrophilic substances are also excluded since they are badly reextracted in dichloromethane/isopropanol. These are essential limitations of this method in systematic toxicological analysis. This method is mainly devoted to alkaline compounds as is the case of most NPS. Several compounds present in the mzCloud™ library were not identified despite a selective fragmentation through DDA. This was due to a mismatch with the reference spectra due to co-isolation/co-fragmentation of ions having similar masses (isolation mass window +/− 1 m/z). This problem was solved for 11/16 compounds by injecting hair samples spiked only with problematic compounds. Five molecules remained unidentified, due to a lack of sensitivity (hydroxyalprazolam, hydroxybromazepam, duloxetine, and psilocin) and to co-fragmentation with impurities presenting a close m/z value (4-fluoroamphetamine). The co-fragmentation of interfering ions and the absence of fragmentation for ions with weak intensity are the two main limitations of DDA. These problems could be solved by gradient modification or by using another eluting column, or lastly by applying a more complex pre-analytical treatment.

SoHT QC analysis revealed all illicit substances found by other laboratories except natural cannabinoids and norcocaine in sample n°2 which was present at low levels (20 pg/mg). Natural cannabinoids (THC, THC-COOH, cannabidiol, and cannabinol) as well as other acidic compounds were poorly detected in positive mode. A SoHT QC C was devoid of drugs, except for trimethoprim and caffeine, in agreement with a high specificity.

DDA with HRMS instruments allows a retrospective analysis of previously acquired raw files with implemented spectra databases.

This point is of particular interest for NPS analysis presenting a continuous diversification, and also when using hair as a matrix, because most of the time, the small amount of hair sample allows only one analysis. The ability to increment the library and to identify compounds retrospectively was highlighted in this study with 19 compounds. Through a worldwide shared and updated MS/MS spectra library, the mzCloud™ project aims to gather all the existing and new compounds spectra in a single library with free online access. Laboratories can acquire their own spectra data. Infusion should be done with pure substances following a defined procedure. Raw files are then transferred to mass spectrometric curators who check and calibrate the file before adding it to the library. This approach allows laboratories having HRMS device to perform untargeted screening without having to buy CRMs. This method aims to be extended to other matrices (urine, plasma, and whole blood) and could also be applied to other compound classes (eg, pesticides).

Several methods have been developed allowing the detection of NPS in hair through different approaches. Some studies developed distinct methods for the detection of different chemical classes of NPS. Strano Rossi et al had developed two LC–MS/MS methods for the detection of 24 synthetic cannabinoids and 26 NPS belonging to amphetamines, cathinones, and piperazines.[32] Salomone et al had used three LC–MS/MS methods for drug of abuse (DOA) detection in hair, the first with 11 common DOAs, the second with 26 NPS and the third with 40 synthetic cannabinoids.[33] Few approaches were able to detect simultaneously different compound classes while achieving a good sensitivity. Boumba et al had developed an LC–MS/MS method for the detection of 132 NPS in hair including synthetic cannabinoids, cathinones, fentanyls, piperazines, and amphetamines like drugs with limits of quantification (LOQs) between 1 and 10 pg/mg.[34] These approaches provided a good sensitivity; however they remain targeted, with a limited number of screened NPS. A single method was developed for unknown screening in hair by Broecker et al using liquid chromatography–quadrupole time of flight limit–mass spectrometry (LC–QTOF–MS).[35] The method was validated for 24 compounds of interest, and identification of unknowns relies on a homemade MS2 library containing about 2500 CID spectra of relevant substances in toxicology. This is the largest hair screening method, but the library remains homemade and not accessible for other laboratories. The main advantage of our approach, beyond the simultaneous detection of many NPS classes in hair, is the possibility to freely increment and access the HRMS library. A major limit of untargeted screening is lack of sensitivity compared to targeted screening; in some cases the LOI might not reveal a single exposure for several compounds (eg, drug-facilitated crimes).

The method was tested by analyzing 11 hair samples submitted for toxicological investigation in the laboratory. A total of 144 compounds were identified: 59 illicit drugs (24 NPS), 81 pharmaceuticals, and 4 naturals. The main classes of NPS were detected – cathinones, phenethylamines, fentanyls, and synthetic cannabinoids. Some compounds were detected several times (1 to 9 times). Two compounds added to the mzCloud™ library were successfully detected in real hair samples, nefopam and oxomemazine. Two problematic compounds were detected, clobazam and N-desmethylclobazam. Nineteen compounds were not identified. Among them, 18 had a concentration below the LOI (MDPV, mexedrone, N-ethylpentylone, normephedrone, prazepam, tramadol, alprazolam, cetirizine, citalopram, clobazam, doxylamine, lidocaine, nalmefene, nefopam, nordiazepam, oxazepam, MDA, and MDMA) and the last one was not detected despite a concentration equal to the LOI (metamphetamine = 100 pg/mg). This could be explained by the low amount of hair sample available for this analysis (case n°1: 20 mg instead of 50 mg). Regarding the detection of AKB48-5F and desmethylcarbodenafil in hair, to our knowledge they have not been previously reported in the current literature. These results are helpful to build consumption profiles and tailor patient management. This approach could provide epidemiological data on illicit drugs trend and improve narcotics monitoring.

5 | CONCLUSION

We developed a sensitive and specific untargeted screening method devoted to hair analysis. LC and HRMS parameters were extensively evaluated and optimized for hair analysis. The final screening relies on an LC–HRMS method bridged to the shared mass spectra data base mzCloud™ with Compound Discoverer™ software. Sensitivity was evaluated with 284 molecules including 72 NPS. Twenty compounds absent from the database were added to the library and 19 were successfully identified on previously acquired data files. This method was applied to real hair samples allowing the detection of 24 NPS, including a synthetic cannabinoid (AKB48-5F) described for the first time in hair. This method is mainly focused on basic substances and may miss acidic as well as neutral compounds of interest.

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SUPPORTING INFORMATION

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

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Publication n° 3

Validation of an UPLC-MS/MS method for the determination of sixteen synthetic cannabinoids in human hair. Application to document chronic use of JWH-122 following a non-fatal overdose

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ORIGINAL ARTICLE

Validation of an UPLC-MS/MS method for the determination of sixteen synthetic cannabinoids in human hair. Application to document chronic use of JWH-122 following a non-fatal overdose

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KEYWORDS
Synthetic cannabinoids;
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Summary
Purpose. – Synthetic cannabinoids (SCs) are the largest group of new psychoactive substances (NPS) monitored worldwide. We validate a rapid and sensitive liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) assay for the determination of 16 SCs in human hair samples. This method was successfully applied to document chronic use of JWH-122 in a 42 years-old male included in large-scale cross-sectional study in Paris.
Method. – The SCs JWH-018, JWH-018-5-N(OH-pentyl), JWH-073, JWH-073-4-N(OH-butyl), JWH-122, JWH-200, JWH-019, JWH-250, AM-2201, EAM-2201, BB-22, ADB-PINACA, AKB48-5F, AB-FUBINACA, 5F-AB-PINACA, 5F-PB-22 were extracted from 20 mg of decontaminated, washed and grinded hair by a mixture of hexane/ethyl acetate (1/1, v/v) in alkaline condition in the presence of JWH-018-D11 internal standard. SCs were then separated by a gradient of formate buffer 2 mM in 0.1% formic acid (A) and acetonitrile (B) on Hypersyl Gold PFP column (100 × 2.1 mm i.d., particle size 1.9 µm, ThermoFisher) at 30 °C. The detection was performed on TSQ Vantage triple-quadrupole mass spectrometer (Thermofisher, Les Ulis, France) in positive ionization and multi-reaction monitoring (MRM) modes. A lock of hair (6 cm) was sampled from a patient

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hospitalized for JWH 122 overdose and segmented into 3 segments: A: 0-2 cm (proximal), B: 2-4 cm and C: 4-6 cm. It was then analyzed with the present method in order to determine past use of synthetic cannabinoids.

Results. — The method exhibits a good linearity ($r^2 > 0.996$) over the range 1-25 to 1000 pg/mg, Limits of detection and quantification ranged from 0.3 to 8 pg/mg and from 1 to 25 pg/mg, respectively (CVs $\leq$ 15%). No interference from endogenous substances with the detection of SCs and their IS was observed and the carryover was insignificant. Intraday and inter-day trueness (bias) and precision (CVs) were $\leq$ 8.2%, $\leq$ 14.3%, $\leq$ 8.3% and $\leq$ 14.9%, respectively. Overall recoveries ranged between 61 and 94% and corrected matrix effect from 31 to 110%. JWH-122 hair concentrations found in patient's hair were: (A/B/C: 200/450/430 pg/mg) in agreement with the concentrations found in the literature suggesting chronic use of JWH-122 over the last six months.

Conclusion. — The present LC-MS/MS method proved to be suitable for the detection and quantification of 16 synthetic cannabinoids in human hair.

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Introduction

New psychoactive substances (NPS), including synthetic cannabinoids (SCs), synthetic opioids and synthetic cathinones among other designer drugs continue to be associated with deaths and acute intoxications in Europe and elsewhere around the world. Despite a several banning measures introduced in this area, NPS continue to represent a challenge to current drug policy models. In 2018, the European Monitoring Center For drugs and drug addiction (EMCDDA) was monitoring more than 730 new psychoactive substances with an overall increase in presentations related to SCs and a decrease in presentations related to synthetic cathinones [1]. SCs are a group of drugs that mimic the effects of tetrahydrocannabinol (THC) which activates a receptor in the brain called the cannabinoid receptor type 1 (CB1 receptor) [2]. CB1 receptor is a part of a so-called endocannabinoids signaling system that helps regulate behavior, mood, pain, appetite, sleep and the immune system among other physiological functions [3]. THC main psychoactive effects are characterized by the feeling of being 'stoned' or 'high'. This includes relaxation, euphoria, lethargy, depersonalization, distorted perception of time, impaired motor performance, hallucinations, paranoia, confusion, fear, anxiety, dry mouth, bloodshot eyes, tachycardia (an abnormally fast heart rate), nausea and vomiting [4]. Since the 1990s, SCs were invented and synthesized by scientists to provide insights into disease and develop new medicines based on their similar activity to THC [3]. However, unlike THC which is only a partial agonist of CB1 receptor, CSs are total agonists explaining why they are more potent and toxic than THC itself. [5]. Severe effects of SCs commonly reported include serious cardiovascular toxicity (including sudden death), rapid loss of consciousness/coma, respiratory depression, seizures and convulsions, hyperemesis, delirium, agitation, psychosis, and aggressive and violent behavior [6—16]. Around the 2000s, SCs began to appear in Europe in products called 'Spice', 'K2', 'synthetic marijuana', and they were sold as 'legal highs' in mixture with plant material to be smoked as cigarettes ('joints'). Since, 179 cannabinoids have been identified on the drug market in hundreds of different products [17]. The EMCDDA reported that last year estimates of SCs use among 15- to 34-year-olds ranged from 0.1% in the Netherlands to 1.5% in Latvia [1]. People who use SCs include recreational users, drug abusers and individuals who are subject to drug testing (such as people in drug treatment, prisoners and drivers). Also, SCs are increasingly used by vulnerable groups, such as the homeless people. Part of the problem in controlling SCs lies in their variety, their low cost, and the fact that they are undetectable by standard toxicology screening as they do not cross-react with THC commercial tests. Thus, their identification in different biological and non-biological matrices require reliable analytical methods based on chromatographic techniques coupled with mass spectrometry detection (e.g. GC-MS, GC-MS/MS and LC-MS/MS) [6,18—21]. Even if targeted screenings are highly sensitive and specific, they remain limited by the unavailability of reference standards (RS). Consequently, it is likely that the number of reported cases is underestimated. In recent years, high resolution mass spectrometry (LC-HRMS) methods allowed seeking for SCs without a need for RS as they are based on a shared MS spectra database continuously enriched with newly marketed NPS spectra [22]. When studying drug related intoxications or deaths, the analysis of biological specimens of drug users could be the most accurate and directly linked indicator of drug consumption. While blood and urine testing can provide useful information of recent exposure to drugs, hair testing allows to establish an extensive exposure pattern of past use and offers a wide detection window, up to months prior to sampling as drugs may remain stable in hair for a long time. Hair is also easy to collect (non-invasive), hard to adulterate and can be stored indefinitely [23,24]. The aim of the present study is to develop and

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validate an ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) assay for the determination of 16 SCs in hair. This method was successfully applied to document chronic use of JWH-122 following a non-fatal intoxication in a 42 years-old male.

Experimental

Chemicals and reagents

A 1 mg/mL certified reference materials (CRMs) of JWH-018, JWH-018 N-(5-hydroxypentyl), JWH-018-D11, JWH-019, JWH-073, JWH-073 N(4-hydroxybutyl), JWH-122, JWH-200, AM-2201, JWH-250, AB-FUBINACA, 5F-AB-PINACA and 5F-PB-22 were obtained from Lipomed Standards (Arlesheim, Suisse). ADB-PINACA, AKB48 N-(5-fluoropentyl), BB-22 (10 mg/mL) and EAM-2201 (10 mg/mL) were purchased in ready to use solution from Cayman chemical company (Michigan, USA).

High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, isopropanol and formic acid were obtained from Lichrosolv, VWR CHEMICALS (Ile-De-France, France). Ethyl Acetate and chloroform were provided by Carlo Erba (Val de Reuil, France). Dichloromethane, hexane, sodium phosphate and sodium carbonate were supplied by Sigma Aldrich (Saint Quentin Fallavier, France).

Ultra-pure water (18 MV) was obtained by ultrafiltration using a Q-Pod apparatus (Millipore Corp., Molsheim, France). Formate buffer (containing formate 2 mM and 0.1% formic acid) was prepared in ultra-pure water.

Working solutions, calibration standards, and quality controls

Two sets of working solutions were prepared at four concentration levels (0.001, 0.01, 0.1, and 1 mg/L) by dilution of a 10 mg/L intermediate solution with MeOH. IS working solution (25 ng/mL) was obtained by successive dilution of JWH-018-D11 (1 g/L) solution in MeOH. Calibration curves were prepared by spiking 20 mg blank hair with appropriate volumes of the previously mentioned solutions to produce calibration standards (CSs) containing 1, 2.5, 5, 10, 25, 50,100, 500 and 1000 pg/mg of each SC. Quality controls (QCs) were prepared at 20, 250 and 750 pg/mg by the same way. All solutions were stored at −20 °C until use.

Drug-free hair samples were collected from individuals who did not undergo any medication and were stored at room temperature.

Hair sample preparation

Hair was twice decontaminated using dichloromethane and washed once using warm water (immersion for 2 min/step). Hair was then grinded into a fine and homogeneous powder using a ball mill (MM200, FisherScientific, Illkrich, France). Each 20 mg were incubated in 1 mL of phosphate buffer (pH 5.0) at 95 °C for 10 min in the presence of 25 ng of JWH-018-D11 and appropriate volume of CSs or QCs working solutions when necessary. Liquid–liquid extraction was performed by 4 mL of a mixture of hexane/ethyl acetate (1/1, v/v) after addition of 2 mL of carbonate buffer (pH 9.7). After twenty minutes of stirring and centrifugation steps, the organic phase was evaporated to dryness under gentle air stream. The residue was reconstituted in 100 μL of mobile phase and 20 μL were injected into the chromatographic system.

LC–MS–MS system and conditions

Chromatography was performed on Accela liquid chromatography system (Thermofisher, Les Ulis, France) composed of a CTC analytics autosampler (Thermofisher), a multi-switch column module (MayLab multiswitch 3.0, Vienna, Austria) coupled to a quaternary eluting pump. The chromatographic separation was achieved using a porous Hypersyl Gold PFP column (100 × 2.1 mm i.d., particle size 1.9 μm, ThermoFisher) preceded by a javelin ™ column filter (2.1 mm i.d.) and maintained at 30 °C. Elution of compounds was performed by a gradient of formate buffer 2 mM in 0.1% formic acid (A) and acetonitrile (B) starting from 30% of acetonitrile to 90% at a flow rate of 300 μL/min. The total run was 14 min, including 2 min column re-equilibration.

Compounds were detected by a TSQ Vantage triple-quadrupole mass spectrometer (Thermofisher) equipped with an electrospray ionization (ESI) source set in positive ionization mode. Ion-spray voltage of +4 kV was applied. The capillary temperature was set at 350 °C. Nitrogen (Nitrox UHPLCMS 18, nitrogen generator, Domnick Hunter, Villefranche sur Saone, France) was employed as sheath and auxiliary gas at a pressure of 50 and 20 arbitrary units, respectively. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. The scan time was of 2 seconds. Data were collected in selected reaction monitoring (SRM) mode, with two m/z transitions per analyte. The determination of the optimal MRM transitions and respective collision energies for both quantifier and qualifier ions were carried out by direct injections of 1 μg/mL individual CRM solutions of SCs through the mass spectrometer. Protonated molecular ions [M + H]+ were selected with a mass resolution of ±0.5u (atomic mass unit) and fragmented with different collision energies (CE) to obtain two product ions for each parent drug.

Chromatographic data acquisition was performed using Xcalibur software (v2.2 SP1). Post-analysis processing was carried out using LCQuan software (v 2.7.0 SP1), ThermoFisher Scientific.

MRM transitions and retention times of synthetic cannabinoids investigated in the present study are reported in Table 1.

Analysis of plasma and recovered material from the case report

LC–MS/MS screening methods were performed on plasma and the recovered powder consumed by the patient just before the overdose.

Briefly, 500 μL of plasma were extracted with 4 mL of a mixture of heptane/ethyl acetate (v/v) in the presence of 100 ng/mL of JWH-018-D11. After agitation and centrifugation, the recovered organic phase was evaporated to dryness and the final residue was reconstituted in 100 μL of mobile phase. Three microliters were injected into the chromatographic system. The separation was achieved using a porous Hypersyl Gold column (100 × 2.1 mm i.d., particle

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Table 1 Retention times, MRM transitions and collision energies of SCs and IS.

Compound	RT (min)	Parent drug	Product ion (Quantification)		Product ion (Confirmation)	
	min	m/z	m/z	CE (eV)	m/z	CE (eV)
JWH-018	11.08	342.2	127.1	41	155.1	23
JWH-018-5-N(OH-pentyl)	7.92	358.2	127.1	45	144.1	20
JWH-073	10.51	328.2	127.1	40	155.1	23
JWH-073-4-N(OH-butyl)	7.51	344.2	127.1	47	155.1	21
JWH-122	11.57	356.2	169.1	25	141.1	40
JWH-200	5.62	385.2	127.1	45	155.1	20
JWH-019	11.69	356.1	127.0	47	155.1	22
JWH-250	10.3	336.2	121.1	19	144.1	33
AM-2201	9.85	360.2	155.1	24	232.2	23
EAM-2201	10.72	388.3	183.1	29	153.1	46
BB-22	10.93	385.3	240.2	24	144.1	39
ADB-PINACA	8.27	345.2	215.1	24	300.2	14
AKB48-5F	11.37	384.4	135.2	29	93.1	37
AB- FUBINACA	6.88	369.3	109.1	37	253.2	26
5F-AB-PINACA	6.47	349.3	233.1	26	145.1	43
5F-PB - 22	9.14	377.2	232.1	19	144.0	38
JWH-018-D11 (IS)	11.00	353.2	127.1	44	155.1	25

size 1.9 μm, ThermoFisher). Elution of SCs was performed in 9.5 min by the same gradient used for hair testing. The method was validated over the range 0.2-50 ng/mL and exhibited satisfactory validation parameters (data not shown).

Ten milligrams of the powder were weighted and diluted in 10 mL of methanol to obtain a 1 g/L solution. Three stock solutions at 1, 10 and 100 mg/L were then prepared by successive dilutions in the same solvent. Two hundred microliters of each solution were mixed with 20 μL of JWH-018-D11 (5 mg/L) and evaporated to dryness. The final residue was reconstituted in 200 μL of the mobile phase (the same used above) and 10 μL were injected. Compounds were eluted by an isocratic flow (200 μL/min) of formate buffer 2 mM in 0.1% formic acid (A) and acetonitrile (B) (60:40) in 7 min. JWH-122 was separated by turbulent flow chromatography (TFC) using cyclone extraction column followed by HypersilGold column (100 × 2.1 mm i.d., particle size 1.9 μm, ThermoFisher) and quantified by mass spectrometry on TSQ Quantum Access Max (ThermoFisher). The method derived from a previously published methods for methoxetamine analysis [25], and was fully validated over the range 100-5000 ng/mL (data not shown).

Method validation procedure

The method was validated for selectivity, carryover, linearity, limits of detection and quantitation (LOD and LOQ), trueness, precision (repeatability and intermediate precision), matrix effect and recovery.

Selectivity, carry over

Drug-free hair samples and samples spiked at the LOQ were analyzed in accordance with the described procedure to investigate whether endogenous matrix constituents interfered with the assay. Assay selectivity was defined by evidence of non-interference at retention times and ion channels of synthetic cannabinoids.

An extracted blank hair sample was also analyzed immediately after running three replicates of the highest CS (1000 pg/mg) to monitor the carryover.

Linearity

Six calibration curves including a blank reagent sample and eight CSs over the range 1 to 1000 pg/mg were assayed over a six-day period. Quantification was achieved by plotting the peak area ratios of each analyte to the internal standard. Back-calculated concentrations of the CSs have to be within 85–115% of the nominal concentrations.

Limits of detection (LOD) and quantification (LOQ)

The LOD was defined as the lowest concentration for which the full MS–MS spectrum could be identified with a signal to-noise ratio greater than 3.

The LOQ was defined as the lowest concentration for which trueness was between 80 and 120% (bias $\leq$ 20%) and precision (coefficient of variation CV) of $\pm$ 20% or less was obtained over six measurements.

Trueness and precision

The trueness [(measured value-nominal value)/nominal value], bias and precision (CV) of the method were carried out over 3 days. Each day, one calibration curve and six replicates of each QC level were assayed. The values obtained were processed using analysis of variance (ANOVA), which separated the intra-assay and inter-assay standard deviation and consequently the corresponding CV. The intra-assay CV takes into account the variability of the 6 replicates within each validation day and the inter-assay CV evaluates the

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variability between the three validation days. A trueness within the range 85–115% (bias ≤ 15%) of the nominal values and a precision with a CV of ± 15% were required, except for the LOQ for which a range of 80–120% (bias ≤ 20%) and a CV of ± 20% were accepted, respectively.

Matrix effect and overall method recovery

To investigate analyte recovery, solutions containing synthetic cannabinoids and the IS were:

- spiked into 20 mg of hair sample before the entire extraction procedure;
- spiked after the extraction process to get a final CS of 100 pg/mg.

The mean peak areas for each compound subjected to the complete extraction procedure were compared with those obtained from the extraction bypassed step, with the latter assumed to represent 100% recovery.

Matrix effect (ME) was defined as the ratio of the mean peak area obtained with six different blank hair matrices spiked at 100 pg/mg with SCs and IS after extraction to the mean peak area obtained in a pure solution of the same analytes at the same concentrations in reconstitution buffer. The corrected matrix effect (CME) was obtained by calculating the ratio of ME of each SC to the ME of the IS ($CME = ME_{analyte}/ME_{internal\ standard}$).

Post-process stability

Stability of processed samples was assessed by re-analysis of the three QC levels after conservation 24 h in the autosampler at +4 °C. Analytes were considered stable when the new concentrations were similar to those firstly obtained (CV ≤ 15%).

Application: Case report

A 42 years old man was hospitalized in the intensive care unit (ICU) one and half hour after sniffing a white powder ordered through internet as a SC. At the admission, the patient presented asthenia and faintness which rapidly worsened (within 2 h) by the appearance of a reactive bilateral mydriasis, high blood pressure (171/87 mmHg), abdominal contractures, palpitations, a constrictive thoracic gene with an irradiation to the jaw and back, renal pains and anuria. The patient had a clear and coherent speech (Glasgow score 15) with no sign of neurological localization or a meningeal syndrome but he described intense pain in the flanks and the stomach with a sensation of contractures and acute cramps. He was discharged within 2 days without sequelae. Over the last year preceding his poisoning, the patient has been followed in addiction clinic for drug addiction first to ketamine and then, to other types of recreational drugs including SCs and cathinones.

A large toxicological assessment was requested. The powder involved in the overdose was recovered for identification. In order to understand patient's behavior regarding drugs use, a lock of hair (6 cm) was also sampled and segmented into 3 segments of 2 cm: A (root, proximal): 0-2 cm, B: 2-4 cm and C (distal): 4-6 cm. Each segment reflects a 2 months exposure period based on average hair growth rate of 1 cm/month [24].

Results and discussion

The validation settings of SCs are summarized in Table 2. The method exhibits a good linearity (determination coefficient $r^2 > 0.996$) over the range 1–1000 pg/mg, except for BB-22, AKB48-5F, AB-FUBINACA and 5F-PB-22 with LOQ at 10 pg/mg, and 5F-AB-PINACA with LOQ at 25 pg/mg. To compensate for heteroscedasticity, a weighted least squares model with a weighting factor 1/x was applied for all compounds. Inter-day bias and CVs for the back-calculated concentrations of CSs ranged from 0.35 to 3.67% and from 1.00 to 5.65%, respectively. LOQs ranged from 1 to 25 pg/mg with CVs ≤ 15%. Blank and zero samples as well as the sample spiked with other relevant drugs and metabolites did not reveal any interference on the MRM transitions of SCs or the IS. Carry-over in a blank sample directly injected after a 1000 pg/mg QC sample was below the limit of detection. (data not shown). Respective LODs were 0.3, 3 and 8 pg/mg accordingly (Table 2). Intraday and inter-day trueness (bias) and precision (CVs) were ≤ 8.2%, ≤ 14.3%, ≤ 8.3% and ≤ 14.9%, respectively.

Overall recoveries ranged between 61 and 94% and corrected matrix effect from 31 to 110%. Significant signal extinction due to matrix effect (≤ 43%) was observed for JWH-018-5-N(OH-pentyl), JWH-250, AB-FUBINACA and 5F-PB-22. Nevertheless, the developed method achieves sufficient validation criteria regarding accuracy, precision and LOQs.

This method proved to be suitable for the determination of JWH-018, JWH-018-5-N(OH-pentyl), JWH-073, JWH-073-4-N(OH-butyl), JWH-122, JWH-200, JWH-019, JWH-250, AM-2201, EAM-2201, BB-22, ADB-PINACA, AKB48-5F, AB-FUBINACA, 5F-AB-PINACA, 5F PB -22 in human hair. It has successfully been applied since 2015 to study the prevalence of SCs in high-risk populations from Paris and its suburbs [26]. Among the 318 hair samples screened within the period 2015-2017, JWH-122 is the only SC detected so far.

In the present case study, preliminary toxicological investigations show negative results for amphetamine, cannabinoids (THC), cocaine, opioids and acetaminophen in patient's urine. The analysis of plasma by LC-MS/MS reports the presence of JWH-122 at 1.3 ng/mL. The recovered powder has also been identified as pure JWH-122 product (purity: 100%). JWH-122 hair concentrations were: (A/B/C: 200/450/430 pg/mg) (Fig. 1), and was detected in combination with methylone (A/B/C: 21735/3000/2000 pg/mg), cocaine (A/B/C: 1540/2455/1555 pg/mg) and its metabolites BZE (430/880/365 pg/mg), EME (75/235/80 pg/mg), cocaethylene (6/5/6 pg/mg) as well as other pharmaceuticals (dextromethorphan, doxylamine) and cutting agents (levamisole, hydroxyzine and cetirizine) (Table 3). These findings confirm the polydrug abuse status of this patient.

According to the EMCDDA 2019 report, synthetic cannabinoids are the largest group of new substances seized and monitored in Europe. However, as most of commercial immunoassays do not cross-react with the extremely wide range of existing SCs, their prevalence is likely to be underestimated.

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Table 2 Validation parameters of SCs in hair.

Compound	r^2	LOD (pg/mg)	LOQ (pg/mg)	CV (%)	Trueness (bias, %)						Precision CV (%)						Corrected Matrix effect (ME_{SC}/ME_{IS}) (%)	Recovery (%)
					Intra-day			Inter-day			Intra-day			Inter-day				
					Low $n=18$	Mid $n=18$	High $n=18$	Low $n=18$	Mid $n=18$	High $n=18$	Low $n=18$	Mid $n=18$	High $n=18$	Low $n=18$	Mid $n=18$	High $n=18$	QC 100 pg/mg	
JWH-018	0.997	0.35	1	15.0	4.1	4.2	6.7	4.6	4.7	5.1	9.8	7.3	6.5	1.2	4.7	13.7	81	74
JWH-018-5-N(OH-pentyl)	0.996	0.35	1	14.0	1.4	3.2	1.2	2.5	1.9	0.1	8.1	8.8	5.4	9.2	14.1	13.8	39	76
JWH-073	0.997	0.35	1	12.0	1.7	3.5	1.8	1.3	3.6	2.3	14.3	8.6	9.4	11.3	5.0	11.1	74	73
JWH-073-4-N(OH-butyl)	0.996	0.35	1	13.0	4.6	3.3	1.4	4.7	1.9	2.2	9.9	9.2	9.9	1.2	13.4	10.5	68	93
JWH-122	0.997	0.35	1	11.0	0.1	1.4	1.1	0.6	0.3	0.3	6.1	5.0	8.2	7.1	13.1	9.4	92	71
JWH-200	0.997	0.35	1	7.0	3.1	6.8	2.4	1.6	8.3	1.5	10.9	4.6	8.8	14.4	13.3	11.2	74	80
JWH-019	0.997	0.35	1	13.3	0.1	1.8	3.7	0.5	1.2	4.2	7.6	9.1	10.4	7.5	10.3	6.4	103	68
JWH-250	0.997	0.35	1	10.5	2.2	2.4	2.9	2.4	1.7	1.9	8.6	9.1	9.9	13.0	10.1	9.4	42	77
AM-2201	0.996	0.35	1	13.2	1.5	0.9	3.3	0.6	1.7	3.5	10.3	6.8	8.4	11.8	13.3	2.2	54	79
EAM-2201	0.998	0.35	1	10.5	5.3	1.0	4.8	5.8	0.7	4.5	11.1	8.7	8.5	5.6	5.7	12.7	83	78
BB-22	0.996	3	10	6.4	2.2	0.3	1.9	3.3	1.4	2.5	5.4	7.5	7.5	11.9	9.3	8.4	81	79
ADB-PINACA	0.996	0.35	1	11.3	3.5	8.2	5.3	3.9	8.1	6.8	4.7	8.5	9.6	14.3	0.8	12.6	106	74
AKB48-5F	0.999	3	10	5.6	4.9	6.0	3.5	5.3	3.7	2.7	6.4	8.2	7.9	14.2	10.2	14.3	74	71
AB-FUBINACA	0.997	3	10	5.4	1.1	7.0	5.6	7.6	7.4	3.8	9.1	6.3	7.9	9.3	11.8	14.9	43	61
5F-AB-PINACA	0.996	8	25	8.6	[a]	2.1	7.0	[a]	1.3	6.5	[a]	10.8	6.9	[a]	7.6	14.9	110	61
5F_PB-22	0.996	3	10	9.5	2.5	2.2	3.9	2.7	2.1	2.7	12.4	9.9	7.9	7.2	3.2	12.4	43	61

r^2 coefficient of determination; LOD limit of detection; LOQ limit of quantification; CV coefficient of variation; QC levels for trueness and precision: Low 20 pg/mg; Mid 250 pg/mg; High 750 pg/mg.

[a] QC levels at 20 pg/mg has not been evaluated for 5F-AB-PINACA as the LOQ was 25 pg/mg.

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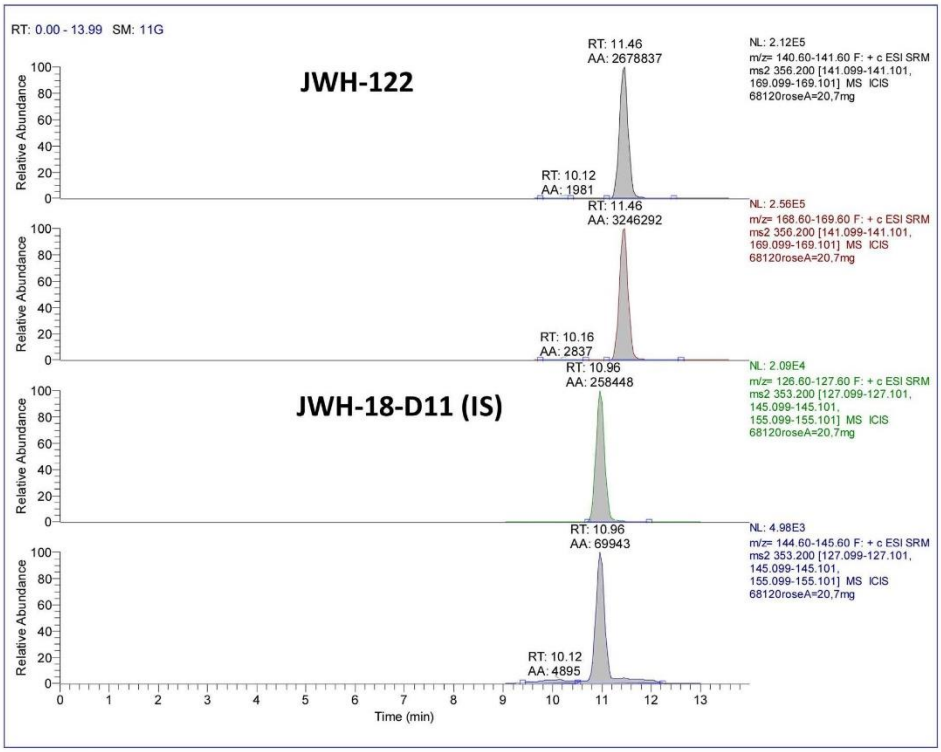


Figure 1. Chromatogram of an extracted segment A of patient's hair showing the two MRM transitions of JWH-122 and JWH-018-D11 (IS).

Table 3 Results of hair analysis for JWH-122 and other detected DOA, pharmaceuticals and cutting agents.

Compound	Hair concentrations(pg/mg)		
	Segment A 0-2 cm	Segment B 2-4 cm	Segment C 4-6 cm
JWH-122	200	450	430
Methylone	21735	3000	2000
Cocaine	1540	2455	1555
Benzoylecgonine (BZE)	430	880	365
Ecgonine methyl ester (EME)	75	235	80
Cocaethylene (CE)	6	5	6
Levamisole	310	2930	1990
Hydroxyzine	1400	110	265
Cetirizine	115	20	20
Dextromethorphane	3540	6385	8370
Doxylamine	3840	80	65

Since 2012, few studies tried to estimate the penetration of SCs into population through hair testing. Most of these techniques used mass spectrometry-based methods, especially LC-MS/MS for reasons of specificity and sensitivity. Ten to 100 mg of hair were needed to achieve acceptable limit of quantification ranging from 0.07 to 20 pg/mg. Liquid-liquid extractions using methanol, NaOH (0.5 or 1 N), ethanol or a mixture of hexane/ethyl acetate were used. The sample preparation lasts between 10 minutes and an overnight. The improvement of extraction recovery by heating the sample at 45 °C or 95 °C was also employed [6,20,26–33].

The low reporting of SCs in these studies could be explained in part by their high potency and thus their use at very low doses, which reduces their detectability,

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especially in hair. It could also be explained by their rapid metabolism leading to lower integration of parent drug into hair. Lastly, the high amount of seized SCs recorded by the EMCDDA and the low prevalence of SC in hair samples may show the effectiveness of regulation to control use as soon as there is enough scientific evidence demonstrating harmful effects. Even though inclusion criteria are broadly different, especially with regard to the studied populations (acute intoxications, psychiatric inpatients, driving relicensing, drug abuse, withdrawal control subjects...etc.), these studies provided objective data to the prevalence of synthetic cannabinoids.

JWH-122 hair concentrations found in the literature ranged from 0.1 to 2800 pg/mg which demonstrated good integration of this SC into the hair matrix.

Our findings suggest repeated exposure to JWH-122 in this patient over the last six months prior to the overdose. High concentration in distal segment (C) with regard to the proximal segment (A) suggests decreasing exposure to JWH-122 over the last six months.

In this case, the overdose in this patient could be explained by a consumption of a high dose of a pure product as shown by the analysis of the recovered powder, rather than an increase in the frequency of consumption.

However, the interpretation of hair results can be challenging and several considerations should be taken into account to achieve unambiguous identification and accurate quantification of SCs in hair. Besides the need to choose the right sampling conditions (area of body to be sampled, orientation of the hair strand...etc.), preparation steps (washing, decontamination, extraction solvents, extraction time...etc.) and the analytical instrument, the most serious pitfalls of hair analysis to avoid are in the interpretation of the results. While a negative hair result does not categorically mean that the person did not use drugs if they have been exposed to a drug infrequently or in low doses, a positive hair findings suggest that a person has been exposed to a drug without excluding external contamination by handling of drug materials [32] or passive exposure to side stream smoke [34], which in case of SCs can lead to concentrations in hair up to 170 and 950 pg/mg, respectively [35]. It is therefore important to highlight these limitations when reporting the results.

As an alternative approach, targeting SCs metabolites in hair to deliver a proof for systemic drug uptake has been proposed. However, many studies showed low concentrations of metabolites compared to parent drugs which significantly reduced detection rates [36–38].

Hutter et al. reported that major metabolic pathways of JWH-122 include monohydroxylation on the naphthoyl moiety, indole moiety or at the N-alkyl chain, as well as dehydrogenation at the N-alkyl chain [39,40]. Uchiyama et al. identified the metabolites, N-(4-pentenyl)-JWH-122 and N-(5-hydroxypentyl)-JWH-122 in a herbal-type product sold in Japan [41].

In hair, Salomone et al. [42] reported the presence of N-(5-hydroxypentyl) metabolite of JWH-122 at 2.5 pg/mg and 0.72 pg/mg in two samples.

JWH-122 concentrations in the same samples were, respectively, 2800 pg/mg and 760 pg/mg. Parent drug-to-metabolite ratio was greater than 1000 in both cases. Unfortunately, N-(5-hydroxypentyl) metabolite of JWH-122 was not screened in our case as the reference standard was not available during the validation process.

On the other hand, many issues surrounding the screening for metabolites in hair may arise because some of them can be formed as degradation products after external contamination of the hair [43], or as an artifact from structurally related SCs, as for JWH-122 metabolite that can be produced by the metabolism of MAM-2201 [44,45]. Therefore, the usefulness of metabolites as markers of systemic uptake has to be considered carefully for each individual SC.

In this context, complementary analysis of biological fluids (e.g. blood, or urine) is strongly recommended for confirmation of active SCs consumption. In our case, JWH-122 was measured in plasma at 1.3 ng/mL. In a set of synthetic cannabinoid cases, Knittel et al. reported JWH-122 blood concentrations ranging from 0.12 to 27.46 ng/mL (mean= 2.83 ng/mL, median= 1 ng/mL, n = 16) [46]. Thus, our finding is in agreement with previously published data, confirming an active use of this SC by the patient and discarding the only possibility of external contamination of hair.

Conclusion

In the present study, the validated UPLC-MS/MS method proved to be suitable for the detection and quantification of 16 synthetic cannabinoids in human hair. The method is linear over a wide range of concentrations, accurate, precise, specific and reached low limits of quantification. Since 2015, authentic hair samples were successfully analyzed with this method in a large cross-sectional study in Paris and its suburbs and allows to document chronic use of JWH-122 following a non-fatal overdose. The results of segmental hair analysis are in agreement with the concentrations found in current literature and suggest chronic use and good incorporation of the drug into the hair matrix.

Disclosure of interest

The authors declare that they have no competing interest.

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Development and validation of liquid chromatography-tandem mass spectrometry targeted screening of 16 fentanyl analogs and U-47700 in hair: Application to 137 authentic samples

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Abstract

Purpose To validate a LC-MS/MS method for the determination of 17 new synthetic opioids (NSOs) in hair including 3-fluorofentanyl, 3-methylfentanyl, acetylfentanyl, acetylnorfentanyl, alfentanyl, butyrylfentanyl, butyrylnorfentanyl, carfentanil, fentanyl, furanylfentanyl, furanylnorfentanyl, methoxyacetylfentanyl, norcarfentanil, norfentanyl, ocfentanil, sufentanil and U-47700, and to apply it to 137 authentic samples.

Method

Twenty milligrams of hair were decontaminated in dichloromethane and underwent liquid extraction. 10 µL of the reconstituted residue were injected onto the system. The separation was performed in 12 minutes in a gradient mode at a flow rate of 300 µL/min using a Hypersyl Gold PFP column (100 x 2.1 mm i.d., 1.9 µm) maintained at 30 °C. Compounds were detected in positive ionization and MRM modes using a TSQ Endura mass spectrometer (ThermoFisher). The method was validated according to EMA guidelines.

Results

LLOQ ranged from 1-50 pg/mg, and the calibration ranged from the LLOQ-1000 pg/mg. Intra- and interday accuracy (bias) and precision were < 15%. Extraction recoveries of parent drugs and metabolites ranged between 74-120% and 7-62 %, respectively. Matrix effect ranged from 59-126% (CVs ≤ 12.9%). Fentanyl was found in 6 cases at concentrations ranging from <1- 1650 pg/mg (n= 14 segments). Five fentanyl analogs were quantified in 2 cases: 3-fluorofentanyl [25-150 pg/mg, n=5], furanylfentanyl [15–500 pg/mg, n=5], methoxyacetylfentanyl [500–600 pg/mg, n=2], acetylfentanyl [1 pg/mg, n=2], carfentanyl [2.5–3 pg/mg, n=2].

Conclusion

This fully validated method allowed to test for the first time 3-fluorofentanyl and norcarfentanil in hair among 15 other NSOs, and bring new data regarding 3-fluorofentanyl and methoxyacetylfentanyl hair concentrations.

Keywords: opioids, 3-fluorofentanyl, methoxyacetylfentanyl, carfentanil, hair analysis, LC–MS/MS

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Introduction

In recent years, new synthetic opioids (NSOs) have been increasingly implicated in overdose deaths, and have caused what is commonly known as the "opioid crisis" responsible for more than 130 deaths per day [1]. In the United States (US), the overdose rate of NSOs represented an annual growth rate of 71% between 2013 and 2017, mainly among men aged 25-44 years-old [2]. In 2017, the sharpest increase in overdose deaths was related to fentanyl analogs with more than 28,400 out of 70,237 (40.4%) drug related deaths [2,3]. In Europe, their use is still geographically localized, especially in northern countries, but their number has grown rapidly. The EU Early Warning System had issued about 50 NSOs including 11 compounds reported for the first time in 2018 [4]. NSOs belong to different classes like 4-anilinopiperidine (fentanyl-like drugs), benzamide (e.g. U-47700, U-49900, AH-7921…etc.) or piperazine derivatives (e.g. MT-45). To circumvent legislation surrounding narcotics, NSOs are available on the Internet under different names like "China White", "Apache", "China Girl", "Dance Fever", "Friend", "Goodfella", "Jackpot", "Murder 8", "TNT", "Tango and Cash", "Synthetic Heroine", "Drop Dead", "Lethal Injection", "Get High or Die Trying"...etc. [5].

The risk of life-threatening poisoning or deaths by NSOs is due to: 1) high potency at μ-opioids receptors (10 up to 10,000 more potent than morphine) and their toxicity at very low dose [6]; 2) routes of administration by injection, snorting, or inhalation that allow high bioavailability into the central nervous system; 3) ease of synthesis and purchase at low cost; 4) adulteration especially with heroin; 5) the fact that they are undetectable by standard screening methods [7]. The European Monitoring Center for Drugs and Drug Addiction (EMCDDA) and Europol have issued eight joint investigations on fentanyls (acetylfentanyl, acryloylfentanyl, furanylfentanyl, 4-fluoroisobutyrylfentanyl (4F-iBF), tetrahydrofuranylfentanyl (THF-F), carfentanil, methoxyacetylfentanyl and cyclopropylfentanyl) that have directly been involved in more than 250 deaths between 2015 and early 2018 [7]. Reported overdose cases include people who unknowingly buy adulterated heroin, other illicit drugs or pain relievers [4,8]. Different strategies like self-reported data, wastewater or syringe residue analysis are used to study the penetration of NSOs in populations. On the other hand, the analysis of biological fluids is necessary to formerly confirm the use of drugs and directly link it to the population of users itself.

Blood, plasma and urine are the most commonly analyzed matrices as they provide useful information of recent exposure (few hours up to days after consumption) and are easy to collect. In contrary, drug testing in hair as an alternative matrix allows establishing an extensive exposure pattern of past use, and offers a wide detection window, up to months prior to sampling. Hair is also a rot-proof material that does not decompose and

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can be kept for a long period at ambient temperature and retrospectively analyzed. Unlike conventional matrices like blood or urine, hair is not affected by adulteration (e.g. dilution) or short-term abstinence in order to produce false negatives results. Finally, the parent drug usually represents the target analyte in hair, (unlike in urine), since it integrates better into the keratin matrix than metabolites due to its high hydrophobicity and thus allows an easier identification. The screening for the parent drug can also avoid misinterpretation in case of common metabolites of different drugs.

Prevalence of NSOs remains underreported as not all laboratories have the capacity to detect the most uncommon designer drugs [4]. In this context, more reliable and sensitive methods dedicated to the identification and quantification of NSOs are needed. Recently, liquid or gas chromatography coupled with mass spectrometry (MS) based methods for NSOs screening and quantification were extensively reviewed [9,10]. Targeted screening are the most used techniques for their high sensitivity and specificity. Most are based on solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by a LC-MS/MS experiment. However, these techniques have some drawbacks: they are limited by the unavailability of reference standards (RSs) [11–15], and the enrichment of the library database by addition of new multi reaction monitoring (MRM) transitions of new compounds often requires further method optimization. Moreover, targeted screening does not allow retrospective processing of MS data to identify unknown compounds. As an alternative, untargeted screening using high resolution mass spectrometry (HRMS) allows seeking for a wide number of structurally different compounds without a need for RSs as they are based on library searching (e.g. MzCloud®) continuously enriched with spectra of newly marketed products [16–18]. Unfortunately, this kind of methods is still not available in most forensic laboratories [9].

To our knowledge, only few targeted screening methods for NSOs identification and quantification in hair have been published so far [19–22]. In this study, we describe the development and validation of targeted LC-MS/MS assay for the detection and quantification of 16 fentanyl analogs and metabolites, and one non-fentanyl derivative (U-47700) in hair, and demonstrate its suitability in routine practice by the screening of 137 authentic hair samples collected from high-risk populations in Paris and its suburbs.

Materials and method

Chemicals and reagents

The reference standards of alfentanil, carfentanil, furanylfentanyl, furanylnorfentanyl, butyrylnorfentanyl, acetylnorfentanyl, norcarfetanil, norfentanyl, methylfentanyl, furanylnorfentanyl, methoxyacetylfentanyl, fentanyl-*d5*, acetylfentanyl-*d5* and norfentanyl-*d5* were purchased from LGC Standards (Molsheim, France);

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sufentanyl, fentanyl, and butyrylfentanyl from Lipomed AG (Arlesheim, Switzerland); 3-fluorofentanyl, ocfentanil and acetylfentanyl from Cayman Chemical Company (Ann Arbor, MI, USA); U-47700 from Chiron AS (Trondheim, Norway); acetonitrile, dichloromethane, hexane, ethyl acetate, formic acid and methanol from Sigma Aldrich (Paris, France) in MS or high-performance liquid chromatography (HPLC) grade; sodium carbonate and hydrogen carbonate from Prolabo (Paris, France). Ultra-pure water (18 MΩ) was obtained by ultrafiltration with a Q-Pod (Millipore Corp., Molsheim, France). Formate buffer containing 2 mM ammonium formate in 0.1% formic acid was prepared in ultra-pure water and stored after each analysis at +4°C away from light for a maximum of one week.

Calibration standards and quality controls

Eight calibration standards (CSs) containing a mixture of screened fentanyls at 1, 2.5, 5, 10, 50, 100, 500, and 1000 pg/mg of hair were prepared by spiking stock solutions of reference standards with appropriate volume into a 20-mg drug-free hair powder. Quality controls (QCs) were prepared at concentrations of 5 (or 20), 250 and 750 pg/mg in the same way. Drug-free human hair was obtained from healthy subjects who are not medically treated.

Hair sample preparation

Hair locks were sampled from the posterior vertex, and whenever possible were cut into 3 segments of 2cm corresponding to an exposure timeframe of about 6 months prior to sampling based on hair growth of 1cm/month. Short hair (<2 cm) was analyzed in bulk. Each segment was considered separately as it reflected a different timeframe. Hair was decontaminated twice using dichloromethane (immersion for 2 min in each step). It was then grinded into a fine and homogeneous powder using a ball mill (MM200, FicherScientific, Illkrich, France). Each 20 mg segment was incubated in 1 mL of phosphate buffer at pH 5.0 at 95°C for 10 min, in the presence of an appropriate amount of deuterated internal standards (IS: fentanyl-D5, acetylfentanyl-D5 and norfentanyl-D5). After spiking the corresponding volume of CS or QC working solutions when necessary, liquid-liquid extraction was performed with 4 mL of a mixture of hexane/ethyl acetate (v/v, 1:1). After agitation and centrifugation for 20 min, the organic phase was recovered and the aqueous phase underwent a second extraction by 4 mL of chloroform/isopropanol (v/v, 80:20). The two organic phases were merged and evaporated to dryness. The residue was reconstituted in 80 µL of mobile phase and 10 µL were injected onto the chromatographic system.

Whenever the concentrations in real samples exceeded the upper limit of quantification, the final extracts were diluted with the mobile phase and re-injected onto the system.

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LC–MS/MS conditions

The system derived from a previously described method [23]. Chromatography was performed on a Dionex Ultimate 3000 pump (ThermoFisher, Les Ulis, France) using a Hypersyl Gold PFP column (100 x 2.1 mm i.d., particle size 1.9 µm) preheated at 30 °C. The mobile phase is a gradient of acetonitrile (A) and 2 mM sodium formate buffer in 0.1% formic acid (B) starting from 20% (A) to 90% (A) in 10 min at a flow rate of 300 µL/min. The total run time was 12 min. Compounds were detected by a TSQ Endura triple-quadrupole mass spectrometer (ThermoFisher) equipped with an electrospray ionization (ESI) source set in a positive mode with ion spray potential at +3.5kV. Capillary temperature was set at 350°C. Nitrogen (Nitrox UHPLCMS 18, nitrogen generator; Domnick Hunter, Villefranche sur Saone, France) was employed as sheath gas at 35 arbitrary pressure unit. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in MRM mode, with two *m/z* transitions per analyte. The target scan time was 1.3 s, resulting in at least 10-12 data points across the peak above half height. Data acquisition and processing were performed using Xcalibur and LC-Quan softwares (both ThermoFisher®).

Retention times, MRM transitions with corresponding collision energies for the screened NSOs and their ISs are shown in Table 1.

Method validation procedure

Selectivity

Drug-free blank hair samples (n=6) were analyzed to determine whether endogenous hair constituents interfere or not at retention times and ion channels of NSOs. Absence of interfering compounds was accepted if the response was less than 20% of the lower limit of quantification (LLOQ) for the analytes and 5% for the IS

Carry-over

A replicate of blank sample was analyzed immediately after the highest CS (upper limit of quantification ULOQ) to determine the carry-over. Results for blank sample following ULOQ should not be greater than 20 % of the LLOQ and 5% for the IS.

Linearity

Six calibration curves were prepared with eight CSs ranging from LLOQ to 1000 pg/mg. Quantification was achieved by plotting the peak area ratios of NSOs to their respective IS. Back-calculated concentrations of the

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CSs should be within ±15% of the nominal concentrations, except for the LLOQ for which it should be within +20%, and at least 75% of the CS must fulfill this criterion.

Limit of detection (LOD) and lower limit of quantification (LLOQ)

The LOD is the lowest concentration for each NSO that can be detected with a signal-to-noise ratio greater than 3. The LLOQ was the lowest concentration of each NSO achievable with an accuracy and precision of ± 20 %.

Accuracy and precision

The accuracy (bias) and precision (coefficient of variation CV) of the assay were determined at three QC levels (low 5 or 20 depending on the LLOQ; mid 250; high 750 pg/mg). For the intraday assay, 6 replicates of each QC were processed the same day. For the inter-day assay, 6 replicates of each QC level were processed on three different days. The concentrations obtained were analyzed using analysis of variance (ANOVA), which separated the intraday and inter-day standard deviation and the corresponding coefficients of variation (CV). An accuracy within the range 85-115% (bias of ±15%) of the nominal values and a precision with a CV of ±15% were required.

Matrix effect and overall method recovery

Matrix effect (ME) was defined as the ratio of the mean peak area obtained by analyzing 6 different blank hair matrices spiked after extraction with ISs and NSOs at two concentrations (100 and 500 pg/mg) to the mean peak area obtained in an aqueous solution at the same concentrations. ME was expressed as a normalized value to that of the IS (IS-normalized ME) by dividing the ME of the analyte by the ME of the corresponding IS. The CV of the IS-normalized ME calculated from 6 batches of matrix should not exceed 15 %.

Overall method recovery was defined as the ratio of the mean peak area obtained by analyzing six different blank matrices spiked before extraction with ISs and NSOs to the mean peak area obtained in an aqueous solution at the same concentrations. The determination of method recovery remains optional for mass spectrometry techniques.

Dilution effect

3 replicates of QC samples at the concentration of 3000 pg/mg were analyzed after 1/4 dilution, and the precision of the result had to be within ±15% of the nominal value.

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Analysis of real hair samples

One hundred thirty-seven hair samples (134 samples screened during 2019 and 3 hair samples previously screened positive for fentanyl) were considered to verify the applicability of the present method after undergoing screening for common opioids, cocaine, amphetamines and NPS using a previously described method [23]. Cannabis has not been sought systematically as its screening requires a different extraction procedure in acidic conditions and a need for a second strand of hair, which was not available in all cases.

Hair was sampled in patients admitted to different departments of Paris hospitals and its suburbs. Patients were mainly hospitalized for: *1)* drug-dependence or *2)* suspected acute intoxication.

No consent from patients was requested as hair testing is part of a routine analysis in France, and was asked by practitioners in order to have more information about past use of drugs of abuse and other psychotropic medicines in their patients.

Results

Validation of the analytical method

The validation settings of the 17 NSOs are summarized in table 2. No interfering signals from the matrix (6 drug-free hair samples) were observed at the retention time where NSOs were expected to elute. Contamination by carry-over from a 1000 pg/mg QC to blank sample was not detected.

The method exhibits a good linearity with determination coefficient $r^2 > 0.993$. A weighting factor 1/x was adopted for all NSOs to compensate for heteroscedastic distribution of the calibration points. The LODs and LLOQs were 0.3 and 1 pg/mg for fentanyl (F), sufentanyl, alfentanil, ocfentanyl, acetylfentanyl (AF), methoxyacetylfentanyl (MeO-F), 3-methyl fentanyl (3-methyl-F) and butyrylfentanyl (butyryl-F); 0.8 and 2.5 pg/mg for 3-fluorofentanyl (3-FF), carfentanil (CaF), furanylfentanyl (Fu-F) and U-47700; 1.5 and 5 pg/mg for norcarfentanil (N-CaF) and furanyl norfentanyl (Fu-NF); 3 and 10 pg/mg for acetyl norfentanyl (A-NF) and butyryl norfentanyl (butyryl-NF); and 20 and 50 pg/mg for norfentanyl (NF). Coefficients of variation at the LOQs ranged between 6.1 and 14.7%. Intra- and inter-day accuracy (bias, %) and precision (CV, %) were: 0.7–10.9; 3–11.7; 0.6–10.9; 0.6–15, respectively. Extraction recoveries of parent drugs ranged between 74 and 120% (median: 94.5%) while for the metabolites Fu-NF, N-CaF, A-NF, But-NF and NF, lower extraction efficiencies were achieved (range: 7-62 %, median: 36.5). Normalized matrix effect (N-IS ME) ranged from 59 to 126% (mean: 91.8%; median: 94.2 %) with CVs $\leq$ 12.9%.

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The 1/4 dilution of QC samples (3000 pg/mg) showed acceptable CV (≤15%) and allowed to validate concentrations ranging between the ULOQ and 3000 pg/mg found in some samples.

Application to authentic hair samples

The 134 samples belong to 69 males (M) and 65 females (F) (sex ratio: 1.06), most were hospitalized in addiction clinics (n=39) or emergency departments (n=34). The remaining 61 patients were hospitalized or seen for different clinical investigations (e.g. nephrology for renal failure, dermatology for Lyell syndrome, etc.). Sixty seven patients (40M, 27F, sex ratio: 1.48) tested positive for at least one opioid: heroine (n=3), 6-acetylmorphine (n=5), acetylcodeine (n=2), morphine (n=12), codeine (n=34), oxycodone (n=6), noroxycodone (n=1), pholcodine (n=6), tramadol (n=43), methadone (n=10) and its main metabolite EDDP (n=9), buprenorphine (n=1), nalmefene (n=2), naltrexone (n=1) and fentanyl (n=3). Fentanyl was also detected in the three reanalyzed hair samples, alone (n=1) or in combination with five fentanyl analogs (3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl, acetylfentanyl and carfentanil) (n=2) and at least one of common opioids (except case No. 2): heroine, 6-AM, codeine, morphine, acetylcodeine, methadone, tramadol, and oxycodone. Table 3 summarizes the 6 cases tested positive for fentanyl and its analogs in hair.

The concentrations of fentanyl found in 14 hair segments ranged from < 1 pg/mg to 1650 pg/mg (median: 14.5). The first case (No. 1) was from a 4 year-old boy hospitalized for suspected intoxication and was given fentanyl as a treatment among other medicines. The two other samples were collected from 2 adults: a 56 years male who had experienced heart attack (case No. 2) and a 22 years-old female (case No. 3) treated for drug abuse (mainly for opioids and benzodiazepines). No information was given in the medical records about fentanyl use in these two patients. Two out of the three hair samples retrospectively reanalyzed were from the same opioid user (43 years, male) whose hair was taken one month (M1, case No. 4) and one year (Y1, case No. 5) after an opioid poisoning. In the first strand of hair (M1, segments: A 0-2cm, proximal; B 2-4 cm; C 4-6 cm), 3-fluorofentanyl (A/B/C: 150/80/60 pg/mg), furanylfentanyl (40/20/15 pg/mg) and fentanyl (37/25/3 pg/mg) were detected. The second hair analysis (Y1, segments: A 0-2cm, proximal; B 2-4 cm) showed the presence of 3 new fentanyl analogs: methoxyacetylfentanyl (A/B: 500/600 pg/mg), carfentanil (A/B: 2.5/3 pg/mg) and acetyl fentanyl (A/B: 1/1 pg/mg), along with the previously detected fentanyl analogs: 3-fluorofentanyl (A/B: 25/80 pg/mg), furanylfentanyl (A/B: 310/500 pg/mg) and fentanyl (A/B: 620/760 pg/mg). This case report has recently been reported in the literature [13]. The last reanalyzed sample (No. 6) was taken in a suspected poisoning case of a 45 year-old female in whom hair testing was requested to study past use of drugs as blood testing taken late after her admission at the intensive care unit was not informative (only clonazepam, metoclopramide and

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levetiracetam were detected at low to normal concentrations). Hair analysis revealed the presence of fentanyl in the two analyzed segments (A 0-2cm, proximal; B 2-4 cm) at 1500 and 1650 pg/mg, respectively. Other opioids (e.g. tramadol) along with different pharmaceuticals were also identified, but unfortunately, no more information related to the origin of these compounds (treatment or abuse) were given.

An example of the MRM chromatograms obtained at LLOQ of each compound are shown in Fig 1

Discussion

The present LC-MS/MS targeted screening method allowed the determination of 16 fentanyl analogs and one unrelated fentanyl compound (U-47-700) in hair, with satisfactory validation criteria. The selection of NSOs to be included in the validation process was mainly conditioned by the availability of reference materials at the time of the validation (Early 2019), but was also based on the most relevant compounds that have been involved in series of life threatening intoxications or deaths, as reported by the EMCDDA and Europol in their joint investigations on fentanyl analogs [7].

The present method showed high specificity and sensitivity for NSOs parent drugs determination in hair with low limits of detection (≤ 0.8 pg/mg) and quantification (≤ 2.5 pg/mg). Apart from norfentanyl that showed a higher LOD (20 pg/mg) and LLOQ (50 pg/mg), furanylnorfentanyl, norcarfentanil, acetylnorfentanyl, butyrylnorfentanyl showed acceptable sensitivity (LOD ≤ 3 and LLOQs ≤ 10 pg/mg).

The use of deuterated ISs like acetylfentanyl-D5, fentanyl-D5 and norfentanyl-D5 allowed to compensate the matrix effect for acetylfentanyl, fentanyl and norfentanyl, respectively. As acetylfentanyl-D5 behaved in the same way as most of the quantified fentanyl analogs in terms of signal extinction and recovery yield, it was considered as a suitable IS for their quantification. For 3-fluorofentanyl, 3-methylfentanyl, butyrylfentanyl, carfentanil, furanylfentanyl, sufentanyl and U-47700, a moderate signal extinction was observed. Nevertheless, this signal extinction was reproducible and had no impact on the accuracy and the precision of the method which allowed to achieve satisfactory LODs (≤ 0.8 pg/mg) and LLOQs (≤ 2.5 pg/mg).

To our knowledge, only limited targeted screening methods for NSOs identification and quantification in hair have been published so far [19–22].

Boumba et al., [19] described a liquid chromatography tandem mass spectrometry (LC–MS/MS) to identify 132 NPS including acetylfentanyl, 3-methylfentanyl (-cis and -trans), butyrylfentanyl, AH-7921 and U47-700. LOD was 1 pg/mg, except for U47-700 (10 pg/mg), and limit of reporting positive cases was set at 100 pg/mg. The method was applied to 23 medico-legal cases where NPS use was suspected or proved. In 2 cases, acetylfentanyl was detected but not quantified.

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Salomone et al., [20] described an UHPLC–MS-MS method able to detect 10 fentanyl analogs along with tramadol, oxycodone and hydrocodone in hair. LODs varied between 0.1-0.3 pg/mg and calculated LLOQs varied between 0.3-0.9 pg/mg. The method was applied to 34 real hair samples collected in New York City from subjects who had reported past-year non-medical opioid use. 17 samples tested positive for at least one target analyte. Among these, furanylfentanyl (44 pg/mg) and fentanyl (2 and 6 pg/mg) were quantified in 2 samples. Simultaneously, 4-ANPP, both a precursor and metabolite of several fentanyl derivatives was detected (1 and 3 pg/mg).

Busardò et al., [21] analyzed fentanyl and 22 analogs and metabolites in hair by LC–MS/MS. LODs were between 0.003-0.007 pg/mg and LOQs were between 0.011-0.021 pg/mg. Fentanyl, norfentanyl and 4-ANPP were detected in 2 cases each. Hair concentrations were 2540 and 2800 pg/mg for fentanyl, 15.1 and 149 pg/mg for norfentanyl, and 10.4 and 11.2 pg/mg for 4-ANPP.

More recently, Ramírez Fernández et al., [22] validated an UPLC-MS/MS assay for the analysis of 13 fentanyl analogs and 3 non-related fentanyl compounds (U-47700, U-49900 and AH-7921) in segmented hair. LLOQs ranged from 0.15-1 pg/mg. The method was applied to 17 authentic hair samples (body or head hair) from US fentanyl analogs users. Concentrations ranges were as follow: fentanyl 2–ULOQ (500) pg/mg (n= 16), norfentanyl 1–38 pg/mg (n= 14), acetylfentanyl 0.6–ULOQ (250) pg/mg (n= 7), furanylfentanyl 2–123 pg/mg (n= 5), tetrahydrofuranylfentanyl 0.5–63 pg/mg (n = 1) and valerylfentanyl 2.1–ULOQ (50) pg/mg (n = 1).

All described methods for NSOs hair analysis were based on LC-MS/MS which became a gold standard, especially in forensic toxicology when sensitivity is required like for fentanyl analogs detection. Our method allowed the detection and quantification of 17 NSOs, among previously added NPS like synthetic cannabinoids [24]. We analysed a relevant number of hair samples (n=137) collected mainly in addicted persons or those who were hospitalized for suspected poisoning. In 2 samples belonging to an opioid user (cases No 4 and 5), different fentanyl analogs including 3-fluorofentanyl, methoxyacetylfentanyl, carfentanil, acetylfentanyl, furanylfentanyl, and fentanyl were detected. To our knowledge, this was the first time that 3-fluorofentanyl and methoxyacetylfentanyl concentrations in segmented hair were reported [13]. Toxicological data regarding 3-fluorofentanyl are missing from the literature. Given the structural similarity between 3-fluorofentanyl and its 2 isomers (2- and 4-fluorofentanyl), both were scheduled as narcotic and have been involved in series of fatalities in the US and Europe (especially 2-fluorofentanyl since 2016), it is expected that 3-fluorofentanyl has similar high potency, dependence and abuse liability [25,26].

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Methoxyacetylfentanyl was found at high concentrations in hair, suggesting chronic exposure. However, more data are needed to accurately interpret these findings as this is the first time this fentanyl derivative has been reported. From a toxicological point of view, this analog has been involved in 28 deaths across Europe and the United States in 2017 [27].

Methoxyacetyl norfentanyl, a metabolite of methoxyacetylfentanyl was not screened in our method as the reference standard was not available. Busardò et al. have reported that this metabolite was not detected whether in blood or urine when methoxyacetylfentanyl was taken, and thus, it was not considered as a suitable marker of methoxyacetylfentanyl consumption [21].

Acetylfentanyl and carfentanil were found in very low concentrations in patient's hair which seemed to reflect a single or few exposures. The interpretation of these low hair concentrations remains tricky as these compounds could also be present as cutting agents of other drugs of abuse like NSOs or heroin. In inpatient detoxification program, Palamar et al. have reported acetylfentanyl hair concentrations ranging from 2.1 to 3200 pg/mg (median: 26 pg/mg) showing good integration of this compound into the hair matrix [28]. In a case of carfentanil related death, hair concentration reported ranged from 54 to 166 pg/mg, suggesting a repeated exposure over the studied period of 9 months, without excluding contamination by sebum and postmortem fluids [29]. As expected, acetyl norfentanyl and norcarfentanil, the respective metabolites of acetylfentanyl and carfentanil were not detected as the concentrations of parent drugs were very low in our study.

An increasing exposure pattern for furanylfentanyl from sample (M1) to sample (Y1) demonstrated chronic exposure to this compound and its good incorporation in hair. In the study of 34 hair samples from a nightclub and festival attendees from the US, Salomone et al. [20] reported one positive case for furanylfentanyl at 44 pg/mg, which with regard to our results may probably reflect occasional exposure.

Fentanyl was detected in 6 cases with concentrations raging from < 1–1650 pg/mg showing good incorporation into hair. In the previous study of Salomone et al., two hair samples were positive for fentanyl at very low concentrations (2 and 6 pg/mg), probably reflecting an occasional exposure. Norfentanyl was not detected neither in Salamone's study nor in ours, probably due to a high parent drug to metabolite ratio (fentanyl/norfentanyl). In fact, in Busardò's study [21], fentanyl/norfentanyl ratio was very high (up to 170 fold), explaining probably why norfentanyl detection was missing in our cases. In the study of Ramírez Fernández et al, fentanyl and norfentanyl were the most detected opioids in hair with concentrations ranging from 13.5 up to ULOQ (500 pg/mg) for fentanyl (n=16) and from 1 up to 38 pg/mg for norfentanyl (n=15). A

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wide range of parent drug to metabolite ratio (up to 55 times) from one person to other and depending on the concentrations seemed to occur.

4-ANPP (or despropionylfentanyl), both a precursor and metabolite of fentanyl [12] and other analogs like furanylfentanyl [30], methoxyacetylfentanyl [31] and acetylfentanyl [32] was proposed as an alternative marker of fentanyl intake than nor-metabolites, even if the few available data showed controversial findings. In the two cases reported by Busardò et al., 4-ANPP was quantified at 10.4 and 11.2 pg/mg, respectively, making it an inefficient and non-specific marker of consumption according to the authors. In contrary, in the 2 hair samples positive to only fentanyl (6 pg/mg) or both furanylfentanyl and fentanyl (44 and 3 pg/mg) in Salomone's study, 4-ANPP was detected and quantified at concentration as high as 3 pg/mg. Regarding these results, it seems that more data are needed to know whether this metabolite can be used as a biomarker of these fentanyl analogs. We did not include this compound in the validation as we could not purchase the reference material during the dedicated period of validation, but we could detect it in the two hair samples (case N° 4 and 5) using a previously validated untargeted LC-HRMS screening method [18].

Finally, one should notice that even if opioids crisis has gained an important level in the US and some European countries, its implication in drug-related intoxications and deaths in France remains unclear. From our study, only 3 out of 134 cases (2.2%) of drug abuse were positive to fentanyl during 2019, some cases corresponding to a prescribed treatment.

Conclusion

The present study described a fully validated LC-MS/MS method dedicated to the analysis of 16 fentanyl analogs and U-47700 in hair. It brought for the first time the possibility to test for 3-fluorofentanyl and norcarfentanil and describes new data regarding 3-fluorofentanyl and methoxyacetylfentanyl concentrations and their good incorporation into the hair matrix.

From an epidemiological point of view, it seems that the diffusion of the screened NSOs in the studied populations was rare in 2019, with only 3 positive cases detected out of 134 analyzed samples. Nevertheless, as the NPS market is continuously changing, it appears crucial to keep the screening library updated by adding new compounds belonging to NPS.

Compliance with ethical standards

Conflict of interest: there are no financial or other relations that could lead to a conflict of interest.

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Ethical approval: the analyses of drugs were requested by practitioners as a part of medical investigations in France. About several tens of strands of scalp hair were noninvasively collected from the patients with their informed consent.

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Table 1 Retention times, multiple reaction monitoring transitions (MRM) and collision energies of the 17 NSOs and their internal standards (ISs)

Compound	IS	RT (min)	Parent drug	Product ion (Quantifier)		Product ion (Qualifier)	
		min	*m/z*	*m/z*	CE (eV)	*m/z*	CE (eV)
3-Fluorofentanyl (3-FF)	AF-D_5	7.16	355.5	188.2	22	105.2	34
3-Methylfentanyl (3methyl-F)	AF-D_5	7.66	351.2	202.1	23	105.1	34
Acetylfentanyl (AF)	AF-D_5	5.50	323.2	188.0	18	105.1	32
Acetyl norfentanyl (A-NF)	AF-D_5	1.96	219.1	136.1	17	84.1	17
Alfentanil	AF-D_5	6.0	417.4	268.3	17	197.2	26
Butyrylfentanyl (Butyryl-F)	AF-D_5	7.86	351.2	188.1	24	105.2	38
Butyryl norfentanyl (Butyryl-NF)	AF-D_5	3.96	247.1	84.1	18	55.1	18
Carfentanil (CaF)	AF-D_5	7.60	395.4	335.3	18	240.1	21
Fentanyl (F)	F-D_5	6.60	337.0	105.1	23	188.2	36
Furanylfentanyl (Fu-F)	AF-D_5	7.07	375.5	188.2	21	105.2	33
Furanyl norfentanyl (Fu-NF)	AF-D_5	3.5	271.1	188.1	16	84.1	16
Methoxyacetylfentanyl (MeO-AF)	AF-D_5	5.22	353.1	188.1	21	105.1	35
Norcarfentanil (N-CaF)	AF-D_5	3.63	291.1	259.1	10	231.2	14
Norfentanyl (NF)	NF-D_5	3.04	233.1	177.1	14	55.1	33
Ocfentanil	AF-D_5	5.89	371.2	188.1	23	105.3	37
Sufentanil	AF-D_5	8.28	387.3	238.1	18	111.1	34
U-47700	AF-D_5	6.46	329.2	172.9	27	144.9	46
Fentanyl-D_5 (F-D_5)	-	6.60	342.2	105.1	23	188.2	36
Acetylfentanyl-D_5 (AF-D_5)	-	5.50	328.2	188.1	22	105.1	35
Norfentanyl-D_5 (NF-D_5)	-	3.01	238.2	182.1	15	84.2	17

RT retention times, *CE* collision energy

Table 2 Validation parameters of the 17 NSOs

Compound	r^2	LOD	LLOQ		Accuracy (% bias)						Precision CV (%)						N-IS ME (%)				Recovery (%)	
				CV (%)	Intra-day			Inter-day			Intraday			Interday								
					Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	CV	High	CV	Low	High
					5[a]	**250**	**750**	**5[a]**	**250**	**750**	**5[a]**	**250**	**750**	**5[a]**	**250**	**750**	**100**	**%**	**500**	**%**	**100**	**500**
F	0.999	0.3	1.0	8.6	4.4	3.6	0.7	4.5	3.5	0.7	4.7	5.0	6.2	9.1	8.6	0.6	101.7	2.8	112.7	4.1	110	84
Alfentanil	0.998	0.3	1.0	9.5	3.8	5.7	3.5	3.3	5.0	3.6	7.8	5.7	5.8	8.9	12.4	5.9	94.2	2.9	108.5	2.8	117	92
Sufentanil	0.997	0.3	1.0	12.2	7.3	7.1	1.6	7.3	7.0	0.2	6.2	4.0	9.5	7.1	14.1	5.4	59	9.1	71.7	10.9	111	77
Ocfentanil	0.998	0.3	1.0	7.5	4.1	4.4	5.0	3.4	0.7	4.2	3.4	4.0	6.7	12.3	15.0	13.7	93.7	3.8	111	3.9	119	92
AF	0.999	0.3	1.0	10.5	1.9	2.7	0.4	0.1	0.4	0.4	5.8	4.8	4.9	6.9	8.3	1.0	97.2	4.3	108.9	2.7	116	91
MeO-AF	0.998	0.3	1.0	8.7	6.0	7.7	6.5	6.0	5.2	6.4	3.9	6.3	7.4	10.0	13.0	7.5	93.7	3.7	111	3.6	117	91
3-methyl-F	0.997	0.3	1.0	9.5	4.1	3.8	5.7	1.1	2.2	5.7	5.9	3.8	7.3	14.6	15	13.9	76.7	6.5	87.1	2.1	120	88
Butyryl-F	0.996	0.3	1.0	11.8	2.7	3.9	4.5	2.2	2.0	4.6	5.3	11.7	8.5	9.2	14.8	8.7	61.2	5.3	71.9	3.2	117	83
3-FF	0.997	0.8	2.5	14.7	4.7	2.7	3.7	1.0	2.0	1.2	5.8	11.5	8.0	13.7	6.3	13.0	73.8	7.9	76.2	12.3	109	82
CaF	0.996	0.8	2.5	6.1	2.1	3.8	4.3	0.0	2.5	2.1	5.8	11.1	9.5	7.0	14.1	14.7	62.9	8.6	73.7	5.5	111	80
Fu-F	0.998	0.8	2.5	7.6	7.9	7.6	3.2	8	7.7	3.2	4.1	7.0	11.1	12.4	13.3	12.2	64.2	11.5	68.3	12.9	97	74
U-47700	0.995	0.8	2.5	10.3	3.0	2.8	3.1	1.0	2.6	1.7	7.0	7.3	10.0	11.1	6.8	10.3	66.9	11.2	71	4.4	103	76
Fu-NF[a]	0.997	1.5	5	12.4	4.9	4.9	3.9	1.4	4.9	1.9	7.4	8.8	7.0	14.3	14.9	13.9	109.0	5.7	113.4	2.4	46	38
N-CaF[a]	0.993	1.5	5	11.5	2.3	8.6	5.6	0.1	9.6	6.6	9.0	5.8	6.9	7.4	4.0	11.2	123.6	7.2	126.7	5.8	44	35
A-NF[a]	0.997	3	10	8.4	2.1	3.6	4.6	1.0	3.2	0	7.2	6.3	9.0	7.7	11.1	15.0	105.8	1.8	107.6	4.2	10	7
Butyryl-NF[a]	0.996	3	10	9	2.2	10.9	2.3	0.6	10.9	2.4	7.4	3.0	6.6	7.3	6.7	6.3	94.3	6.7	98.6	3.1	62	52
NF[a]	0.995	20	50	7.7	n/a	6.1	4.7	n/a	5.9	3.3	n/a	6.4	7.3	n/a	14.3	13.6	111.6	11.6	113.5	5.6	33	30

r^2 coefficient of determination, LOD limit of detection, LLOQ lower limit of quantification, CV coefficient of variation,

F fentanyl, AF acetylfentanyl MeO-AF methoxyacetylfentanyl, 3-methyl-F 3-methylfentanyl, butyryl-F butyrylfentanyl, 3-FF 3-fluorofentanyl, Ca-F carfentanyl, Fu-F furanylfentanyl, Fu-NF furanylnorfentanyl, N-CaF norcarfentanil, A-NF acetylnorfentanyl, NF norfentanyl.

All concentrations in pg/mg.

[a] low QC level at 20 pg/mg for Furanylnorfentanyl, Norcarfentanil, Acetylnorfentanyl, Butyrylnorfentanyl, and Norfentanyl

N-IS-ME: normalized matrix effect (ratio of the ME of the analyte to the ME of IS). CV calculated from 6 different batches of hair

Tables 3 NSOs detection and quantification in real hair samples (n=6)

Case No.	Cause of admission	Year of the case study	Age	Sex	Segments (cm)	NSOs in hair	Concentrations per. Segment (pg/mg)	Associated drugs detected in hair
1	Suspected poisoning in children	2019	4	M	A 0–2.5 (bulk)	F NF	20 ND	**Codeine, morphine**, ketamine, norketamine, midazolam, hydroxymidazolam, labetalol, amitriptyline, nortriptyline, nicardipine, lidocaine, MEGX
2	Suspected poisoning (Heart attack)	2019	56	M	A 0–2; B 2–4	F NF	9–7	Venlafaxine, norvenlafaxine, acetaminophen, acebutolol, diacetolol, lidocaine, midazolam
3	Drug abuse	2019	22	F	A 0–2; B 2–4; C 4–6; D 6–11; E 11–16; F 16–21; G 21–26	F NF	(DE/F/G: <1–3–3–4) ND	**Morphine, codeine, oxycodone, methadone, tramadol**, ketamine, metizolam, diazepam, prazepam, nordiazepam, lorazepam, clobazam, norclobazam, zopiclone, zolpidem, alimemazine, citalopram, norcitalopram, propranolol
4	Drug abuse	2016	42	M	A 0–2; B 2–4; C 4–6	F NF 3-FF Fu-F	35–27–3 ND 150–80–60 40–20–15	**Heroine, 6-AM, codeine, acetylcodeine, morphine, methadone, tramadol**, dextromethorphan,

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								methamphetamine, amphetamine, MDMA, MDA, 2C-E, cocaine, BZE, EME, CE, norcocaine, AEME, ketamine, norketamine, lidocaine, levamisole, sildenafil, tadalafil, prazepam, hydroxyzine, cetirizine, amitriptyline, nortriptyline, etifoxine, diltiazem
5	Drug abuse	2017	43	M	A 0–2; B 2–4	F NF 3-FF Fu-F Fu-NF AF A-NF CaF N-CaF MeO-AF	620–760 ND 25–80 310–500 ND 1–1 ND 2.5–3 ND 500–600	**Methadone, tramadol**, dextromethorphan, methamphetamine, amphetamine, MDMA, MDA, 2C-E, cocaine, BZE, EME, CE, norcocaine, ketamine, norketamine, lidocaine, levamisole, sildenafil
6	Suspected poisoning	2018	45	F	A 0–2; B 2–4	F NF	1500–1650 ND	**Tramadol**, methylphenidate, nefopam, lidocaine, prilocaine, acetaminophen, hydroxyzine, cetirizine, metoclopramide

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6-AM 6-acetylmorphine, MDMA 3,4-methylenedioxy-methamphetamine, MDA 3,4-methylenedioxy-amphetamine, 2C-E 2,5-Diméthoxy-4-éthyl-phénéthylamine, BZE benzoylecgonine , EME ecgonine methyl ester, AEME anhydroecgonine methyl ester, F fentanyl.

ND : not detected

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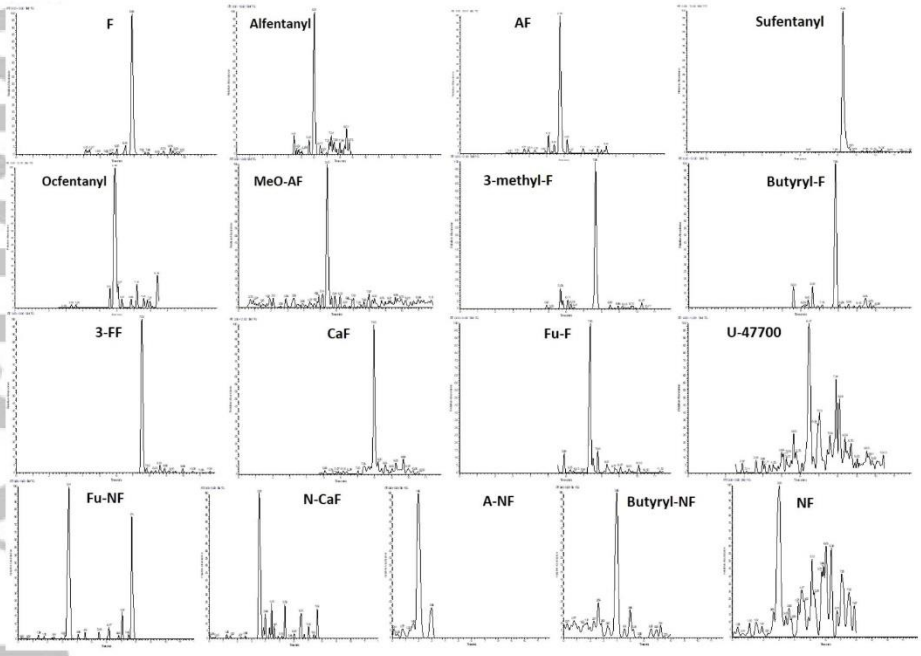


Fig. 1. MRM chromatograms of the 17 NSOs at the corresponding LLOQ

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2. Discussion

Dans le 1er volet de cette partie des travaux, nous avons évalué une technique de dépistage rapide de drogues classiques et de NPS (***cannabinoïdes de synthèse*** de type JWH-18, UR-144, AB-PINACA), **cathinones** de type **pyrovalérone** (α-PVP), ***dérivés de fentanyl***, et ***benzodiazépines de synthèse***) par chimiluminescence sur biopuces (dite BAT ou Biochip Array Technology). Les résultats obtenus sur 161 échantillons de sang (n = 80) et d'urine (n = 81) prélevés dans différentes circonstances cliniques (intoxications, addiction, médecine de travail…etc.) ou médicolégales (recherche des causes de la mort, soumission chimique, délit routier…etc.) ont été comparés à ceux obtenus par LC-MS/MS ou LC-HRMS afin de vérifier la fiabilité du dépistage et définir ses limites.

Cette méthode est complètement automatisable et ne nécessite qu'une simple dilution de l'échantillon et sa centrifugation. Les résultats sont rendus en moins de 20 minutes et comportent la recherche de 19 panels de drogues par matrice. Chaque panel regroupe plusieurs molécules ou métabolites présentant la même structure que le chef de fil contre lequel l'anticorps est dirigé. Ces caractéristiques confèrent un aspect très pratique à cette méthode qui permet de répondre à des situations d'urgences toxicologiques en un temps court et inclut la recherche d'un large éventail de composés les plus fréquemment rencontrés lors des intoxications aigues par les drogues et certains médicaments psychotropes. En toxicologie médicolégale, cette technique permet un premier criblage des échantillons afin d'orienter la recherche ultérieure et d'éviter des analyses inutiles sur des échantillons négatifs.

Plusieurs échantillons contenant des NPS ont été dépistés positifs grâce à cette technique. Cela inclut des dérivés stimulants de type pyrovalerone (α-PVP, MD-PHP, pentedrone, n-ethylpentylone et hexedrone), des dérivés opioïdes (fentanyl, 3-fluorofentanyl, ocfentanyl) et une benzodiazépine de synthèse (étizolam).

En routine, notre emploi du dépistage par BAT et du criblage ciblé par LC-MS/MS est quasi systématique. Le recours à la LC-HRMS est réservé à des situations particulières, par exemple quand une cause toxique est fortement suspectée alors que les recherches de routines sont négatives, ou lorsque les molécules recherchées ne sont pas incluses dans le criblage ciblé (car absentes des bibliothèques de spectres).

C'est le cas dans cette étude où certaines substances nouvellement apparues sur le marché ont pu être décelées grâce à ce dépistage, et qui n'auraient jamais pu être identifiées avec le criblage ciblé seul (car la bibliothèque LC-MS/MS ne les comportait pas).

L'évaluation de la fiabilité des 2402 résultats acquis montre une concordance avec les résultats de la spectrométrie de masse dans plus de 90% des cas (50% des panels ayant montré 100% de concordance). De même, la spécificité de l'anticorps employé et la sensibilité de la méthode étaient supérieures à 90% pour la majorité des panels (n=14).
Néanmoins, certains résultats FP ont été rendus par cette méthode. Nous citons l'exemple du panel « α-PVP » qui fournissait des résultats positifs à ce test alors que les confirmations par LC-MS/MS et LC-HRMS étaient négatives. L'investigation approfondie de ce genre de discordance a pu mettre en évidence une réaction croisée entre l'anticorps dirigé contre l'α-PVP et le tramadol et/ou la venlafaxine (ainsi que son métabolite la norvenlafaxine) quand les concentrations de ces dernières excédaient 2000 ng/mL. La similitude structurelle entre ces molécules et l'α-PVP semble à l'origine de cette réaction croisée jamais décrite auparavant. Il en est de même pour le dépistage de drogues classiques où le panel des amphétaminiques était le moins performant à cause des réactions croisées connues avec les amines putréfactives (ex : cadavérine, putrescine) ayant positivé certains échantillons de sang post-mortem (13/16 FP)

Par ailleurs, des résultats faussement négatifs (FN) ont aussi été décelés lors de cette comparaison, mais les concentrations mesurées par LC-MS/MS étaient souvent en dessous des seuils établis par le fabriquant, excepté certains échantillons d'urines rendus négatifs alors qu'ils contenaient des concentrations considérables de cathinones (ex : méphédrone à 2500 ng/mL, 3-MMC à 13020 ng/mL ou 4-MEC à 3075 ng/mL). Ces FN ont été facilement expliqués, l'anticorps dirigé contre la pyrovalerone ne croisait pas avec les substances de type cathinones classiques, et de ce fait ces molécules ne sont pas recherchées par cette méthode, comme le précise le fabriquant dans sa notice. Néanmoins, malgré une évolution du marché des NPS qui a vu surgir la sous-classe des pyrovalerones au dépend des cathinones classiques, la présence et l'utilisation de ces dernières au sein des populations spécifiques d'usagers (ex : chemsex) reste considérable, et leur recherche demeure indispensable comme nous allons le voir plus loin (partie II).
Malgré des seuils de positivité (sensibilité) très acceptables de cette méthode, ce type d'analyse ne doit en aucun cas être utilisé seul dans des situations où la recherche de traces de substances est demandée (ex : soumission chimique)

Enfin, il faut souligner que cette méthode est la première technique d'immunoanalyse automatisée qui propose un kit de dépistage de drogues dont des NPS dans le sang total post-mortem, une matrice très complexe et difficile à analyser [60].

L'une des techniques de criblage non ciblé en plein essor actuellement est la LC-HRMS, car elle permet de rechercher un nombre considérable de substances tout en s'affranchissant de l'utilisation des standards (étalons) de référence, ce qui lui confère un aspect pratique et très économique. Afin de permettre une meilleure connaissance du nombre et des types de NPS consommés au sein de plusieurs populations (patients intoxiqués, pharmacodépendants…etc.), nous avons mis en place et validé une méthode d'identification par LC-HRMS de type Orbitrap et l'avons appliquée à 317 composés d'intérêt en toxicologie dont 72 NPS.
Cette méthode est dédiée principalement à l'analyse des cheveux qui présentent un intérêt particulier dans les études d'exposition à long terme comme nous l'avons déjà évoqué dans la revue bibliographique. Les données spectrales acquises sont comparées à la bibliothèque de spectres mzCloud™ (https://www.mzcloud.org/), en libre accès pour les utilisateurs de cette technologie et comporte actuellement plus de 7 millions de spectres correspondant à plus de 18000 composés, et est enrichie régulièrement. La 2e particularité de cette technologie est sa capacité à retraiter les données spectrales d'une façon rétrospective ; cette fonctionnalité permet le retraitement ultérieur des données, et donc la détection de composés non présents dans la bibliothèque au moment de la première analyse (c'est le cas des NPS nouvellement apparus). Cette fonctionnalité permet également d'éviter un second échantillonnage, ce qui est très pratique quand il s'agit des cheveux, où la quantité fournie est souvent insuffisante. Cette caractéristique qui n'est pas partagée avec la LC-MS/MS montre la supériorité de la LC-HRMS.

Plusieurs paramètres d'optimisation ont été testés depuis la préparation de l'échantillon jusqu'au retraitement des données acquises en utilisant 19 composés de référence.
Cinquante milligrammes de cheveux étaient nécessaires pour obtenir une limite d'identification suffisante des composés. Bien que cette quantité paraisse élevée en comparaison des méthodes de criblage ciblé (utilisant souvent 20 mg), elle demeure inférieure à celles employées en criblage non ciblé par HRMS où les quantités s'échelonnent entre 150 et 200 mg.

Avec la technologie Orbitrap, un temps maximal d'injection des ions dans la trappe (MIT : Maximal Injection Time) approprié est un paramètre indispensable pour acquérir un spectre de masse informatif. Lorsque la fenêtre maximale d'injection (MIW : Maximal Injection Window, un 2e paramètre important) diminue, peu d'ions rentre dans la trappe (c-trap), ce qui confère un temps d'injection (MIT) élevé par ion, et vice versa. De la même manière, un 3e paramètre appelé temps dynamique d'exclusion (DET : Dynamic Exclusion Time) (temps pendant lequel l'injection de l'ion est suspendue) est un paramètre majeur en HRMS. Ce temps d'exclusion permet d'éviter une fragmentation répétée d'une même molécule, et de ce fait, laisse le temps pour la fragmentation d'autres molécules présentes à faibles concentrations dans l'échantillon.

Le mode de détection choisi est le Data-Dependent Analysis (DDA), un mode qui permet de ne fragmenter que les composés ayant une intensité du signal (une concentration) élevée (généralement les 5 plus élevés). Cette façon de procéder permet de générer des données spectrales moins fournies et donc faciles à retraiter, mais présente l'inconvénient de passer à côté des composés d'intérêt ayant des intensités (donc des concentrations) plus faibles.
Afin de limiter la fragmentation de composés ayant une intensité élevée et qui ne présentent aucun intérêt en toxicologie, une liste d'exclusion comportant les composants de la phase mobile a été utilisée ce qui a permis d'améliorer la sensibilité de la méthode. Cette sensibilité était suffisante pour détecter des concentrations aussi faibles que celles décrites dans la littérature pour les 19 composés testés, à l'exception du bromazépam, zopiclone et JWH-18 (un CS) qui n'ont pas pu être détectés du fait de leur faibles concentrations (correspondant probablement à une prise unique).
Ce type de situation où la recherche de traces est nécessaire (ex : soumission chimique) représente probablement la limite majeure à l'utilisation de la LC-HRMS.

Afin de vérifier la validité de la méthode, nous l'avons appliqué à 11 échantillons de cheveux, à partir de cas cliniques (3 intoxications présumées et 9 suivis d'addictologie), ce qui a permis d'identifier 144 composés dont 24 NPS tels que le cannabinoïde de synthèse AKB-48-5F, détecté pour la première fois dans les cheveux. 19 composés n'ont pas été détectés par manque de sensibilité, parmi lesquels on note 4 NPS : MDPV, mexedrone, N-ethylpentylone, et normephedrone. Parallèlement, l'analyse des contrôles de qualité fournis par la société internationale de l'analyse des cheveux (SOHT : Society Of Hair Testing) dans le cadre d'un contrôle inter-laboratoires a permis grâce à cette méthode de détecter la majorité des composés présents dans les échantillons. Seul le THC (non recherché par cette méthode à cause de son

caractère acide) et la norcocaine (présente à une concentration très faible de 20 pg/mg) n'ont pas été détectés. Enfin, nous avons également contribué à l'enrichissement de la bibliothèque américaine mzCloud^{TM} par l'ajout et la validation de 19 composés non présents dans la base de données, dont certains NPS (ex : 1-propionyl-LSD, 2-oxo-3-hydroxy-LSD, étizolam, HU-210).

Cette méthode présente des atouts majeurs pour étudier les profils des consommateurs de NPS ou leur métabolisme. Sa limite majeure reste son manque de sensibilité dans certaines circonstances liées aux expositions faibles, d'où l'intérêt de la compléter par le criblage ciblé.

Dans ce dernier volet, nous présentons la deuxième approche analytique utilisée au cours de ces travaux et qui est basée sur le criblage ciblé de NPS. Ce travail analytique vient compléter l'arsenal analytique existant au laboratoire dédié à l'analyse de NPS.
La première méthode concerne l'identification et le dosage de 16 cannabinoïdes de synthèse (CS), et la deuxième l'analyse et la quantification de 17 nouveaux opioïdes de synthèse (NOS), les deux appliquées à la matrice capillaire. Ce choix était motivé par deux observations inquiétantes : *1)* la part des CS dans le marché de drogues, qui représentent le plus grand groupe de NPS surveillé à travers le monde, alors que leurs identifications en France restent faibles, *2)* la crise des NOS qui a ravagé les États Unis ces dernières années (> 20000 morts/ans) et qui a poussé le gouvernement américain à déclarer l'état d'urgence sanitaire.
Ces substances sont moins notifiées sur le continent européen, et l'insuffisance des outils analytiques permettant leur surveillance semblent l'un des arguments avancés derrière cette sous-estimation.
Les deux méthodes ont été validées selon les recommandations de l'Agence Européenne des Médicaments (EMA) et ont été appliquées à l'étude des tendances de consommation de NPS dans différentes populations, ou à la description de cas d'intoxications ou de dépendance aux drogues.

Depuis 2012, quelques études ont estimé la prévalence des CS à l'aide de différents outils analytiques en utilisant les cheveux (10 à 100 mg) comme matrice. La plupart de ces études utilisaient le criblage ciblé par LC-MS/MS pour des raisons de sensibilité et de spécificité, et le prétraitement des échantillons comportait une extraction liquide-liquide à l'aide de différents solvants et parfois un chauffage à différentes températures (45 à 95°C) [61–65].

Depuis 2015, notre méthode a été appliquée à l'étude de la prévalence des CS chez des populations à risque. Parmi les 318 prélèvements de cheveux analysés entre 2015 et 2017, seul le JWH-122, un CS de la classe des indoles a été détecté. Nous reviendrons sur cette faible prévalence observée des CS dans la partie II consacrée à ce sujet. Ce cas identifié correspond à une intoxication aigue chez un polyconsommateur de drogues de 42 ans hospitalisé aux urgences une heure et demi après avoir consommé une poudre achetée sur internet. Il a présenté une symptomatologie de type mydriase bilatérale, hypertension artérielle, palpitations, gène thoracique, douleurs rénales et anurie, et a été libéré deux jours après son admission sans séquelles. Un prélèvement sanguin, capillaire et un échantillon de la poudre consommée nous ont été adressés pour analyse. l'analyse capillaire segmentaire a révélé la présence de JWH-122 dans les 3 segments analysés (A/B/C ; 200/450/430 pg/mg), montrant une exposition chronique à ce CS dans les 6 mois précédant le prélèvement. Ces concentrations sont comprises dans l'intervalle très large de concentrations de JWH-122 rapportées dans la littérature (0,1 à 2800 pg/mg). Ainsi, l'intoxication aigue chez ce consommateur régulier semble évoquer la piste d'une erreur de dosage (quantité ingérée) par le consommateur d'un produit pur (pureté évaluée à 100%), une situation très fréquente lors des intoxications aigues, plutôt qu'une augmentation de sa consommation. Cette observation est confortée par la tendance à la baisse des concentrations capillaires mesurées (concentration proximale plus faible que la concentration distale). L'analyse capillaire par un criblage plus large des substances psychoactives a permis de confirmer le statut de polyconsommateur du patient en révélant son usage de la methylone, un autre NPS de la classe des cathinones, ainsi que d'autres drogues classiques (THC, cocaïne), médicaments psychotropes et produits de coupe (doxylamine, dextrométhorphane, lévamisole, hydroxyzine et cétirizine).

Malgré les performances des outils analytiques développés actuellement, qui permettent d'atteindre des niveaux de sensibilité très bas, de l'ordre du picogramme, l'interprétation des résultats de l'analyse capillaire en général, et celles des CS en particulier demeure un défi pour tout analyste. Il est admis qu'un résultat d'analyse des cheveux négatif ne signifie pas forcément que le sujet n'avait pas consommé le produit recherché, car une ou quelques expositions ponctuelles de certains produits peuvent ne pas marquer (intégrer) les cheveux, et ainsi ne pas être détectées. Un résultat négatif peut aussi provenir d'un manque de sensibilité de la méthode employée, ou d'une faible intégration dans les cheveux des substances recherchées. De même, un résultat positif, notamment dans le cas des CS ne signifie pas forcément une consommation du produit, car la concentration mesurée peut provenir d'une

contamination externe des cheveux par la manipulation des produits par le consommateur (qui contamine par la suite ses cheveux), ou par le fumage passif. Les concentrations de certains CS évoquant des contaminations externes peuvent avoisiner les 1000 pg/mg [66]. Pour pallier à ces biais d'interprétation, certains auteurs ont proposé la recherche des métabolites pour écarter la piste de contamination. Cependant, la faible intégration des métabolites à cause de leur hydrophilie réduit drastiquement leur détectabilité dans les cheveux. C'est le cas du N(5-hydroxypentyl)-JWH122, le métabolite du JWH-122. Dans une étude, ce métabolite présentait une concentration de l'ordre de 2,5 pg/mg pour une concentration de JWH-122 de 2800 pg/mg (ratio parent/métabolite > 1000) [67]. Bien que nous ne disposions pas de ce métabolite lors de la validation analytique, les concentrations retrouvées de JWH-122 chez notre patient (< 500 pg/mg) n'auraient probablement pas permis de détecter ce métabolite. D'autres problèmes liés à l'emploi des métabolites comme marqueurs de consommation sont bien documentés. Certains métabolites peuvent être produits par dégradation du composé parent, même après une contamination externe, d'autres sont des métabolites communs à plusieurs composés, c'est le cas encore du N(5-hydroxypentyl)-JWH122, métabolite du JWH-122 et du MAM-2201, un autre CS. Il est de ce fait indispensable d'interpréter les résultats des analyses capillaires avec précaution, notamment celles des CS.

Quoi qu'il en soit, la présence de JWH-122 dans le sang de notre patient (concentration à 1,3 ng/mL) a formellement objectivé la prise, et a écarté la piste de la contamination externe.

Récemment (2020, donnée non encore publiée), l'utilisation de cette méthode analytique a permis de décrire un second cas d'exposition non intentionnelle d'un consommateur de cannabis à 9 CS (JWH-019, JWH-200, JWH-250, AM-2201, BB-22, 5F-PB-22, 5F-APINACA, ADB-PINACA, AB-FUBINACA). Les concentrations capillaires étaient comprises entre < 3 et 50 pg/mg, confirmant ainsi la fiabilité de la méthode développée et l'usage rare mais existant de ces produits en France (le résumé du cas clinique a été accepté comme communication orale au congrès national de la société française de toxicologie analytique qui aura lieu à Grenoble en décembre 2020).

La deuxième méthode de criblage ciblé par LC–MS/MS est dédiée à l'analyse de 16 dérivés de fentanyl (3-fluorofentanyl, 3-méthylfentanyl, acetylfentanyl, acetylnorfentanyl, alfentanyl, butyrylfentanyl, butyrylnorfentanyl, carfentenil, fentanyl, furanylfentanyl, furanylnorfentanyl, methoxyacetylfentanyl, norcarfentanil, norfentanyl, ocfentanil et sufentanyl) et le U-47700 (un opioïde de la classe des benzamides) dans les cheveux. Cette méthode a été appliquée à 137

échantillons de cheveux réalisés chez différents groupes d'usagers potentiels de drogues (intoxiqués) ou confirmés (pharmacodépendants) pour estimer la prévalence des NOS. Un criblage large a aussi été réalisé sur ces échantillons afin de vérifier l'association à d'autres drogues de synthèse et stupéfiants classiques. La sélection des NOS inclus dans la présente méthode était basée sur les composés ayant déjà été impliqués dans des intoxications aigues ou des décès selon les rapports de l'OEDT et d'Europol [68], mais ce choix était aussi conditionné par la disponibilité des étalons de référence au moment de la validation (début 2019).

La revue de la littérature scientifique actuelle ne fait apparaitre que 4 méthodes analytiques dédiées à l'identification et au dosage de 4 à 22 NOS dans les cheveux. Ces méthodes basées toutes sur la LC-MS/MS avaient des limites de quantification variant entre 0.021 et 10 pg/mg. Comparativement, notre méthode présente une sensibilité satisfaisante avec une LDQ ≤ 10 pg/mg, excepté le norfentanyl (LDQ de 50 pg /mg) due à son caractère polaire et sa mauvaise intégration dans les cheveux. Il faut souligner que les LDQ extrêmement faibles (0.021 pg/mg) annoncées dans certaines études semblent théoriques car les mesures de répétabilité et de reproductibilité exigées par les normes internationales de validation des méthodes nécessitent la fabrication de contrôles de qualité à la LDQ (donc à 0.021 pg/mg) ce qui semble inatteignable, voire impossible avec les outils de mesures actuels dont disposent les laboratoires. Ces études ont permis d'identifier et de quantifier le fentanyl et 6 de ses dérivés avec des concentrations très variables: fentanyl (2-2800 pg/mg), norfentanyl (1-149 pg/mg), 4-ANPP (1-11,2 pg/mg), furanylfentanyl (2-123 pg/mg), acetylfentanyl (0,6- >250 pg/mg), tetrahydrofuranylfentanyl (0,5-63 pg/mg), valerylfentanyl (2,1- > 50 pg/mg) [69–72]

Notre méthode a permis d'identifier et quantifier le fentanyl (<1- 1650 pg/mg), et 5 de ses dérivés : 3-fluorofentanyl (25-150 pg/mg), methoxyacetylfentanyl (500-600 pg/mg), carfentanil (2,5-3 pg/mg), furanylfentanyl (15-500 pg/mg) et acetylfentanyl (1 pg/mg).

Les concentrations de fentanyl et d'acetylfentanyl semblent comparables à celles précédemment rapportées, celles du furanylfentanyl semblent parfois plus élevées et reflètent donc des prises plus régulières, tandis que les concentrations du 3-fluorofentanyl, methoxyacetylfentanyl et carfentanil sont les premières jamais rapportées dans la littérature.

Bien que toutes les méthodes décrites ci-dessus (incluant la nôtre) présentent des performances analytiques très intéressantes en termes de sensibilité et de spécificité, ce qui permet d'étudier des profils très variés de consommateurs occasionnels ou réguliers, leur limite majeure reste le

nombre faible de composés recherchés, qui ne donne qu'une idée très sommaire de la tendance de consommation des NOS. A rappeler que l'augmentation du nombre de NOS déclarés sur la période 2016-2019 au système d'alerte de l'UNODC était estimée à 110%, et qu'en 2019, 62 produits étaient déjà sous surveillance [73]. Cette observation montre à quel point ces méthodes doivent être régulièrement enrichies par l'ajout d'autres substances, et montre l'intérêt majeur des techniques de criblage non ciblé comme méthodes complémentaires.

PARTIE II : Evaluation de la consommation des nouveaux produits de synthèse

1. Rationnel de l'étude

Les préoccupations concernant l'utilisation croissante des NPS et leurs effets néfastes ont conduit à un besoin croissant de surveillance de ces substances. Néanmoins, l'ampleur de leur utilisation à l'échelle mondiale demeure mal connue. Jusqu'à présent, il n'y a pas eu d'estimations sur la prévalence de l'utilisation des NPS dans la population générale. Un aperçu est fourni par l'Eurobaromètre de 2014 réalisé auprès d'un peu plus de 13 000 jeunes adultes âgés de 15 à 24 ans. Cette enquête déclarative a révélé que 8% des répondants avaient utilisé un NPS au moins une fois. Les niveaux d'utilisation les plus élevés ont été enregistrés en Irlande (9%), en Espagne, en France (8% chacun) et en Slovénie (7%), les plus faibles étant signalés par Malte et Chypre (0%) [74].

Au même titre, aucune estimation à l'échelle européenne n'existe pour l'utilisation de NPS parmi les populations à haut risque, et seuls quelques pays disposent d'estimations nationales. En Belgique, la prévalence des NPS durant l'année précédente (2016) chez les consommateurs de drogues était en moyenne de 26% [75]. En République tchèque, un tiers des consommateurs de drogues auraient utilisé une cathinone ou de la phénéthylamine au moins une fois. En Hongrie en 2014, plus de deux tiers (68%) des usagers d'héroïne bénéficiaires du programme de seringues ont déclaré s'injecter des NPS. Au Royaume-Uni, 8,9 % des participants à une enquête de surveillance anonyme en 2014 ont signalé avoir injecté de la méphédrone pendant l'année précédente. En Ecosse, une étude a exploré l'utilisation des NPS dans plusieurs populations (patients atteints de pathologies psychiatriques, sans-abri, jeunes consommateurs, injecteurs de drogues et les hommes ayant des relations sexuelles avec des hommes HSH, n= 424). Les NPS les plus utilisés étaient les cannabinoïdes de synthèse (41%), les benzodiazépines de synthèse (41%), les NPS stimulants (21%) et la méphédrone (19%).

Dans une enquête allemande, 9% des élèves de 15 à 18 ans ont déclaré avoir fumé des produits à base de CS et 3% déclarent avoir consommé des « sels de bain » (cathinones) au moins une fois dans leur vie [8]. En suisse, la prévalence de NPS dans une étude menée chez les consommateurs d'amphétamines a été estimée à 37% [76]. Enfin, dans certains pays européens (ex : Luxembourg), aucune preuve n'a soutenu l'utilisation des NPS par les consommateurs de drogues classiques [75].

Malheureusement, les données françaises relatives à la prévalence des NPS sont très rares. Une étude déclarative réalisée par le réseau d'addictovigilance français a notifié 874 cas d'usage de NPS entre 2009 et 2017. Les cathinones (3-MMC, 4-MEC, mephedrone) et les CS étaient les substances les plus signalées. Malheureusement, seulement 17% (148) de ces cas ont été confirmés analytiquement [77]. Une autre étude réalisée en 2012 en région parisienne sur 3489 seringues usées montre la présence de la 4-MEC dans 23% des cas, mais ce NPS était le seul recherché parmi les 23 substances analysées [78].

Depuis quelques années, l'OEDT diversifie son arsenal de surveillance des drogues en adoptant une approche multi-indicateurs de collecte de données, car aucune mesure ne peut fournir une vision d'ensemble sur ce phénomène. Nous citons l'exemple des données issues des analyses des eaux usées ou des résidus de seringues utilisées, les données des services d'urgence des hôpitaux, des enquêtes en ligne ou celles des services des forces de l'ordre. Bien que ces méthodes améliorent considérablement les connaissances sur les populations d'usagers et leurs modes de consommation, le manque de définition commune des NPS et d'échantillons représentatifs, les différences de législation entre les pays et le nombre croissant de ces substances viennent alourdir les disparités entre elles. De plus, elles présentent des biais liés à l'aspect déclaratif, ou à cause du lien indirect suggéré entre l'outil de surveillance utilisé (ex : eaux usées, seringues…etc.) et la population étudiée [79]. Seules des études basées sur des mesures réalisées dans des échantillons biologiques issus de ces populations permettraient d'établir un lien direct, et fournir ainsi une idée précise des profils de ces consommateurs.

Bien que le sang et les urines représentent les matrices les plus employées dans les différentes études de par leur popularité et leur simplicité de collecte, leur fenêtre de détection courte des composés (24h à quelques jours) limite l'interprétation aux expositions les plus récentes. D'autre part, les cheveux ont prouvé leur utilité comme matrice alternative et complémentaire pour les études des expositions à long terme grâce à leur caractère cumulatif des produits, leur stabilité et leur vitesse de pousse. Nous porterons une attention particulière à cette matrice dans les travaux présentés ci-après.

Cette deuxième partie sera donc consacrée à l'étude des tendances de consommation des NPS chez des usagers à haut risque en Ile-de-France. Afin d'évaluer l'état de l'art sur le sujet, nous avons réalisé une revue de la littérature scientifique. Notre choix s'est porté sur l'évaluation de la prévalence des cathinones et dérivés, l'une des familles les plus importantes de NPS. Nous nous sommes limitées aux études effectuées par analyse capillaire pour des raisons

d'homogénéité avec nos travaux entrepris dans cette partie (***publication n° 5***). Cette introduction laissera place à la première étude observationnelle de prévalence des NPS et des drogues classiques en Ile de France entre 2012 et 2017. Les populations visées par cette étude sont principalement les usagers à risque de surdosage admis dans les services de réanimation et les pharmacodépendants (***publication n°6***).

Le deuxième travail correspond à une sous-étude de l'essais clinique « Ipergay » initié par l'Agence Nationale de Recherche contre le SIDA (ANRS). Elle vise à évaluer l'utilisation de NPS et drogues classiques parmi les pratiquants du chemsex (ou HSH) bénéficiant d'un traitement antirétroviral préventif (PrEP), en comparant les résultats issus de l'analyse capillaire à l'auto-questionnaire déclaratif (***publication n°7***).

Deux exemples issus d'une description de cas clinique et médicolégal (***publications n°8 et n°9***) illustreront les observations faites au cours de ces études de prévalence.

Publication n° 5

Prevalence and Surveillance of Synthetic Cathinones Use by Hair Analysis: An Update Review

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REVIEW ARTICLE

Prevalence and Surveillance of Synthetic Cathinones Use by Hair Analysis: An Update Review

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Abstract: New psychoactive substances (NPS) have emerged in a threatening way in the last decades. They are sold via the internet or head shops with several names (bath salts, Research chemical, RCs, Legal Highs) and forms (pills, tablets, powder...etc.), and are labelled ambiguously to escape governmental legislation. Designer drugs belong to different chemical classes, but cathinones derivatives presented the most prevalent group. In 2013, this group accounted for 30% of NPS seizures in Europe with more than 450 different compounds, including 101 new molecules reported for the first time in 2014. The increased number of NPS as being sold in parallel market has led several countries to adopt different strategies either on individual surveillance of new emerging drugs or more efficiently on generic control regrouping a wide number of isomers and structurally similar compounds. The identification of these substances is a challenge for toxicologists, which requires sensitive and specific analytical methods based on LC-MS/MS or GC-MS. The usefulness of hair as an alternative matrix for prevalence studies was proved since it offers an overview on drug exposure with a large detection window over weeks or even months and years according to the length of the hair strand. However, as for many drugs of abuse, prevalence studies on cathinones derivatives use are still scarce. Self-reported use or case reports provide most of the available data.

The aim of this paper is to provide an update review on prevalence and surveillance of synthetic cathinones use conducted by hair analysis, excluding case report.

Keywords: New psychoactive substances, designer drugs, bath salts, hair, alternative biological matrix, prevalence, mass spectrometry, synthetic cathinones.

1. BACKGROUND

New psychoactive substances (NPS) derived from conventional drugs of abuse, like amphetamine or 3, 4-methylenedioxymethamphetamine (MDMA) to mimic their effects. They are sold via internet or head shops with several names like "bath salts" or "Research chemical, RCs" labelled ambiguously "not for human consumption" to escape governmental legislation. Since the 2000th, their emergence presented a public health concern and both governmental and non-governmental institutions raised their control to prevent their relevant toxicities [1]. NPS belong to different chemical classes including phenetylamines and cathinones, piperazines and piperidines, tryptamines, arylalkylamines, synthetic cannabinoids…etc. [2, 3] They appeared onto the market by a chemical modification of controlled drugs to make new substances that were not prohibited. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported 46730 seizures of NPS amounting to more than 3.1 tons in 2013 which represent a seven fold increase in reported seizures between 2008 and 2013 [1, 4]. This increased number led several countries to adopt a new strategy based not only on placing every new designer drug under control but on generic control regrouping a wide number of new emerging isomers and structurally similar molecules [5]. Synthetic cathinones, a beta-ketone amphetamine-like derivatives represent the most prevalent group of new psychoactive substances (Table **1**) [4, 6]. They were found naturally in leaves of Catha edulis plant (Khat) grown in the Horn of Africa and the Arabian peninsula where fresh leaves were chewed for their stimulant effect [7]. Cathine or katin was the first alkaloid identified from Khat by Fluckinger and Gerock in the late nineteenth century followed by cathinone a few years later [2, 8]. The first cathinones synthesized by the chemical process were ephedron (methcathinone) in 1928 and mephedrone (4-methylmethcathinone) in 1929 [2]. The structural similarities of these compounds with amphetamine initially led to a great interest for their medical use. They act as amphetamine-like central nervous system stimulant by increasing synaptic concentrations of biogenic amines (dopamine, noradrenaline, and serotonin) via two mechanisms: *1)* inhibition of monoamine uptake transporters and *2)* release of the neurotransmitters from intracellular stores. However, regarding serotonin system, there have been some controversial data about their potency [9-13]. Thus, ephedron was marketed as an antidepressant in the Soviet Union (USSR) and later in the 1980s bupropion, another cathinone-like compound, was used for the same pathology.

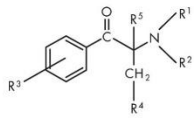
Nevertheless, only a limited number of cathinones were evaluated for their therapeutic potential in an isolated part of the world and no subsequent evidence have resulted [14] leading to their disappearance from medical practice.

These molecules reappeared in the 2000th in the market through the internet or head shops under imaginative names and different forms [15]. They were as important as conventional drugs because of their similar effects, their low cost, and their legal status. Moreover, the purity of the MDMA and cocaine powders was decreasing in that period leading to an enhancement of their use [16, 17]. Misuse of NPS was not a new phenomenon since it was first observed with methcathinone in the 1970s [2]. This molecule was consequently listed to schedule I of the United Nations (UN) convention on Psychotropic Substances that already included cathine and cathinone. In 2013, synthetic cathinones group accounted for 30%

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Table 1. General structure of a cathinone derivative (from: (http://www.emcdda.europa.eu/publications/drug-profiles/synthetic-cathinones.Cited 2017 Jan 27).



R^1	R^2	R^3	R^4	R^5	Name
H	H	H	H	H	Cathinone
Methyl	H	H	H	H	Methcathinone (ephedrone)
Methyl	Methyl	H	H	H	N,N-Dimethylcathinone (metamfepramone)
Ethyl	H	H	H	H	N-Ethylcathinone (EC)
Methyl	H	H	Methyl	H	Buphedrone
Ethyl	H	4-Methyl	H	H	4-Methyl-N-ethylcathinone
Methyl	H	4-Methyl	H	H	Mephedrone (4-MMC; M-CAT)
Ethyl	Ethyl	H	H	H	Amfepramone
t-Butyl	H	3-Cl	H	H	Bupropion
Methyl	H	3,4-Methylenedioxy	H	H	Methylone (βk-MDMA)
Ethyl	H	3,4-Methylenedioxy	H	H	Ethylone (βk-MDEA)
Methyl	H	4-Methyl	Methyl	H	Butylone (βk-MBDB)
Methyl	H	4-Methoxy	H	H	Methedrone (βk-PMMA)
Methyl	H	4-F	H	H	Flephedrone (4-FMC)
Methyl	H	3-F	H	H	3-Fluoromethcathinone (3-FMC)
{pyrrolidino}		H	H	H	α-Pyrrolidinopropiophenone (PPP)
{pyrrolidino}		4-Methyl	H	H	4-Methyl-α-pyrrolidinopropiophenone (MPPP)
{pyrrolidino}		4-MeO	H	H	4-methoxy-α-pyrrolidinopropiophenone (MOPPP)
{pyrrolidino}		4-Methyl	Propyl	H	4-Methyl-α-pyrrolidino-hexanophenone (MPHP)
{pyrrolidino}		4-Methyl	Ethyl	H	Pyrovalerone
{pyrrolidino}		4-Methyl	Methyl	H	4-Methyl-α-pyrrolidino-butyrophenone (MPBP)
{pyrrolidino}		4-Methyl	H	Methyl	4-Methyl-α-pyrrolidino-α-methylpropiophenone
{pyrrolidino}		3,4-Methylenedioxy	H	H	3,4-Methylenedioxy-α-pyrrolidinopropiophenone (MDPPP)
{pyrrolidino}		3,4-Methylenedioxy	Ethyl	H	3,4-Methylenedioxypyrovalerone (MDPV)

of NPS seizures (1.1 tons) with more than 450 NPS including 101 new molecules reported for the first time in 2014 in Europe [4]. Synthetic cathinones are consumed through different administration routes including insufflation (snorting), oral intake and swallow of powder wrapped in cigarette paper (bombing). Intravenous, subcutaneous, intramuscular, and rarely rectal or eye insertions (eyeballing) were also reported [18, 19]. A subclass of cathinones with pyrrolidinophenone structure has appeared in the late 1990s [20]. Methylenedioxypyrovalerone (MDPV) and α-pyrrolidinovalerophenone (α-PVP) are the most identified molecules of this group [21, 22].

Another group of new designer drugs is represented by piperazines with benzyl- and phenylpiperazine subgroups. 1-benzylpiperazine (BZP), 1-(3-chlorophenyl) piperazine (m-CPP)

and 1-(3-trifluoromethylphenyl) piperazine (TFMPP) were the most frequent and well- known compounds [23-26].

Many issues with NPS identification were frequently encountered in toxicological laboratories. One can underline the fact that these substances do not react with common immunoassay used for conventional drugs screening. The switch to a more efficient method such as gas or liquid chromatography coupled with mass spectrometry detection (GC/MS and LC-MS/MS, respectively) often requires reference standards (RS) which are very expensive and difficult to obtain. Consequently, underestimated number of reported cases can be assumed [24]. Furthermore, as the main compounds found in conventional matrices like urine were primary and secondary metabolites which were often unavailable as RS, and the lack of well-described transformation patterns have led to limited use of this matrix [6].

As an alternative, hair testing has been proposed to overcome all the issues related to NPS identification in conventional matrices (blood and urine), like a short detection time window or the presence of metabolites. Hair is a valuable matrix since it allows the study of drug exposure over several weeks or even months or years according to the length of the analyzed hair strand [3, 27, 28].

Moreover, in hair unlike in urine the parent drug which is a more lipophilic molecule than the metabolites usually represents the main incorporated analyte leading to an easier identification [6].

The diversity of new psychoactive substances that appeared onto the market in the last few years and their spectacular widespread had prompted the development of many sensitive and reliable analytical method for their identification and quantitation in different matrices, especially in hair (29-42). These different methods when applied to real hair samples allow determining the prevalence or at least a surveillance of the synthetic cathinones use worldwide.

2. PREVALENCE OF NPS IN HAIR: REVIEW OF LITERATURE

As for many drugs of abuse, prevalence studies on NPS use are scarce. Self-reported use in user surveys and case reports provide most of the available data (12, 15, 22, 23, 28, 39, 40, 43-45). Nevertheless, some studies on the prevalence of synthetic cathinones evaluated by hair analysis are available. The list of the compounds screened in each study is given in Table **2**. Table **3** summarizes all the reported studies. These studies are conducted worldwide:

2.1. Major Studies

2.1.1. In Switzerland

The usefulness of hair analysis for retrospective prevalence studies was studied by Rust *et al.* (24). To check for NPS presence, authors re-analyzed all hair samples from 2009 to 2010 that originally tested positive for amphetamines and/or MDMA (n=325) in driving-ability assessment using a qualitative LC-MS/MS method. Active users of amphetamine and/or MDMA are included, as well as those who stopped consuming and those who might have switched to something else. In total, 293 men (90.1%) and 26 women (8%) were included. the sex of participants was unknown in six cases. The age distribution starts at 18 years old (minimum age for a driver license in Switzerland) to up to 55 years-old but the highest number of cases was in the group of the 25-30 years-olds. After a three-step washing procedure with water (2min, 15mL), acetone (2min, 10mL) and hexane (2min, 10mL), a weighted hair

Table 2. NPS screened in each study.

Authors	Countries	Synthetic cathinones screened	Method	Refs.
Rust *et al.*	Switzerland	Mephedrone, methylone, butylone, ethylone, MDPV, cathinone, methcathinone, 4-fluoroamphetamine (4-FA), BZP, mCPP, TFMPP, methylphenidate, ketamine	LC-MS/MS	[24]
Salomone *et al.*	Italy	Methylone, ethcathinone, 4-FA, buphedrone, PMA, amfepramone, PMMA, butylone, mephedrone, ketamine, 6-APB, 4-MEC, pentedrone, 5-MAPB, MXE, mCPP, α-PVP, MDPV, 2C-B, bupropione, trazodone, PCP, 4-MeO-PCP, diphenidine, 25H-NBOMe, 2CP, 25C-NBOMe, 25I-NBOMe	LC-MS/MS	[6]
Palamar *et al.* Salomone *et al.*	USA	Mephedrone, 3-methylmethcathinone, 4-methylethcathinone, methylone, 4-fluoroamphetamine (4-FA), 3,4 methylenedioxypyrovalerone (MDPV), pentedrone, ethcathinone, alpha-pyrrolidinovalerophenone (α-PVP; a.k.a.: "Flakka"), butylone, buphedrone, 25I-NBOMe, 25 C-NBOMe, 25 H-NBOMe, 25B-NBOMe, 2 C-P, 2 C-B, 1-(benzofuran-5-yl)-N-methylpropan-2-amine (5-MAPB), 5-(2-aminopropyl) benzofuran (5-APB),6-(2-aminopropyl)benzofuran (6-APB), para-methoxymethamphetamine, para-methoxyamphetamine, amfepramone, meta-chlorophenylpiperazine (mCPP), and bupropione, plus five dissociative drugs, namely methoxetamine (MXE), phencyclidine (PCP), 4-methoxyphencyclidine (4-MeO-PCP), diphenidine and ketamine.	LC-MS/MS	[50,51]
Aldamowicz *et al.*	Poland	143 NPS (see reference)	LC-MS/MS	[5]
Lendorio *et al.*	Spain	Methylone, mephedrone, methedrone, MDPV, FMC, fluoromethamphetamine, mCPP, TFMPP	LC-MS/MS	[37]
Martin *et al.*	France	Mephedrone	GC-MS	[43]
Kim *et al.*	Korea	Amphetamine, phentermine, methamphamine, cathinone, cathine, methcathinone, fenfluramine, desmethylselegiline, MDA, MDMA, MDEA, norketamine, mescaline, 2-CB.	GC-MS	[42]
Barroso *et al.*	Portugal	TFMPP, mCPP and MeOPP	GC-MS	[25]

Table 3. Summarize of the prevalence studies on synthetic cathinones identified by hair testing.

Countries	Number of hair sample (N)	Population Characteristics					NPS Positive cases (N)	Number of identified NPS (N)	Top 3 of the most identified NPS	Prevalence (%)	Refs.
		Sex (%)			Age range, mean (Y)	Context /circumstances					
		M	F	UK							
Switzerland	325	90.1	8	1.9	[18- up to 55]	Amphetamines users	120	7	Ketamine mCPP 4-fluoroamphetamine	37	[24]
Italy	77	>70	NS	NS	[26-56] m = 36.3	MDMA and KET abusers (A) or negative tested subjects (B)	6 (4M + 2F)	8	MXE Mephedrone Methylone	21.7 (A) 1.8 (B) 7.8 (A)+(B)	[6]
USA	80	NS	NS	NS	[18-25]	Nightclubs attendees	26 (14M+12F)	6	Butylone Methylone MXE	32.5	[50,51]
Poland	NS	91	9	-	[16-50] m = 24.5	DUID, road accidents, drugs possession or use, violence, theft, rape, kidnapping, suicide, intoxications	112	21	3-MMC α-PVP Pentedrone	10.6	[5]
Spain	16	NS	NS	NS	NS	Amphetamines Positive specimens, Trazodone treatment	10	1	mCPP	------	[37]
France	67	NS	NS	NS		Suspected mephedrone abusers	13	1	Mephedrone	19.4	[43]
Korea	141	NS	NS	NS	NS	Suspected drug abusers	0	0	-------	--------	[42]
Portugal	NS	NS	NS	NS	NS	Drug addicts (post-mortem), psychiatric evaluation, trazodone treatment	NS	NS	NS	NS	[25]

Y:years; UK : unknown ; NS : not specified ; M : male; F: female; m :mean; DUID : driving under the influence of drug.

sample of 20-30 mg was cut into small snippets and extracted in two steps with methanol (5mL, 16h, ultrasonication) and methanol acidified with 50µL hydrochloric acid 33% (3mL, 3h, ultrasonication). After evaporation, the residue was reconstituted with 50µL of methanol and 500µL of ammonium formate 0.2mM in water. Deuterated analogs were used as internal standards. The analytes were separated on a Phenomenex Kinetex PFP, 2.6µm column using a gradient elution of 5mM ammonium formate buffer (pH 3.5) with formic acid (eluent A) and methanol containing 5mM ammonium formate (eluent B). The column was set at 40°C. Detection used a Q trap linear ion trap quadrupole mass spectrometer in MRM mode followed by an enhanced product ion scan as dependent scan.

The limits of detection (LOD) were 10 pg/mg for 4-FA, BZP, Ketamine, mCPP, 4-MMC, butylone, ethylone, MDPV, methcathinone, methylone, methylphenidate, and TFMPP. For cathinone, the LOD was 50 pg/mg.

To check the stability of the samples during the storage in the freezer, some positive samples were re-reanalyzed after 11 months. The decrease observed in area ratio of the analytes and internal standard (MDMA-d5) were acceptable.

Seven different NPS were found in 120 cases, corresponding to an NPS prevalence of 37% in amphtamine-user population. Concerning the piperazines drugs, mCPP could be detected in 34 cases (10.5%) and TFMPP in one case. In 11 cases (3%), 4-MMC (mephedrone) was identified. Concerning smart drugs, methylphenidate was found in 16 cases (5%) but in over a half of these cases (54%), a prescription had been issued. In 45 cases (14%) ketamine could be detected. The association of cocaine and ketamine (so called "CK") was found in 89% of ketamine positive cases. 4-FA was identified in 12 cases (4%) and methylone in one case. The antidepressant trazodone was included in order to distinguish a direct mCPP intake from a trazodone treatment. Indeed, five mCPP positive cases were also positive to trazodone.

Concerning mephedrone positive cases, the author compared their findings with Martin *et al.* and Shah *et al.* [40] studies where the presence of mephedrone in hair was investigated. Martin *et al.* performed a GC-MS method for the determination of mephedrone in 67 hair samples from subjects with presumed mephedrone consumption. Thirteen samples were tested positive for mephedrone (19.4%). Unlike the LC-MS/MS method, GC-MS allowed chromatographic separation of 4-MMC from its isomer 3-MMC and thus confirmed mephedrone intake. Shah *et al.* followed a second approach to distinguish between these two isomers, based on mephedrone metabolism. They developed and validated a LC-MS/MS method for the quantitative analysis of mephedrone and its two metabolites 4-methylephedrine and 4-methylnorephedrine in human hair. Five out of 154 samples in the United Kingdom (3.2%) were tested positive for mephedrone with only one case suitable for quantification. Therefore, in Rust's study based on a LC-MS/MS method without mephedrone metabolism approach, the presence of 3-MMC could not be distinguished from mephedrone.

2.1.2. In Italy

Salomone *et al.* [6] described a 5.5 min UHPLC-MS/MS method to determine 26 stimulants and psychedelic and 5 dissociative drugs in 77 real hair specimens. The study was carried-out in 2013 and 2014. Samples were taken either from proven MDMA and ketamine abusers (Group A, 23 samples) or from drivers previously tested negative for conventional drugs in license recovery (Group B, 54 samples). All the subjects from the group B were male and young (<25 years) while the group A was mixed without specifying the exact number of male and female.

All samples were re-analyzed by the described LC-MS/MS method to verify the potential presence of NPS, not previously targeted. Only a proximal hair segment 0-6 cm was analyzed. Shorter head hair and pubic, axillary or chest hair were analyzed in their full length.

About 25 mg of hair was twice washed with dichloromethane and methanol (2mL, vortex mixed for 3 min). The hair was dried at room temperature with gentle nitrogen flow then cut into small segments (1-2mm). 0.3 ng/mg of internal standards was added in each sample and then were incubated in 1.5 mL of methanol at 55°C for 15h without stirring. The organic phase was collected and 1 µL was directly injected into the analytical system. If the real sample concentrations exceed the highest calibration point, the final extracts were diluted with methanol and re-injected into the system. Separation was performed in a Zorbax Eclipse Plus C18 column (100mmx.1mm, 1.8µm) set at 45°C and the elution solvents were water/formic acid 5mL (eluent A) and acetonitrile/methanol 80: 20 plus formic acid 5mM (eluent B) in a gradient mode. MS/MS detection used a QTRAP mass spectrometer equipped with an electrospray ion source in MRM mode. For method validation, selectivity, specificity, linearity range, detection and quantification limits (LODs and LOQs), intra-assay and inter-assay precisions and accuracies were investigated. Carryover, matrix effect and recovery were also evaluated. LOD ranged from 0.9 pg/mg to 17 pg/mg, while LOQ was between 1.8 and 35 pg/mg. the upper limit of quantification (highest calibration point) was 1000 pg/mg. The matrix effect appeared to be significant only for ethcathinone (>25%). Modest deviation from the 15% acceptance limit was observed during the evaluation of intra-day precision for trazodone and inter-day accuracy for 4-MEC. All the other parameters were satisfactory according to the international standard validation.

Five samples from group A were found positive for at least one compound, corresponding to a 21.7% of prevalence in MDMA and ketamine users group. MXE was identified in three samples and mephedrone in two samples while other compounds were detected in one sample: 4-MEC, methylone, α-PVP, 4-FA, MDPV and diphenidine. One subject was positive for both 4-MEC and mephedrone while one subject turned out positive for six NPS. In group B, only one subject was found positive for methylone. In summary, eight different NPS have been identified. Four NPS positive subjects were men while two women were detected positive. The age ranged between 26 and 56 years old (mean = 36.3 years, median= 32.5 years). The measured levels for most of the drugs were in the range of picograms of drug per milligram of hair, suggesting sporadic exposure, or low incorporation rate of these drugs in keratin matrix. Authors emphasized on limited literature data concerning the detection of new designer drugs in hair, leading the interpretation of NPS concentration still ambiguous and underlying the issues to differentiate two couples of isomers: mephedrone/3-MMC and 5-APB/6-APB by LC-MS/MS.

This study investigated the diffusion of selected NPS among a special population group, especially in association with MDMA and ketamine. According to the author, mephedrone and methoxetamine, are likely the most common NPS within the Italian territory in the present day. Other sporadic findings (4-MEC, methylone, 4-FA …etc.) indicate that new substances are simultaneously consumed in the local territory. No NBOMe-series compounds were found through this analysis, possibly because of the delayed spread of these recent drugs among Italians or more likely because these substances are active at very low doses, reducing the detectable levels in hair, especially in case of single or episodic exposure.

2.1.3. In USA

Although usage data were limited on self-reported surveys and cases reports [46-49], NPS gained prevalence within the US. In recent papers, Palamar *et al.* [50, 51] have studied the prevalence of NPS in high-risk population using hair testing. Six hundred seventy nine nightclub and festival attendees were surveyed from July through September of 2015. Participants were surveyed outside of 10 different venues (including two dance festivals) over 21 days. They were eligible if they identified as age 18-25 years and accepted to participate to the randomly selected study. Of the 679 participants surveyed, 80 (11.8%) accepted to provide a sufficient hair sample to undergo the analysis. Hair samples were analyzed in their full length with an average length of 9.9cm (median 9.0cm). Thirty six samples were head hair, 3 were leg hair and in 18 cases, the sampling site was not recorded. A minimum of 20 mg was needed to perform the analysis. Among positive specimens, 40.4% (n = 23) were provided by female participants. Donors mainly identified as White (n = 35, 61.4%), while other ethnic groups included Hispanic (n = 12, 21.0%), Asian (n = 4, 7.0%) or other/mixed (n = 6, 10.6%).

The collected specimens were tested using three previously published and validated methods based on UHPLC-MS/MS. One was used to screen each sample for 11 common drugs of abuse or metabolites [52]. The second method was addressed to detect 26 NPS, primarily synthetic cathinones and psychedelic substituted phenethylamines. Salomone *et al.* used the same method for the Italian prevalence study. The last one was used to screen for synthetic cannabinoids [3]. Altogether, each hair sample was screened for 82 drugs and metabolites. No sample tested positive for synthetic cannabinoids. Among positive samples (n = 57), 31 tested positive exclusively for at least one traditional drug.

Twenty six samples tested positive for at least one NPS. Two cases were positive for NPS only, while in the remaining 24 cases, one or more traditional drugs were associated to NPS. Among all NPS positive cases, nine samples were positive for two or more NPS. Positive specimens were almost equally distributed between males (n = 14; 53.8%) and females (n = 12; 46.2%). The most common NPS detected was butylone, which was found in 25 samples, often at high concentrations (range <7–4,900 pg/mg, mean = 440 pg/mg, median = 21 pg/mg). Other multiple detection included methylone (five cases; range <6–98 pg/mg), MXE (four cases; range 3–19 pg/mg), 5/6-APB (one case; 82 pg/mg), α-PVP (one case; 6 pg/mg) and 4-FA (one case; 29 pg/mg). Eight samples

tested positive for five or more drugs. Traditional drugs detected in association with NPS were MDMA (17 cases), THC (15 cases), cocaine (15 cases), AMP (8 cases), ketamine (6 cases) and methamphetamine (1 case). While butylone was detected in a wide range of concentrations, with nine samples above 100 pg/mg, the measured levels for the remaining NPS were interestingly below 100 pg of drug per milligrams of hair, suggesting either sporadic exposure to these substances, or low rate of incorporation into the keratin matrix.

This study shows that NPS were detected in a large number in a high-risk population of nightclubs and dance festival attendees in New York City. Methylone, butylone and MXE were detected exclusively or more often concurrently in 25 out of 26 cases which tested positive for at least one NPS. In a previous study, the authors showed that many nightclubs and dance festival attendees reporting MDMA use tested positive for methylone and/or butylone, with 4 out of 10 self-reported MDMA/ecstasy/Molly users testing positive for these drugs after reporting no use of synthetic cathinones or unknown pills or powders [50]. NPS are often identified at festivals as being sold in place of traditional drugs primarily ecstasy/Molly. Therefore, it is not unlikely that most of positive findings would be unexpected by the users and can be attributed to the unaware intake of NPS.

It should be noted that prevalence of synthetic cathinones use is constantly shifting in the USA. In 2009, five synthetic cathinones were reported to the National Forensic Laboratory Information System (NFLIS). By comparison, in 2015, 35 different synthetic cathinones were reported to NFLIS. Methylone, alpha-PVP, and ethylone accounted for 91% of the 20 most frequently identified synthetic cathinones reported from January 2013 through December 2015. Methylone decreased from 2013 to 2015 in all regions, and ethylone increased during the same period in all regions [50, 53].

2.1.4. In Poland

Adamowicz *et al.* [5] have studied the prevalence of NPS in biological material including blood, urine, vitreous humour, body tissues and hair samples. The study was carried out from 2012 to 2014 and most of the relevant analyzed cases concerned driving under the influence of drugs (DUID), road accidents, drugs possession or use, violence, theft, rape, kidnapping, suicide, as well as intoxications. One thousand fifty eight samples, mainly blood, were analysed for the presence of NPS. Unfortunately, no details were provided on the real number of hair specimens analyzed. The majority of NPS users were men (91%), women, accounting for only 9%. Users ranged in age from 16-50 years-old with an average of 25.4 years and a median of 24.5 years.

The analysis was carried-out by LC-MS/MS but no pretreatment procedure for hair samples was provided. The author precise that analytes were isolated from the matrix either by precipitation with acetonitrile (for blood) or by extraction with n-butyl chloride for others specimens. Separation was performed on a Kinetex C18 (100x4.6mm, 2.6µm), 100A° column and Zorbax SB-C18 (2.1x50mm, 1.8µm) column, based on previously published method [54].

One hundred and twelve cases were positives for NPS along the 3-years corresponding to an overall prevalence of 10.6%, 75 being detected the last year (2014). The prevalence of all NPS in 2014 (17.6%) was similar to that of amphetamine the same year (16.2%). the drugs found belonged to following classes: cathinones (88%), synthetic cannabinoids (5%), phenethylamines (3%), piperazines and piperidines (3%) and arylalkylamine (1%). The most common detected compounds (number of cases in brackets) were: 3-MMC (n=50), α-PVP (n=23), pentedrone (n=16), MDPBP (n=12), UR-144(n=7), ethcathinone (n=5), mephedrone (n=5), MDPV (n=4), 4-MEC (n=3), buphedrone (n=3), 2-DPMP (n=3), methylone (n=2) and 2C-B (n=2). In single cases, 2-MMC, 2C-P, eutylone, 25I-NBOMe, mCPP, ephedrone, MPA and 5-APB were found. 3-MMC was first seen in 2013 (11 cases; 2.9% of the 2013 samples) and its frequency increased considerably in the next year (39 cases; 9.1% of the 2014 samples). α-PVP was first seen in 2014 while pentedrone, MDPBP and UR-144 were observed throughout the three-years period. The authors explained the increased trend in 3-MMC by the fact that this compound has quickly replaced mephedrone following the report of several fatal intoxications since 2010. They argued that this pattern is characteristic of the countries such as Poland in which the law is based on individual lists of controlled substances, unlike in countries that applied a generic legislation where new isomers were automatically controlled (e.g. United Kingdom), and in which mephedrone remains popular. These results were slightly in contrast of what had been reported by the Chief Sanitary Inspector in Poland, where UR-144 and pentedrone were the most identified NPS in Legal Highs preparations through the three year-period (2012-2014) and that 3-MMC could not be identified in a considerable way until 2014. One NPS was the only compound detected in 35% of all cases, and two or more NPS were present in 19% of cases. NPS (one or more) were identified in 65% of the cases with other conventional drugs (like amphetamines, cannabinoids, cocaine and benzodiazepines), demonstrating a polyconsumption pattern. Only five of the 21 NPS detected were controlled in Poland at the time of this study: 2C-B since 2005, mephedrone since 2010, MDPV, 4-MEC and methylone since 2011. Other NPS (except 2-MMC) are controlled since 2015.

The author concluded that the popularity of various NPS is changing over time and that the most commonly detected in Poland are cathinones 3-MMC, α-PVP, pentedrone and MDPBP, and synthetic cannabinoid UR-144. These five compounds constitute over 75 % of all identifications. He emphasized that the analysis of these compounds should be a part of the routine course of examination.

2.2. Other studies

2.2.1. In Spain

Lendoiro *et al.* [37] developed and validated a LC-MS/MS method for amphetamine-type stimulant analysis in hair including selected synthetic cathinones and piperazines. Sixteen hair samples were collected, seven specimens with a previously positive result for classic amphetamine derivatives (amphetamine, n=7; methamphetamine, n=3; MDA, n=2; MDMA, n=7) and 9 hair specimens from patients under daily trazodone treatment. No details were provided about the demographic characteristics of the subjects included in this study. Each hair sample was washed with 2 mL of dichloromethane three times during 2 min. After that, samples were completely dried in a heater at 40°C. Thirty mg of hair were powdered, and then incubated with 2 mL 0.1% HCl in methanol for 1 h at 60°C. Sample was centrifuged (4000 rpm, 10 min) and the supernatant was evaporated to dryness under nitrogen at 35°C. The residue was reconstituted in 2 mL of 2% formic acid (FA) in water. Extraction was performed using solid phase extraction Strata X-C cartridges eluted with 2 mL of dichloromethane, propanol and ammonium hydroxide. Gradient elution was performed using an Atlantis T3 (2.1 mm x 50 mm, 3 µm) column set at 35°C. Ammonium formate 2mM with 0.1% FA (pH=3) (A) and acetonitrile (B) were used as mobile phase. Detection was operated by a triple quadrupole in a MRM mode. The method was validated for selectivity (no interferences), linearity (2-20 to 2000-4000 pg/mg), limits of detection (0.2 to 5 pg/mg) and quantification (2 to 20 pg/mg), accuracy (93.4-109.4% of target concentration), imprecision (CV<11.6%), extraction recovery (40.5-92.1%), matrix effect (24.1-227.2%), process efficiency (9.8-165.7%) and stability in the autosampler (-14.5% of loss).

Ten hair samples were positive for mCPP with a concentration between 341 and 4000 pg/mg. However, in all cases, trazodone was also identified showing a licit origin of mCPP.

2.2.2. In France

Martin *et al.* [43] developed a specific and accurate method for mephedrone analysis and applied it to 67 hair samples originated from persons with a suspected history of mephedrone abuse. Hair strand was decontaminated with methylene chloride and cut into small pieces. Fifty mg were incubated overnight in a buffer pH 7.0 at 40 °C. Deuterated MDMA was used as internal standard. The extraction was performed by using ethyl acetate after alkalinisation with sodium hydroxide (NaOH, 1 N). The dry extract was derivatized using a mixture of heptafluorobutyric anhydride/ethyl acetate (100: 50, v/v), evaporated and dissolved in ethyl acetate (25 ml). Injection was set in splitless mode, and the chromatographic separation was achieved on a 5-MS capillary column. Detection was done in single ion monitoring mode. The linearity ranges from 0.2 to 50 ng/mg, with a good correlation coefficient. The LOD and LOQ were 0.08 and 0.2 ng/mg respectively. The intra-day and inter-day precision (CV) were less than 4%, and the accuracy (bias) was less than 15%. The extraction recovery was greater than 76% and no interferences, or carryover were observed.

Thirteen samples were tested positive to mephedrone (19.4%), with concentrations ranging from 0.2 to 313.2 ng/mg (mean = 26.8 ng/mg). These high levels show the good integration of mephedrone into keratin and are comparable to those of the other stimulants, at least in case of repeated abuse.

2.2.3. In Korea

A GC-MS method was developed and validated by Kim *et al.* [42] for the determination of 13 psychotropic phenyl alkylamine derivatives in human hair including cathinone (Khat) and methcathinone. All these drugs except selegiline are regulated under the Controlled Substances Act in Korea. One hundred and forty one hair samples received from suspected illicit drug abusers between October and November 2006 from the Narcotics Department at the Seoul District Prosecutors' Office were analyzed. Demographic characteristics of the participants, primarily sex and age distributions were not provided.

Twenty mg of each sample were washed with distilled water and acetone and cut into small fragments. They were incubated in 0.25 M methanolic HCl solution under ultrasonication at 50 °C for 1 h. The resulting solutions were evaporated to dryness, derivatized using trifluoroacetic anhydride (TFAA) at 70 °C for 30 min, and analyzed by GC/MS. Separation was achieved on a capillary column (DB-5MS, 30m, 0.25mm i.d., 0.25mm) with a defined temperature program and splitless injection mode. Detection and quantification were set in selected ion monitoring mode (SIM). The linear ranges were 0.02-25.0 ng/mg for amphetamine, phentermine, cathinone, fenfluramine, desmethylselegiline, MDMA, and 2C-B; 0.05-25.0 ng/mg for methamphetamine, methcathinone, and mescaline; 0.05-12.5 ng/mg for MDA; and 0.1-25.0 ng/mg for MDEA and norketamine, with good correlation coefficients ($r^{(2)} > 0.9985$). The intra-day, inter-day, and inter-person precisions were within 12.7%, 14.8%, and 16.8%, respectively. The intra-day, inter-day, and inter-person accuracies were between -10.7 and 13.4%, -12.7 and 11.6%, and -15.3 and 11.9%, respectively. The recoveries were in the range of 76.7-95.6%. The limits of quantifications (LOQs) for cathinone and methcathinone were 0.02 and 0.04 ng/mg respectively and below 0.08 ng/mg for all the other compounds. Sixty seven samples turned out positive for amphetamine, 79 for methamphetamine, two for phentermine, two for MDMA, only one for MDA and one for norketamine. Cathinone and methcathinone were not detected in any sample. This study was probably conducted too early to identify NPS, and showed that in 2006, NPS were not still very widespread, at least in Korea.

2.2.4. In Portugal

Barroso *et al.* [25] have developed and validated a GC-MS method for the determination of TFMPP, mCPP and MeOPP in human hair. Authentic samples belonging to drug addicts were collected at the beginning of autopsies performed at the National Institute of Legal Medicine, Lisbon, Portugal. Hair samples were also collected from living subjects under psychiatric evaluation for child custody issues at the same institution, and from persons undergoing treatment with trazodone. Patient's age and sex have not been described. Hair was washed sequentially with dichloromethane, deionized water, and methanol. The last wash was stored for further analysis. Twenty mg of hair cut into small pieces were extracted by incubation in sodium hydroxide 1M at 50 °C for 40 min. The extracts were cleaned up using mixed-mode solid-phase extraction. The analytes were derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide with 5% trimethylchlorosilane and analyzed by GC-MS in SIM mode. The method was linear from 0.05 (lower limit of quantitation LLOQ) to 4 ng/mg, with correlation coefficients higher than 0.99 for all the compounds. Precision and accuracy were within the limits criteria (CV and bias <15%, <20% for LLOQ) for all compounds. Surprisingly, no results from the real hair have been reported in this study.

DISCUSSION

It appears from all these different studies that:

- the interest of hair as an alternative matrix in this kind of studies is confirmed, because unlike conventional matrices (blood, urine), hair offers a wide detection window which can range from a few weeks to several month, and therefore, allows the evaluation of occasional or repeated exposure to one or more NPS;
- the development of new analytical methods such as chromatography coupled to mass spectrometry, which are more sensitive and specific than other detection, has made it possible to monitor the emergence of these new compounds. Nevertheless, this development should progress to follow the exponential evolution of the NPS market. High-resolution mass spectrometry is probably a good solution in order to identify new NPS still unknown in the laboratory libraries;
- NPS consumption phenomenon is observed among particular target group, especially young people and within a high-risk population like MDMA abusers, clubbers and dance party attendees;
- the identified molecules are different from one country to another (with a few exceptions), mainly related to the specificity of the legislation in terms of narcotic regulation in each country. We note that most of these compounds are still not scheduled in the United Nations (UN) convention on Psychotropic Substances
- these studies target different populations, with different sociodemographic characteristics (age, sex, circumstances…etc.), and are therefore difficult to compare. Moreover, they do not allow the real evaluation of prevalence in general population.

CONCLUSION

More prevalence studies, especially using hair analysis are needed to monitor the evolution of the NPS phenomenon, which is a real public health concern nowadays.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Publication n° 6

Prevalence of New Psychoactive Substances(NPS) and conventional drugs of abuse (DOA) in high risk populations from Paris(France) and its suburbs A cross sectional study by hair testing(2012–2017)

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Prevalence of New Psychoactive Substances (NPS) and conventional drugs of abuse (DOA) in high risk populations from Paris (France) and its suburbs A cross sectional study by hair testing (2012–2017)

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ABSTRACT

Background: The aim of the present study is to describe the prevalence of NPS and conventional DOA in Paris and its suburbs over a six-year period using hair testing approach.
Method: Hair was sampled in patients admitted to different departments of Paris hospitals between 2012 and 2017. Two high-risk populations were mainly considered: *1)* drug-dependent and *2)* acutely intoxicated patients. Segmental hair analysis was performed by validated LC–MS/MS method to screen for DOA and 83 NPS.
Results: 480 patients (280 M/200 F, 15–70 years) were included. 141 patients tested positive for NPS (99 M/42 F; median age: 33). NPS prevalence was 29%, that of amphetamines, cocaine and opioids were 32%, 38.5% and 52%, respectively. 27 NPS were identified, 4-MEC and mephedrone (number of cases n = 24 each) were the most detected cathinones. JWH-122 (n = 1) was the only detected synthetic cannabinoid while ketamine (n = 104) was present in numerous NPS users (67%). 3-fluorofentanyl (n = 1), furanylfentanyl (n = 1), N-ethylpentylone (n = 2), pentedrone (n = 2), mexedrone (n = 1), methcathinone (n = 3), 6-APDB (n = 2), TFMPP (n = 2), 2-CE (n = 1), 3,4-MD-αPHP (n = 1) and dextromethorphan (n = 27) were identified for the first time in hair. Users were found to have more than one NPS in 53% of cases, mostly in combination with conventional DOA. The number of detected NPS rose from 5 in 2012 to 42 in 2017. A broad range of hair concentrations (0.001–318 ng/mg) was found, but the low median concentrations seem to show an occasional exposure more than chronic use.
Conclusion: NPS screening should be assessed in routine clinical practice, especially in high-risk populations.

1. Introduction

New psychoactive substances (NPS) are derived from regulated drugs of abuse (DOA) such as amphetamines, cocaine, ketamine or fentanyl after slight chemical alteration, and are ambiguously labeled as bath salts, research chemical (RCs) or legal highs to circumvent the legislation surrounding narcotics. They are increasingly available over the internet and widespread use is of concern. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (2019) is actually monitoring more than 670 NPS with about one additional NPS being reported every week in Europe, illustrating how complex this market has become (European Monitoring Centre for Drugs and Drug Addiction [EMCDDA], 2018). NPS include several groups of drugs belonging to 4 major classes: synthetic cannabinoids (SC), synthetic cathinones and other synthetic stimulants, new synthetic opioids (NSO), and synthetic benzodiazepines. The increased number of recorded NPS has led several countries to adopt different strategies either by targeting the producers and retailers, or by applying generic bans to a wide number of structurally similar compounds. Part of the problem in controlling the proliferation of these substances lies in their variety, their ease of synthesis and purchase, low cost, and the fact that most are undetectable by standard toxicology screening (Khaled et al., 2016). Cases of severe toxicity and deaths caused by NPS are increasingly reported in the scientific literature (Gaillard et al., 2013; Gerace et al.,

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2014; Poklis et al., 2016; Papaseit et al., 2017; Rojek et al., 2014; Wikström et al., 2010; Wyman et al., 2013) but we note that self-reported use or case reports provide most of the current data (Bretteville-Jensen et al., 2013; Kelly et al., 2013; Kikura-Hanajiri et al., 2013; Simonato et al., 2013; Palamar et al., 2015, 2016).

The identification of NPS in biological or seized materials requires reliable analytical methods based either on gas or liquid chromatography coupled with mass spectrometry detection (GC–MS and LC–MS/MS). This requires expensive reference standards (RS) that are often unavailable and difficult to obtain. Consequently, due to the limited number of NPS screened by forensic laboratories, it is likely that the number of reported cases is underestimated. Recently, a new approach based on liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), with the use of a shared MS spectra database and without a need for RS has emerged (Concheiro et al., 2015; Fabresse et al., 2019; Maurer and Meyer, 2016; Pleil and Isaacs, 2016; Scheidweiler et al., 2015), allowing qualitative determination of these compounds which can be secondarily confirmed and quantified by targeted screening using LC–MS/MS.

The usefulness of hair as a complementary matrix to blood or urine to study the exposure to many substances is well known: most substances are incorporated into hair mainly from the bloodstream, remain stable and can be detected from a few weeks up to months or years depending on hair length. Moreover, hair sampling is non-invasive, easy and does not require any medical training.

In the last decade, different strategies have emerged in order to study the prevalence of DOA. Some are based on wastewater analysis to estimate the consumption of drugs by the measurement of residues in sewage while other approaches evaluate specific population like the analysis of residual content of used syringes in injection drugs users (Karolak et al., 2010; Néfau et al., 2015). Both of these strategies provide an indirect link between drugs consumption and the population of consumers itself.

Besides that, the study of NPS prevalence using biological materials and especially alternative matrices as hair allow direct evaluation of drug exposure of an individual or a population by analyzing their own samples. Despite their great interest, this kind of approaches remain scarce (Adamowicz et al., 2016; Rust et al., 2012; Salomone et al., 2014, 2017).

The aim of this study is to evaluate the prevalence of NPS in high risk populations from Paris and its suburbs over a six-year period using hair testing, and to compare it with that of conventional DOA within the same populations.

2. Material and methods

2.1. Inclusion criteria

Adult patients (age ≥ 15 years) successively admitted from January 2012 through December 2017 to Paris hospitals and its suburbs were included in this study. Four groups were identified according to the department to which they were admitted: addiction departments (AD), emergency departments (ED), forensic departments (FD) and other departments (OD).

No consent from patients was needed as hair testing is part of a routine clinical investigation in France, and was asked by practitioners in order to have more information about patients' use of DOA.

2.2. Analytical method

The analysis was performed by a liquid chromatography tandem mass spectrometry technique (LC–MS/MS) derived from a previously published methods (Alvarez et al., 2016a; Larabi et al., 2018a).

2.2.1. Chemical and reagents

Reference and certified standards were purchased from 3 suppliers: LGC Standards (Molsheim, France), Sigma–Aldrich (Paris, France) and Lipomed AG (Arlesheim, Switzerland) in 1 mg/mL vials or as a powder to be reconstituted with a convenient solvent (e.g. methanol or acetonitrile). Acetonitrile, dichloromethane, hexane, ethyl acetate, formic acid and methanol were supplied by Sigma–Aldrich (Paris, France) in mass spectrometry or high-performance liquid chromatography (HPLC) grade. Sodium carbonate and sodium hydrogenocarbonate were from Prolabo (Paris, France). Ultra-pure water (18 MV) was obtained by ultrafiltration using a Direct-Q UV3 apparatus (Millipore Corp., Molsheim, France). Formate buffer (containing 2 mM ammonium formate in 0.1% formic acid) was prepared in ultra-pure water and stored after each analysis at +4 °C away from light for a maximum of one week.

2.2.2. Calibration standard and quality control

Eight calibration standards (CS) containing a mixture of all screened compounds at 1, 2.5, 5, 10, 50, 100, 500, and 1000 pg/mg of hair were prepared by spiking stock solutions of reference standards with appropriate volume into a 20-mg blank human hair. Quality controls (QC) were prepared by the same way at concentrations of 5, 20, 250 and 750 pg/mg.

Blank human hair was obtained from children who are not medically treated, after having requested a signature of the consent to their parents, and were stored at room temperature away from light until the analysis.

2.2.3. Hair sample preparation

Hair was decontaminated twice using dichloromethane and washed once with warm water (immersion for 2 min in each step). It was then grinded into a fine and homogeneous powder using a ball mill (MM200, FicherScientific, Illkrich, France). Each 20 mg were incubated in 1 mL of phosphate buffer at pH 5.0 at 95 °C for 10 min, in the presence of an appropriate amount of deuterated internal standards (IS). After spiking the corresponding volume of CS or QC working solutions, liquid–liquid extraction was performed by 4 mL of a mixture of hexane/ethyl acetate (v/v: 1/1) after addition of 2 mL of carbonate buffer at pH 9.7. After agitation and centrifugation for 20 min, the organic phase was recovered and evaporated to dryness. The residue was reconstituted in 80 µL of mobile phase and 10 µL were injected in the chromatographic system.

2.2.4. LC–MS/MS conditions

Chromatography was performed on Accela liquid chromatography pump (Thermofisher, Les Ulis, France) coupled with a CTC auto sampler (Thermofisher), using a Hypersil GOLD PFP column (100 x 2.1 mm, 1,9 µm) preheated at 30 °C. The column is preceded by a javelin filter (2.1 mm i.d.). The mobile phase is a gradient of acetonitrile and 2 mM formate buffer in 0.1% formic acid starting from 20% of acetonitrile to 90% in 10 min at a flow rate of 300 mL/min. The total run time was 12 min. Compounds were detected by a TSQ Vantage triple-quadrupole mass spectrometer (Thermofisher) equipped with an electrospray ionization (ESI) source set in a positive mode. An ion-spray voltage of +3.5 kV was applied. The heated capillary temperature was set at 350 °C. Nitrogen (Nitrox UHPLCMS 18, nitrogen generator, Domnick Hunter, Villefranche sur Saone, France) was employed as sheath and auxiliary gas at a pressure of 50 and 20 arbitrary units, respectively. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in selected reaction monitoring (SRM) mode, with two m/z transitions per analyte. Chromatographic data acquisition was performed using Xcalibur software (v2.0.7 SP1, Thermofisher), and quantification was carried out by using LCquan software v2.5.

MRM transitions with corresponding collision energies for the screened compounds are shown in supplementary material 1.

Table 1
validation parameters of some detected NPS in hair according to the EMA guideline on bioanalytical method validation.

Validation settings of some NPS	4-MEC	Mephedrone	MDPV	Butylone	DXM
1. Linearity (pg/mg)	1–1000	1–1000	1–1000	1–1000	1–1000
R^2	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
2. LOD (pg/mg)	0.5	0.5	0.5	0.5	0.5
3. LOQ (pg/mg)	1	1	1	1	1
4. Matrix Effect (%)	< 5	< 5	< 5	< 5	62
5. Extraction recovery (%)	66	70.1	87	81.9	85.8
6. Accuracy: trueness bias (B %) + precision (CV %)					
	QC 5 pg/mg				
Intraday CV (%)	5.4	14.4	7.0	8.8	9
Interday CV (%)	10.8	7.6	4.0	9.3	14.4
Trueness B (%)	100.3	101.2	104.6	103.4	102.0
	QC 20 pg/mg				
Intraday CV (%)	7.0	7.9	7.5	6.4	8.6
Interday CV (%)	4.5	8.2	11.7	11.8	3.2
Trueness B (%)	100	96.8	98.2	103.5	104.0
	QC 250 pg/mg				
Intraday CV (%)	9.9	8.1	9.2	8.7	9.2
Interday CV (%)	12.5	14.3	2.0	9.9	2.3
Trueness B (%)	99.3	96.4	96.2	102.6	99.2
	QC 750 pg/mg				
Intraday CV (%)	12.9	3.2	5.1	5.9	8.6
Interday CV (%)	5.7	6.7	8.3	14.6	10.4
Trueness B (%)	104.8	107.5	106.8	99.8	105.8

2.3. Hair analysis

Non-cosmetically treated hair was sampled within 24 h of hospitalization and systematic toxicological analysis (STA) was performed to screen conventional DOA and 83 NPS, as shown in supplementary material 1. As natural cannabinoids (THC, cannabidiol and cannabinol) are acidic compounds, their screening requests the use of a different extraction procedure on a second strand of hair, which was not available. Thus, their research was not performed in the present study. Whenever possible, hair was cut into 3 segments of 2 cm to explore user's exposure profiles in the last 6 months based on hair growth of 1 cm/month. Short hair (< 2 cm) was analyzed in bulk. Each segment was considered separately as it reflected a different timeframe.

2.4. Method validation and statistics

The method was validated according to the European Medicines Agency (EMA) guideline, in terms of *linearity*, *limits of detection* and *quantification* (LOD and LOQ), *specificity*, *matrix effect*, *extraction recovery*, within-day and between-day *accuracy* (bias) and *precision* (coefficient of variation).

Statistics and illustrations were performed using "Tableau Publique" 10.5.3 and GraphPad Prism 5.01 softwares.

3. Results

3.1. Method validation

No interferences were observed from constituents of drug free human hair at the retention times and the MRM (Multi Reaction Monitoring) transitions of the detected compounds. The calibration curves were linear from 1 to 5 pg/mg to 1000 pg/mg. They exhibited a good linearity (coefficient of determination $R^2 > 0.99$). Different weighting strategies were tested and 1/x was found to be the most appropriate to achieve the best response function. The LOD and LOQ were between 0.5 and 2 pg/mg, and 1 and 5 pg/mg, respectively. All CVs were < 15%. The data indicated satisfactory criteria regarding method accuracy (Bias and CV < 15%). Matrix effects were insignificant (< 5%) and the extraction recovery was adequate and enabled to achieve good accuracy and sensitivity. Carry over was less than 0.05% for each analyte when we injected a blank sample after the highest calibration standard (1000 pg/mg). Table 1 summarizes validation parameters of some detected NPS.

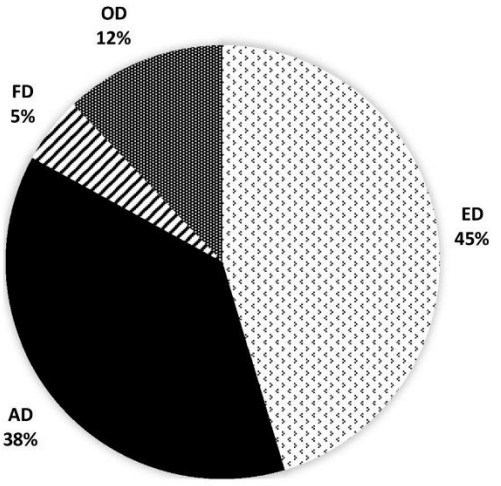


Fig. 1. Frequency of NPS detection according to the admission department (AD: addiction departments, ED: emergency departments, FD: forensic departments, OD: others departments).

3.2. Demographics

3.2.1. Overall studied population

Four hundred eighty patients (280 M, 200 F) were included. Most were admitted either to ED (n = 238; 143 M, 95 F) or to AD (n = 86; 65 M, 21 F). FD group represents 14% of patients (n = 69; 21 M, 48 F) while the remaining 18% of patients were admitted to OD (n = 87,

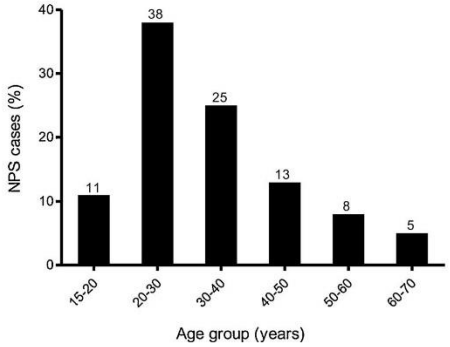


Fig. 2. Age distribution of NPS users.

58 M, 42 F). The mean age was 39 years [range: 15–70 years; median: 36 years].

3.2.2. Population of NPS users

141 patients tested positive for NPS corresponding to 99 M and 42 F (sex ratio = 2.35). When adjusting positive results according to gender, one in four female (42/200) and one third of male patients (99/280) tested positive for NPS. 64 cases (45%, 42 M, 22 F) were recorded in ED and 53 cases (38%, 43 M, 10 F) in AD. FD and OD recorded 7 (5%, 2 M, 5 F) and 17 cases (12%, 12 M, 5 F), respectively (Fig. 1).

The mean age of NPS users was 33 years [range: 17–70 years; median: 33 years] and the highest number of positive cases (n = 50, 35%) was observed in the 20–30 age group (Fig. 2). In comparison, the median age of cocaine, amphetamines and opioids users within the same populations were 36, 38 and 40 years old, respectively.

3.3. Prevalence of NPS

The prevalence of NPS was 29% (141/480): AD 11%, ED 13%, FD 1.5%, OD 3.5%. Prevalence of conventional DOA were: amphetamines: 32% (152/480: AD 12%, ED 13%, FD 3%, OD 3%), cocaine: 38.5% (185/480: AD 13%, ED 18%, FD 3%, OD 4%), licit and illicit opioids: 52% (248/480: AD 11%, ED 25%, FD 7%, OD 9%). 54% of NPS users had been exposed to more than one NPS in the same period (at least 2 detected NPS in the same segment), mostly in combination with cocaine (66%), amphetamines (65%) or opioids (64%). NPS alone were found in 32% of patients.

27 different NPS were identified (AD: 23, ED: 12, FD 2, OD: 8) (Fig. 3). Mephedrone: [range: 0.005–169 ng/mg hair, 24 cases (C), 56 positive segments (S)] and 4-MEC (0.001–97.3 ng/mg hair, 24C, 47S) were the most detected cathinones, followed by methylone (0.008–21.7 ng/mg hair, 15C, 35S) and MDPV (0.001–1.5 ng/mg hair, 7C, 16S). In the piperazine group, TFMPP (0.003-0.03 ng/mg hair, 2C, 5S), and m-CPP (0.07 ng/mg hair, 1C, 1S) were found. JWH-122 (0.2-0.43 ng/mg hair, 1C, 3S) was the only synthetic cannabinoid detected during this study. Other NPS were detected, some for the first time: 3-fluorofentanyl (0.06-0.15 ng/mg hair, 1C, 3S), furanylfentanyl (FU-F: 0.015-0.04 ng/mg hair, 1C, 3S), N-ethylpentylone (NEP: 0.015-0.64 ng/mg hair, 2C, 4S), pentedrone (0.018-0.65 ng/mg hair, 3C, 6S), 2-CE (0.07-0.25 ng/mg hair, 1C, 3S), mexedrone (0.03-0.05 ng/mg hair, 1C, 3S), methcathinone (0.011-0.485 ng/mg hair, 3C, 6S), 6-APDB (0.05-0.18 ng/mg hair, 2C, 5S), TFMPP (0.003-0.03 ng/mg hair, 2C, 5S), and 3,4-MD-αPHP (qualitative analysis, 1C, 3S). Table 2 summarizes the quantitative results for all detected NPS in comparison with hair concentrations found in the literature.

KET was found in 104C, most cases in the ED and AD groups (48C and 40C, respectively). In 10C, treatment with KET during the hospitalization was recorded. DXM was identified in 27C, mostly (71%) in ED and AD (10C each) in combination with DOA (70%), and with no recorded prescription.

The number of detected NPS increased from 2012 (9% of total

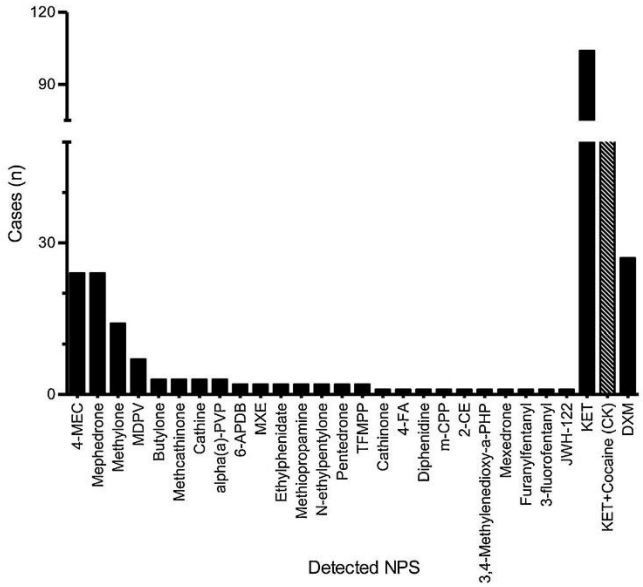


Fig. 3. Number of detected NPS in hair (abbreviations are reported in Supplementary material 1).

Table 2
Concentration range of the detected NPS (mean, median), number of hair positive segments and reported concentrations from the current literature (N1: number of cases, S: number of positive segments, N2: number of hair samples, ng/mg: nanograms per milligram of hair, NQ: not quantified, NA: not applicable, (*): concentrations found under trazodone treatment).

Substance	NPS and related compounds								
	Cases (N1)	Positive Segments (S)	LOQ [ng/mg]	Concentrations range [ng/mg]	Mean [ng/mg]	Median [ng/mg]	Reported hair concentrations [ng/mg]	Hair samples (N2)	References
KET	104	245	0.001	0.001–318	6.24	0.040	0.6–489	51	Leong et al., 2005
							0.11–11.4	6	Salomone et al., 2015
NKET	48	116	0.001	0.001–35	1.94	0.036	0.8–196.3	51	Leong et al., 2005
							0.02–0.71	6	Salomone et al., 2015
4-MEC	24	47	0.001	0.001–97.3	9.97	0.23	30	1	Alvarez et al., 2016a
							0.003	1	Larabi et al., 2018a
Mephedrone	24	56	0.001	0.005–169	6.05	0.08	0.1–87	24	Kintz, 2017
							0.2–213.2	13	Martin et al., 2012
Methylone	14	35	0.001	0.008–21.7	0.99	0.06	< 0.006–0.098	5	Salomone et al., 2017
MDPV	7	16	0.001	0.001–1.5	0.38	0.22	0.005	1	Larabi et al., 2018a
							0.12	1	Alvarez et al., 2016a
							1	1	Salomone et al., 2016
							1–22	1	Namera et al., 2013b
α-PVP	3	7	0.001	0.001–0.14	0.049	0.018	0.2–1.2	1	Namera et al., 2013b
							7.5–9.4	4	Namera et al., 2013a
Butylone	3	6	0.001	0.001–0.17	0.041	0.026	0.007–4.9	25	Salomone et al., 2017
Cathine	3	7	0.001	0.012–1.57	0.500	0.040	0.57 to 23.9	23	Sporkert et al., 2003
Ethylphenidate	2	6	0.0025	0.11–0.485	0.240	0.200	3.06	1	Lelong et al., 2018
MPA	2	6	0.001	0.19–0.91	0.550	0.550	0.38	1	Lelong et al., 2018
MXE	2	6	0.1	0.13–2.93	0.880	0.290	0.007–0.027	3	Salomone et al., 2016
Diphenidine	1	5	0.0025	0.02–0.12	0.066	0.079	0.079–0.12	1	Alvarez et al., 2016b
JWH-122	1	3	0.001	0.2–0.43	0.29	0.25	0.007–2.8	8	Salomone et al., 2014
4-FA	1	2	0.005	7.5–7.8	7.64	7.64	0.055	1	Salomone et al., 2016
Cathinone	1	1	0.01	0.13	NA	NA	0.11–22.7	23	Sporkert et al., 2003
m-CPP	1	1	0.05	0.07	NA	NA	0.34– > 4 (*)	10	Lendoiro et al., 2017
	NPS detected for the first time in real hair samples								
DXM	27	77	0.001	0.001–8.37	0.81	0.11	NR	—	—
Methcathinone	3	6	0.001	0.011–3.7	0.710	0.040	NR	—	—
6-APDB	2	5	0.0025	0.05–0.18	0.097	0.083	NR	—	—
TFMPP	2	5	0.0025	0.003–0.03	0.015	0.017	NR	—	—
N-ethylpentylone	2	4	0.001	0.015–0.64	0.033	0.027	NR	—	—
Pentedrone	2	3	0.001	0.018–0.65	0.310	0.275	NR	—	—
Mexedrone	1	3	0.0025	0.03–0.05	0.04	0.036	NR	—	—
2-CE	1	3	0.001	0.07–0.25	0.173	0.200	NR	—	—
Furanylfentanyl	1	3	0.005	0.015–0.04	0.025	0.020	NR	—	Larabi et al., 2018b
3-fluorofentanyl	1	3	0.0025	0.060–0.15	0.097	0.08	NR	—	Larabi et al., 2018b
3,4-MD-αPHP	1	3	0.0025	NQ	NQ	NQ	NR	—	—

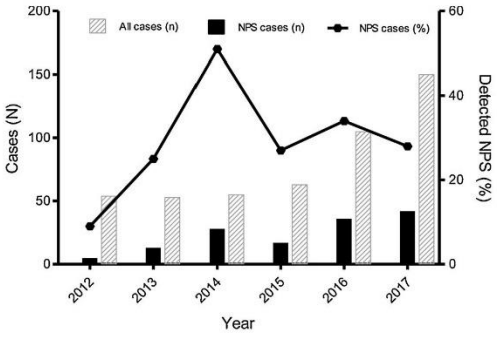


Fig. 4. Trend of NPS detection in Paris between 2012 and 2017.

cases) to 2017 (28%) with a peak of detection in 2015 where more than half of analyzed cases (51%) were positive to designer drugs. (Fig. 4).

4. Discussion

Hair is found to be a suitable matrix to study the prevalence of NPS. Indeed, hair offers a wide detection window (from months to years depending on hair length), is easy to sample and difficult to alter, so it can be kept for a long time and retrospectively analyzed (Kintz, 2015). Hair analysis has clear clinical and forensic applications. In emergency cases, hair is not used to diagnose intoxications, but rather to retrospectively study the exposure profile of poisoned persons. Hair testing has also gained an interesting role in the harm reduction process, which is often employed as a therapeutic approach in drug addiction. The results of hair analysis can be used to show drug users discrepancies that might exist between the compounds they bought, and those really consumed through the analysis of their hair (Larabi et al., 2018b). Finally, hair samples may be of use in forensic cases, notably in drug facilitated sexual assault (DFSA) where the use of some NPS has recently been reported (Hagan and Reidy, 2015; Larabi et al., 2018a). One hundred forty-one patients tested positive for at least one NPS. Men are twice as likely to be tested positive for NPS as women. NPS prevalence ranges from 0.3% in Hong Kong (Tang et al., 2015) to 37% in Switzerland (Rust et al., 2012) (Table 3).

The prevalence we found in Paris especially from high risk populations (29%) was as high as that recently observed by Salomone et al. (2017) in the US (32.5%) in a population of nightclubs attendees or by Rust et al. (2012) in Switzerland (37%) from amphetamines users. In Hong Kong, NPS were surprisingly not predominant (prevalence: 0.3%), despite the proximity to China, the country with the highest number of laboratories manufacturing NPS. (United Nations Office on Drugs and Crime [UNODC], 2017). Even though inclusion criteria are

Table 3
Comparison of major prevalence studies conducted by hair analysis (N1: number of included patients or volunteers, N2: number of NPS positive cases, N3: number of detected NPS, M: male, F: female, UKN: unknown, NR: not reported).

Countries / Authors / Publication Year	(N1)	Population Characteristics					NPS cases (N2)	Detected NPS (N3)	Top 3 of detected NPS	Prevalence (%)	References
		Sex (%)			Age (range, mean)	Type of population					
		M	F	UKN							
France Larabi et al., 2019	480	70	30	0	[16–70] m = 39	Addict follow-up Intoxications DFSA Clinical investigations	141	27	Ketamine 4-MEC Mephedrone (± 3-MMC)	29	—
USA Salomone et al., 2017	80	NR	NR	NR	[18–25] m = NR	Nightclubs attendees	26 (14M + 12F)	6	Butylone Methylone MXE	32.5	Salomone et al., 2017
Poland Adamowicz et al., 2016	NR	91	9	0	[16–50] m = 24.5	DUID, road accidents, drugs possession or use, violence, theft, rape, kidnapping, suicide, intoxications	112	21	3-MMC α-PVP Pentedrone	10.6	Adamowicz et al., 2016
Italy Salomone et al., 2016	77	> 70	NR	NR	[26–56] m = 36.3	MDMA and KET abusers (A) or subjects tested negative (B)	6 (4M + 2F)	8	MXE Mephedrone Methylone	7.8	Salomone et al., 2016
Hong Kong Tang et al., 2015	1036	74	26	0	[18–74] m = 35	Abuse clinics, Emergency department	3	2	PMMA Ketamine	0.3	Tang et al., 2015
Switzerland Rust et al., 2012	325	90	8	2	[18- up to 55] m : NR	Amphetamines users	120	7	Ketamine mCPP 4-FA	37	Rust et al., 2012

broadly different, especially with regard to the studied populations, the prevalence in all reported studies were high compared to conventional DOA. This is despite the fact that NPS are not routinely analyzed by all laboratories. It is likely that prevalence is underestimated.

According to the European School Survey Project on Alcohol and Other Drugs use (ESPAD) conducted in 2015 among 15- to 16-year-old students from 35 European countries, lifetime use of NPS averages 4% (range: 1% in Belgium to 10% in Estonia and Poland) (EMCDDA, 2018). In our study, we found that 11% of NPS users were under 20 years-old, which confirms the use of designer drugs in a young population in Paris. More than a half of NPS users had experienced more than one NPS in the same period (within two-months), more often in combination with conventional DOA (cocaine, amphetamines or opioids). It is important to stress that the use of NPS by opioid abusers is frequent in the studied populations and thus, screening of NPS should be considered in all DOA users and not be kept for patients abusing only stimulant drugs. NPS may be used in isolation: in our study 32% of NPS users had not used any conventional DOA. These consumers would not be detected if only routine drug analysis, which does not include NPS screening, is performed.

As observed in the previously published studies (Table 2), cathinones are the most prevalent group of NPS. However, differences can be seen with regard to substances dominating each local market. This could be related to different policies adopted by each country. Some have adopted an individual ban strategy of new emerging drugs (e.g. Poland), while others work on generic control regrouping a wide number of isomers and structurally similar compounds (e.g. France).

In our study, 4-MEC and mephedrone (4-MMC) are the most detected cathinones, followed by methylone and MDPV. 4-MEC was first notified as a recreational drug in Europe in 2010, but was only scheduled in table III of the 1971 United Nation (UN) Convention on psychoactive substances in 2016, due to many fatal cases and its abuse potential (Expert Committee on Drug Dependence [ECDD], 2016). In our study, 4-MEC was detected from 2013 (n = 2) through 2017 (n = 6) with a peak detection in 2016 (n = 9). In a study conducted by Néfau et al. (2015) in Paris area, 3489 used syringes were collected from injection drug users and analyzed by LC-MSMS for 23 DOA. 4-MEC was present in 23% of analyzed syringes (n = 2016), showing the appearance of this designer drug between the summer and winter of 2012 (Néfau et al., 2015).

Reports of mephedrone use emerged in different countries and regions of middle-east and Europe since 2007 (Kelly, 2011). Up to 2010, it was identified as the most common cathinone used in Europe (EMCDDA, 2019). Due to the choice of LC–MS/MS as reference screening method, we could not distinguish between mephedrone (4-MMC) and its isomer, 3-MMC, another designer drug that appeared later in the market. The two isomers have the same retention time and MRM transitions. Ideally, to distinguish between them, GC–MS analysis should be performed to achieve chromatographic separation (Gerace et al., 2014; Power et al., 2011). Another approach may consist of researching both metabolites of 3-MMC (eg. 3-methylephedrine or 3-methylnorephedrine) and of 4-MMC (eg. normephedrone) (Frison et al., 2016). Due to the absence of 3-MMC metabolites from our library, we used mephedrone for the validation process, even though we could not discriminate the isomers in positive samples. Thus, the presence of 3-MMC should not be excluded in mephedrone positive cases.

According to the EMCDDA 2018 report, synthetic cannabinoids (SCs) are the largest group of new substances seized and monitored in Europe. Surprisingly, this group was not dominant in most of the described studies conducted by hair analysis. Adamowicz et al. (2016) have reported the presence of only 7 cases of UR-144 use among 112 NPS positive cases (6.25%). Other studies have reported low prevalence of SCs, ranging from 1.29% (n = 232) (Cirimele et al., 2014) to 7.82% (n = 179) (Salomone et al., 2012) in different groups of drugs abusers. Concentrations of SCs ranged from 0.1 pg/mg for JWH-122, JWH-122 N-5 − OH and JWH-173 (Kim et al., 2015) to 13,000 pg/mg for MAM-2201 (Schaefer et al., 2013). From 2015, we introduced screening for 18 SCs (LOD and LOQ: 0.5 and 1 pg/mg, respectively), but JWH-122 was the only detected compound so far (range: 200–430 pg/mg), in accordance with the concentrations found in the literature (range: 7–2800 pg/mg) (Salomone et al., 2014). The low reporting of SCs from these studies could be explained in part by their high potency and thus their use at very low doses, which reduces their detectability in hair. Their low reporting could also be explained by their rapid metabolism leading to lower integration of parent drug into hair. In similar studies, the authors set the limit of detection as the minimum criterion to

establish the use of NPS. The results from positive samples with very low hair levels should be interpreted with caution because they do not exclude external contamination by passive smoking or handling materials containing SCs.

The high amount of seized SCs recorded by the EMCDDA and the low prevalence of SCs in hair samples could also show the effectiveness of regulation to control use as soon as there is enough scientific evidence demonstrating harmful effects.

KET and DXM are at the boundary between conventional DOA and NPS. These molecules have been abused for a long time but their use is still growing among NPS consumers. At the international level, ketamine was subject to a series of risk assessments and has found its place among clubbers, and is especially valued for its dissociative experiences like the "K-hole" with "near death" and "out of body" experiences (Dillon et al., 2003; UNODC, 2013). However, its use is often associated with cocaine: we found cocaine in over 70% of KET cases (n = 67), a combination so-called CK (Cocaine-KET), that has already been described in the literature (Rofael and Abdel-Rahman, 2002). DXM is a cough suppressant drug, but is also used recreationally, especially by teenagers. In recent years, it has appeared in MDMA-like (Ecstasy) tablets. When exceeding the maximum dosage, it acts as a dissociative hallucinogen with similar effects to those of ketamine and phencyclidine (PCP). DXM is not listed in the 1961 UN Convention on Narcotics, but several countries like France in 2017 have placed it under control due to its use in a harmful way (Cold and Cough Medicines, 2015; ECDD, 2012; Johnston et al., 2007).

Segmental hair analysis applied in 80% of cases (n = 384) showed a wide range of concentrations of NPS (Table 2). For the most detected NPS, low median values of hair concentrations differentiated two populations: occasional users with hair concentrations in the pg/mg range, and regular consumers with hair concentrations in the ng/mg range. Overall, hair concentrations are similar to those already described in the literature (Alvarez et al., 2016a, 2016b; Kintz, 2017; Larabi et al., 2018a, 2018b; Lelong et al., 2018; Lendoiro et al., 2017; Leong et al., 2005; Martin et al., 2012; Namera et al., 2013a, 2013b; Salomone et al., 2014, 2015, 2016, 2017; Sporkert et al., 2003). Finally, we report the presence of some NSO, like 3-fluorofentanyl (range: 0.06-0.15 ng/mg hair, 1C, 3S), and furanylfentanyl (range: 0.015–0.04 ng/mg hair, 1C, 3S). To our knowledge, this is the first time these fentanyls have been detected and quantified in hair. This is also the case for many other NPS such as N-ethylpentylone (0.015–0.64 ng/mg hair, 2C, 4S), pentedrone (0.018–0.65 ng/mg hair, 2C, 3S), mexedrone (0.03–0.05 ng/mg hair, 1C, 3S), methcathinone (0.011–0.485 ng/mg hair, 3C, 6S), 2-CE (0.07–0.25 ng/mg hair, 1C, 3S), 6-APDB (0.05-0.18 ng/mg hair, 2C, 5S), TFMPP (0.003–0.03 ng/mg hair, 2C, 5S), 3,4-MD-αPHP (not quantified), and dextromethorphan (0.001–8.37 ng/mg hair, 27C, 77S). These data are still difficult to interpret but remain important for future monitoring of NPS concentrations in hair.

5. Conclusion

This study addresses the issue of NPS spread through the identification of the most detected compounds in high risk populations from Paris and its suburbs over a six-year period, and allows the description of demographic characteristics of NPS users. In contrast to self-reported data, that often include reporting biases related to users' ignorance about the products they really consume, hair analysis brings objective data to the prevalence of designer drugs. This study shows that NPS prevalence is as high as that of conventional DOA in the studied populations. Thus, screening for NPS should be performed in clinical practice, especially in high-risk populations.

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Contributors

All authors are responsible for this reported research. I.A- LARABI designed the study, and conducted the statistical analyses. J.C ALVAREZ critically reviewed and revised the study and the manuscript. N. Fabresse and I. Etting performed analytical method development and analysis. L. Nadour managed the database on "Tableau publique" software. G. Pfau, JH Raphalen, P. Philippe and Y. Edel organized hair sampling and provided clinical information regarding included patients. All authors approved the final manuscript as submitted.

Declaration of Competing Interest

No conflict declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.drugalcdep.2019.06.011.

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Publication n° 7

Evaluation of drug abuse by hair analysis and self-reported use among MSM under PrEP: Results from a sub-study of the ANRS-IPERGAY trial.

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ADDICTION SSA SOCIETY FOR THE STUDY OF ADDICTION

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METHOD:	clinical trials
FIELD OF STUDY:	pharmacology
Keywords:	MSM, PrEP, chemsex, harm reduction, hair, cathinones, new psychoactive substances (NPS), HIV, addiction

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Conflict of interest declaration :

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Author contributions:

J.C., R.B., G.P., J.C.A., J.M.M, P.R., B.S and G.Pe. led the study and wrote the manuscript. J.C.A and A.L. performed the hair analysis. R.B, C.C. and L.M. analyzed the data. G.P., E.C, A.P., F.R., L.C. and J.M.M. conducted the study at their sites. All the authors critically reviewed and approved the manuscript.

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Abstract (350 words)

Background: Recreational drug use is on the rise among men who have sex with men (MSM), particularly during chemsex, and associated with many high-risk practices. We used the ANRS-IPERGAY trial in order to qualitatively and quantitatively measure drug use among MSM under PrEP using two different measurement methods, in order to understand and to be able to respond collectively to risky practices.

Method: Among the 429 MSM enrolled in the PrEP ANRS-IPERGAY study we included 69 voluntaries who provided written informed consent and had enough hair collection. We measured drug use by two methods: (1) Detected drugs by hair analysis by collecting samples every 4 months; (2) Reported drug use by self-reported drug consumption using a baseline and every two months online questionnaire.

Results: New Psychoactive Substances (NPS) and conventional drugs were detected in 53/69 (77%) by hair analysis and in 39/69 (57%) by questionnaire. Hair analysis detects more conventional drug and/or NPS use ($p<0.05$). In total, 219 hair segments were analyzed. The most commonly used drugs were cocaine in 47/69 (68%) and MDMA/ecstasy in 31/69 (45%). NPS consumption was detected in 27/53 (51%), associated with cocaine use in 25/27 (93%), MDMA in 20/27 (74%) or ketamine in 19/27 (70%). In total, 1,061 questionnaires were analyzed. The most commonly used drugs were also cocaine in 31/69 (79%), and MDMA/ecstasy in 29/69 (42%). NPS consumption was detected in 16/69 (23%). Whatever the method, drug use was significantly associated with a higher number of sexual partners

in the past two months (p≤0.001), more often casual partners (p≤0.001), more condomless anal sex (p≤0.005), more hardcore sexual practices (fisting, sadomasochistic practices) (p≤0.001), a higher number of STIs and more chemsex (p≤0.05).

Conclusion:

Drug use, particularly NPS, is high in the MSM PrEP population. Self-report drug use by questionnaire remains the reference tool for harm reduction at the individual level due to its easy feasibility and low cost. However, hair analysis is more sensitive, give an objective measure of consumption and is interesting in order to understand the uses and to be able to respond on a collective scale to risky practices with adapted messages.

Key words: cathinones, new psychoactive substances (NPS), chemsex, hair, PrEP, HIV, MSM, harm reduction, addiction.

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Introduction

Drug use is on the rise among men who have sex with men (MSM), particularly in sexual contexts, with the emergence of a new class of drugs, in addition to conventional drugs, called New Psychoactive Substances (NPS). Nearly 100 NPS have appeared in recent years ([1-2]). Both the scientific community and users often lack adequate information on the effects of these new drugs used alone or in combination. Synthetic cathinones are of particular concern because some drugs in this class are used instead of ecstasy without consumers' knowledge as their effects are close to those of ecstasy or other stimulants ([3-4]). Cathinones exposures has been associated with many side effects, both physical and psychological ([5-6-7-8-9]). Users may not always know what drug they are really taking, such as ecstasy that may contain synthetic cathinones ([10]). This drug use is also associated with risky sexual behavior in the practice of chemsex ([11-12-13-14]), characterized by the use of psychoactive drugs during sexual intercourse ([15-16]), which is called « slam » when these drugs are injected. In order to respond individually and collectively to their worrying NPS consumption, it is important to precisely identify the drugs used and to offer these users the most targeted multidisciplinary harm reduction counseling and

care possible. All studies evaluating recreational drug use among MSM are qualitative studies and use a method of measuring drugs consumed by self-questioning with the many subjective biases associated ([14-17]). We used the ANRS-IPERGAY trial to test a new method of measuring drugs consumed by hair analysis that allows for objective, more reliable and accurate qualitative and quantitative measurements of drug use. Hair analysis has become a new routinely tool for detection of xenobiotics intake, including NPS ([18-19]). Many studies investigate the association of hair ART-concentrations with virologic outcomes to determine thresholds of hair concentrations of various ARTs associated with virologic suppression ([20]). Hair analysis may provide a non-invasive, cost-effective adherence assessment of drug abuse. The main advantage of this matrix over traditional ones like urine or blood is its window of drug detection which is dramatically extended to weeks or months or even years according to the length of the strand (1 cm of hair corresponding approximately to 1 month of exposure). In parallel, drug use was also measured by self-questioning. The objective was to qualitatively and quantitatively measure drug use among MSM in PrEP using two different measurement methods, to understand use and to be able to respond collectively to risky practices with appropriate messages.

Methods

Study population

This study was a sub-study of the ANRS IPERGAY trial, which showed efficacy of on-demand PrEP with tenofovir disoproxil fumarate plus emtricitabine in reducing HIV incidence among high-risk MSM ([21-22-23]). Following this result, an open-label study extension was implemented from Nov 4, 2014, to June 30, 2016, to provide all participants with tenofovir disoproxil fumarate plus emtricitabine until PrEP approval (full approval in France in January 2016). Briefly, eligible participants for inclusion in the ARNS IPERGAY trial were HIV-negative men or women having sex with men, aged 18 years or older, and at high risk for HIV acquisition (defined as having condomless anal sex with at least two different partners during the past 6 months). Event-based dosing involved taking two tablets between 2 and 24 h before potential exposure, a third tablet 24 h later and a fourth 24 h after that, with daily dosing until at least two days after the last potential exposure as previously reported ([21]). During the ANRS IPERGAY trial,

participants completed online questionnaires at baseline, annual and biennial visits, and every 2 months (follow-up). Participants were also asked every four months to provide hair samples for antiretroviral monitoring initially. In November, 2015, the ANRS IPERGAY protocol was again amended to implement this substudy of qualitative and quantitative evaluation of the prevalence of conventional drugs and NPS consumption among MSM in French sites only. Public health authorities and ethics committees in France (Committee for the Protection of Persons (CPP), Paris Saint-Louis) approved this amendment. The present sub-study included all participants provided written informed consent.

Sub-study period

The analysis period of the sub-study was defined between the first hair sample collected on October 08, 2013 and the last hair sample collected on May 30, 2016.

Hair analysis

Hair analyses were performed using at least 20 mg of sample. Each hair strand was decontaminated successively by dichloromethane and hot water. One hair sample can give several samples if the hair is long. Thus, when possible (hair length ≥ 3cm and weight ≥ 20mg) a segmental analysis (2cm segments) was carried out, 1 cm corresponding to 1 month of previous drug use. Hair of each segment was cut into small pieces of less than 2 mm. Samples were then extracted by liquid-liquid extraction before being analysed by liquid-chromatography coupled to high resolution chromatography (LC-HRMS, Q-Exactive, ThermoFisher®) and liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS, TSQ Vantage, ThermoFisher®). LC-HRMS untargeted screening was realized by data dependent analysis, compounds were identified thanks to a shared MS^2 spectra library ([24]). Confirmation and quantification were carried out using a LC-MS/MS accredited method (Comité Français d'Accréditation, COFRAC) in Multiple reaction monitoring (MRM) mode. This method previously described ([25-26]) was validated according to European Medicines Agency's guidelines and allow to quantify the conventional drugs of abuse (opiates, cocaine and metabolites, amphetamines and phenethylamines), 86 NPS and therapeutic substances. Segmental hair analysis performed showed different pattern of consumption, ranging from rare intake (concentration < 200 pg/mg) to regular

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intake (>1000 pg/mg). Hair samples of the participants were analyzed every four months to detect drugs (except GHB/GBL and THC because both required another hair sample that was not included in the original design of the ANRS-IPERGAY study and poppers for technical reasons).

Questionnaires

Participants completed online questionnaires at baseline, every 2 months visits and annual visits. Baseline questionnaire collected data on sociodemographic and socioeconomic characteristics (age, educational level, employment status), data on drug abuse and data on sexual behaviour during the previous twelve months. The every two month follow-up questionnaire collected data on drug abuse and sexual behaviour during their most recent sexual intercourse. Annual questionnaire collected data on drug abuse and sexual behaviour during the previous twelve months and during their most recent sexual intercourse. In July 2015, participants were asked to answer an additional section in the every two month follow-up questionnaire and annual questionnaire, which addressed the use of an extended list of psychoactive substances during their most recent sexual intercourse and during the previous two months (cannabis, ecstasy, cocaine, poppers, alcohol, GHB/GBL, ketamine/Special K, Viagra and, as of July 2015, crack, heroin, methamphetamine/speed/crystal meth, LSD or mephedrone/ cathinone/MDPV/NRJ3/4MEC). Quantitative assessment of drugs used was based on different patterns of use, ranging from rare intake (less than once a week) to regular intake (every day). To analyse the data, all questionnaires completed during the sub-study period (October 08, 2013 to May 30, 2016) were taken into account.

Drug consumption definition

In hair samples, consumers were defined as participants with at least one hair sample containing at least one NPS or conventional drug (excluding therapeutics drugs, GHB/GBL, poppers and THC). In questionnaires, consumers were defined as participants with at least one reported drug use (ecstasy, cocaine, ketamine/Special K, GHB/GBL and, as of July 2015, crack, heroin, methamphetamine/speed/crystal meth, LSD or mephedrone/ cathinone/MDPV/NRJ3/4MEC) among all questionnaires collected during the analysis period of the sub-study. Chemsex was defined as reporting to be under the effect of at least one of these substances during their most recent sexual

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intercourse. Recreational drug use is defined by drug use in general, whether in a festive or chemsex context.

Sexual behaviour

Data collected on sexual behaviours included: type of partner (main partner, causal partner, sex party); number of partners during in past 2 months; number of sexual acts in past 4 weeks; type of sexual practice (oral sex, insertive anal sex, receptive anal sex); high-risk HIV exposure (condomless anal sex or not); hardcore sexual practices.

Sexually Transmitted Infection (STI)

Participants were tested every 6 months. Syphilis was diagnosed by a positive serological assay with treponemal and non-treponemal tests. Chlamydial and gonorrhoeal infections were defined by a single positive PCR test or culture from at least one site (throat, urine, or anus). In case of a positive PCR result at multiple sites with the same organism at the same time, a single infection was counted. Data collected on STIs used e-CRF medical data.

Psychiatric disorders

Data collected on psychiatric disorders used e-CRF medical data and were coded with MedDRA (Medical Dictionary for Regulatory Activities Terminology). The SOC associated with these events is Psychiatric disorders, with four levels of severity: Mild, Moderate, Severe and Life threatening.

Statistical analysis

We consider all self-questionnaires between the baseline of the trial and the date of the last hair sample. The percentages of responses to a question were calculated based on the number of participants who could answer the question. To compare the distribution of two groups, we use the Wilcoxon test, to compare proportions we use Chisquare test or Fisher test, depending on the expected effective and to compare paired nominal data we use MacNemar test. The number of people with at least one STI was calculated from the medical data of the e-CRF. The same applies to psychiatric events. All analyses were performed with software R version 3.5.2.

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Results

Recruitment

From February 22, 2012 to June 30, 2016, 429 participants were included in the ANRS IPERGAY trial in 5 French centers and 1 Canadian center. The sub-study was carried out only in French centers for logistical reasons, which represented 381 participants. At least one hair sample was available for 210/381 participants. At the start of the sub-study on 4 January 2016, 44/210 participants were no longer followed in the main study. Of the remaining 166 participants, 106 participants signed the consent form for the sub-study, 41 refused to participate (reasons for refusal were not provided), 1 were infected with HIV and we had missing data for 18. Of the 106 participants included in the sub-study, only 69 had sufficient hair matrix to be analyzed (Fig. 1).

Characteristics of the substudy sample versus study sample at baseline

Total of 5,898 questionnaires corresponding to 429 participants included in the ANRS IPERGAY trial were available for analysis. No significant difference was found between individuals in the drug analysis sub-study and other participants in the Ipergay study (Table 1).

Prevalence of drug use assessed by hair analysis

Using hair analysis, NPS and/or conventional drug were detected among 53/69 (77%) participants. A total of 219 segments corresponding to 137 hair samples were analyzed with a median hair sample per participant of 2 [1;3] among consumers and 1.5 [1;2] among non-consumers, without statistical difference. Thirty-two different molecules were detected in 3 categories: 15 NPS, 11 therapeutic agents and 6 conventional drugs. The most commonly used drugs were cocaine in 47/69 (68%) participants and 3,4-methylenedioxymethampthetamine (MDMA)/ectasy in 31/69 (45%) participants. NPS consumption (especially cathinones) was detected in 27/69 (39%) of them. In these users, NPS use was associated with cocaine use in 25/27 (93%), MDMA in 20/27 (74%) or ketamine in 19/27 (70%) of them, reflecting poly-consumption. Use of NPS and/or conventional drugs is mostly rare (hair concentration < 200 pg/mg). Drugs used on a regular intake were amphetamines and methamphetamines. NPS abuse was rather rare or occasional intake and 3 of them were detected but with the frequency of their intake undefined because they were not quantified (standard not available

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at the time of the detection). Therapeutic agents were detected among 50/69 (72%) participants. The most commonly drugs use were erectile drugs in 25/69 (36%) participants, followed by ephedrine in 18/69 (26%), and among analgesics drugs, tramadol and codeine with 15/69 (22%) and 9/69 (13%) of them (Table 2).

Prevalence of drug use assed by questionnaire analysis

Using questionnaire analysis, NPS and/or conventional drugs were reported in 39/69 (57%) participants. A total of 1,061 questionnaires were analyzed. Ten different drug classes were detected in 3 categories: 8 conventional drugs, 1 NPS and 1 therapeutic drug. The most commonly used drugs are conventional drugs with cocaine in 31/69 (45%), MDMA/ectasy in 29/69 (42%) and GHB/GBL in 25/69 (36%) participants. NPS consumption was reported in 16/69 (23%). The use of NPS and/or conventional drug is mostly rare (less than once a week). Erectile drugs were reported in 34/69 (49%) participants (Table 3).

Similar data between hair and questionnaire were observed for 49/69 (71%) participants. According to Macnemar's test between drug use data from the hair analysis and drug use data from the questionnaire analysis, the hair analysis detects more conventional drug and/or NPS use ($p<0.05$) and there is no difference between the two collection analysis for detecting erectile drugs ($p>0.05$).

Characteristics of the study sample (consumers versus non-consumers).

Total of 1,061 questionnaires corresponding to 69 participants included in our sub-study population were available for analysis: 848 questionnaires among 53 consumers and 213 questionnaires among 16 non-consumers. At baseline, the median [IQR] age of the 53 consumers was 34.5 years [28.3-40.9]; 35 (66%) had completed higher education and 44 (83%) were working. Fifty-one participants (96%) were MSM and 2 (4%) were bisexual, 7 (13%) were circumcised. For the follow-up, the results are derived from the average of the data from all follow-up questionnaires considered during the period of the sub-study. Drug use was significantly associated with a higher number of sexual partners in the past two months ($p\leq0.001$), more often casual partners ($p\leq0.001$), more condomless anal sex ($p\leq0.005$), more hardcore sexual practices (fisting, sadomasochistic practices) ($p\leq0.001$) and a higher number of STIs with at least 1 syphilis-type STI, gonococcal anal/genital/pharyngeal STI or chlamydia

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anal/genital/pharyngeal STI (p≤0.02). There was 1 HCV infection among consumers and no HIV infection in either group. Practice of chemsex was significantly higher among consumers versus non-consumers (p≤0.05). Thirty-two psychiatric disorders were reported in 19 consumers: 8 with 13 moderate disorders (4 depression, 4 sleep disorder, 3 anxiety and 2 nightmare), 13 with 16 severe disorders (8 depression, 2 sleep disorder, 1 affective disorder, 1 alcoholism, 1 anxiety, 1 burnout syndrome, 1 loss of libido and 1 suicidal ideation) and 3 with 3 life threatening disorders (2 suicide attempt and 1 alcoholism). Three psychiatric disorders were reported in 4 non-consumers: 3 with 3 moderate disorders (1 depression, 1 nightmare and 1 emotional disorder) and 1 t with 1 severe disorder (1 anxiety). Rate of psychiatric events in consumers was higher than in non-consumers without any significant difference being established (Table 4).

Discussion

This study describes qualitatively and quantitatively drug abuse, licit or illicit, consumed by MSM, volunteers in PrEP's ANRS-IPERGAY study, through hair analysis and questionnaires. In PrEP studies published so far, drug use among MSM is only assessed using self-administered questionnaires ([14-27-28-29-30]).

The overall prevalence of drug use detected by the hair method (53/69, 77%) in our study is higher than those reported by the questionnaire method in the various PrEP trials among high-risk MSM (43%-77%) ([21-22-27-28]), in surveys conducted among MSM outside PrEP studies (18%-60.4%) ([8-14-31-32-33]) and in our own ANRS-IPERGAY sub-study (39/69, 57%). The difference in the prevalence rates observed between the drugs detected in hair and those reported in the self-questionnaires can be explained by different points: i) Until July 2015, the bimonthly ANRS-IPERGAY questionnaires surveyed only drug use at last sexual intercourse, which may explain some negative responses from participants about this one-time use, but does not eliminate possible use at a time other than last sexual intercourse; ii) the collection of self-administered data presents several possible biases such as social desirability bias, memory bias due to the side effects of drugs consumed, methodological bias with fatigue filling among respondents and cognitive bias due to ignorance of products consumed and altered memory capacities

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of many of the New Psychoactive Substances ([34]); iii) the volatile nature of cocaine could partly explain the higher level of cocaine detected in hair versus reported in the questionnaires. Thus, a participant with a low level of cocaine detected once in the hair may not necessarily have used cocaine.

The overall prevalence of NPS use detected in hair in this MSM population (27/69, 39%) is higher than those reported in literature using hair analysis in other types of population, like nightclubs attendees, amphetamine or ketamine users, drivers under influence of drugs, acutely intoxicated patients In intensive care unit or drug-dependent patients, in which prevalence ranged between 7.8 to 32.5% ([18-35]), showing the high risk taken by this population. Our high prevalence may suggest a lack of knowledge among users of the drugs actually consumed, a deception about the true composition of the products purchased most often on the net, the lack of access to drug testing with likely use of NPS instead of ecstasy (MDMA) without their knowledge. This is of particular concern because of the possible consequences on somatic and neuropsychic health; these drugs can cause mental health problems, including self-harm, anxiety, depression and strong withdrawal effects as craving ([6-36-37-38]). However, the study population remains too small to demonstrate a significant difference in observed rates of psychiatric events between consumers and non-consumers. In addition, psychiatric adverse events were not specifically requested at each visit and study physicians were not psychiatrists.

The overall prevalence of use of therapeutic drugs is high (50/69, 72%) with important rates of erectile drugs, ephedrine, and analgesic drugs (tramadol and codeine) consumption. Ephedrine is often found in methamphetamine or MDMA powders since it is a precursor of these drugs. Tramadol and codeine could be used as cutting agent of the drugs. They pose many potential risks, especially since the correlation between consumer perceptions and test results is often low. The side effects of many drugs used as cutting agents are potentiated by alcohol, which also increases and/or modifies the effects of the drugs.

In our study drug use is not associated with a specific socio-demographic profile in this high-risk, 30-year-old MSM population with a high school education and large majority of them working. Studies have shown that this use is perceived by the majority of MSM as normal behavior and this normalization is established by the perceived pervasiveness of drug use ([39]).

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As already reported in the literature ([14-17-32-40-41-42]), drug use in our sub-study is associated with risky sexual behaviours. Thus, drug users in this MSM population under PrEP have a higher number of partners in the last two months, more often casual partners, with more unprotected sex, more hard practices, and therefore have a higher risk of STIs highlighted in our study by an increased bacterial sexually transmitted diseases in drug consumers during follow-up.

Limitations and bias

This study had biases and limitations: i) The study population represents only voluntary MSM with high-risk HIV behaviours taking PrEP and is not representative of the general MSM population; ii) The capillary matrix used for drug detection has limitations. Many participants had hair too short to be collected and analyzed. Pubic or axillary hair should have been sampled, but we did not include this possibility of a matrix in the consent signed by the participants. Moreover, it is an expansive method ; iii) the small size of our population limits the statistical power; iiii) The late implementation of this sub-study in the main trial resulted in some limitations. First, the majority of hair analyses were performed on samples collected prior to the start of this sub-study and did not allow sufficient hair to be collected to perform the GHB/GBL analysis in addition to other drugs. GHB/GBL remains a very frequently reported drug in this MSM population ([17-42]) and in our study.

Secondly, the list of drugs used by the self-administered questionnaires at each visit was extended from July 2015 with the addition of the NPS, crystal/methamphetamine and Speed/Amphetamine classes, which did not allow a correlation to be established before that date between the drugs detected in the hair and those reported in the questionnaires at the same time, which would have made possible to analyze the possible amnesia effect of certain drugs and the quality control of the drugs consumed. Finally, the CPP had requested that the results not be communicated individually to the participants, removing any possibility of analyzing the impact in terms of risk reduction of the announcement of actual consumption over time associated with counseling according to each product.

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Conclusion

The high rate of drug use, particularly of NPS, raises questions about the somatic and psychological risks taken by MSM already at high risk of HIV infection and STIs. Self-report drug use by questionnaire remains the reference tool for harm reduction at the individual level due to its feasibility and low cost. However, hair analysis are more sensitive with more molecules detected, especially molecules unknown to their users and therefore unexpected in their nature and associations, give an objective measure of consumption, is reproducible and is interesting in order to understand the uses and to be able to respond on a collective scale to risky practices with adapted messages, thus offering this group of patients a targeted multidisciplinary approach that includes psychologists, sexologists, addictologists and sexual health physicians.

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ANNEXES

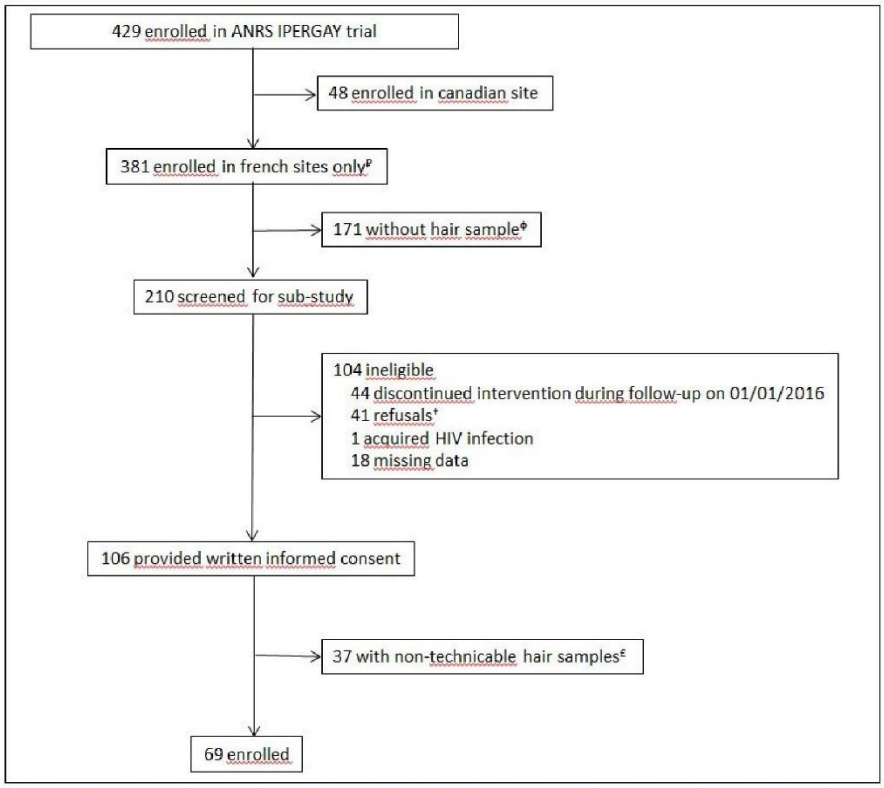


***Figure 1*: Study profile.** ÞFor logistical and financial reasons.[Φ]Too short hair to be collected and analyzed. [†]Reasons for refusal not provided. £No sufficient hair matrix to be analyzed.

Table 1. Baseline characteristics (ANRS IPERGAY study, n=429 participants, 5898 questionnaires).

	Drug Sub-study population n=69 participants n=1,061 questionnaires	Ipergay Not in drug sub-study population n=360 participants n=4,837 questionnaires	*p*
Age (years)	34.7 (28-40.9)	35.2 (29.2-42.8)	0.21
Sexual orientation			
MSM	67 (97%)	347 (96%)	1
Bisexual	2 (3%)	13 (4%)	
Education level			
Postsecondary¥	45 (65%)	261/355 (74%)	0.28
Unemployed	15 (22%)	44/355 (12%)	0.06
Use of recreationnal drugs ∞	29 (42%)	157/356 (44%)	0.85
In the context of Chemsex practice∞	18/67* (27%)	81/236* (34%)	0.3
Sexual risk factors at baseline			
Number of partners in past 2 months†	9 (5-15)	8 (5-17)	0.92
Number of sexual acts in past 4 weeks†	10 (5-16)	10 (5-17)	0.79
Fisting	2/51** (4%)	28/220** (13%)	0.08
Hardcore sexual practices	4/51** (8%)	23/221** (10%)	0.8
Condomless anal sex	18/41*** (44%)	55/185*** (30%)	0.12
Circumcision	8 (12%)	80 (22%)	**0.045**
At least one bacterial sexually transmitted disease in past 12 months‡	23 (33%)	92 (26%)	0.18

Data are n(%), median (IQR), or n/N (%).¥Postbaccalaureate certificate achieved at the end of high school. ∞Recreational drugs used in general in past 12 months or chemsex practice at most recent sexual encounter, including ectasy, crack cocaine, cocaine, crystal (methamphetamine), speed (amphetamine), and GHB/GBL. *Missing answers to questions about most recent sexual encounter. †All types of sexual acts are included (insertive or receptive anal sex and oral sex). ‡Included syphilis serological testing by rapid plasma reagin confirmed with the use of a treponema-specific assay ; *Neisseria gonorrhoeae* and *Chlamydiae trachomatis* were detected by PCR with urine samples and throat and anal swabs. ** Including only participants with questionnaires with a positive answer concerning partners in past 2 months or sexual acts in past 4 weeks. *** Including only participants who had anal intercourse reported.

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Table 2. Drugs detected and pattern of consumption by hair method (n=69 participants, 137 hair samples, 219 hair segments, 2 classifications: drugs common to questionnaire method and other drugs only detected by hair method).

	Drugs detected	Participants with drug detected (/69*), N (%)	Hair samples with drug detected (/137†)	Hair segments with drug detected (/219‡)	Detected but undefined	Rare intake (< 200 pg/mg)	Occasional intake (200-1000 pg/mg)	Regular intake (> 1000 pg/mg)
						(/N hair segments with drug detected)		
Drugs common to the questionnaires method	Cocaine	47 (68%)	82	137	-	75	29	33
	MDMA Ectasy	31 (45%)	56	91	-	50	21	20
	Ketamine	26 (38%)	41	69	-	57	7	5
	Amphetamine Methamphetamine	8 (12%)	13	20	-	5	8	7
	Mephedrone* 4-MEC* Methylone* MDPV Metamfepramone Dimethylone	22 (32%)	28	42	-	29	8	5
	Erectile drugs Sildenafil Tadalafil Vardenafil	25 (36%)	59	99	-	47	36	16
Others	Methoxetamine	4 (6%)	7	10	-	4	5	1
	Methylphenidate	1 (1%)	1	1	-	1	-	-
	Ethylphenidate	5 (7%)	5	10	-	9	-	1
	Methiopropamine	3 (4%)	5	8	-	6	1	1
	PMMA	3 (4%)	7	12	-	1	2	-
	5F-PB22	1 (1%)	1	2	-	2	-	-
	Phendimetrazine	1 (1%)	1	1	1	-	-	-
	Phentermine	1(1%)	1	1	1	-	-	-
	N-Methyl-2AI	1 (1%)	1	2	2	-	-	-
	Analgesic drugs Codeine	9 (13%)	16	26	-	24	2	-
	Nefopam	7 (10%)	11	17	-	16	1	-
	Dextropropoxyfen	4 (6%)	5	6	-	6	-	-
	Pholcodine	4 (6%)	5	7	-	4	1	2
	Namelfene	1 (1%)	1	1	-	1	-	-
	Tramadol	15 (22%)	27	42	-	20	15	7
	Butorphanol	1 (1%)	1	1	-	1	-	-
	Ephedrine$	18 (26%)	23	33	-	28	5	-
	Total conventional drug and/or NPS detected	53 (77%)	110	184	15	89	38	42
	Total therapeutics drugs detected	50 (72%)	12	16	3	10	2	1

Data are n (%). */69 participants. †/137 hair sample. ‡/219 Hair segments. Conventional drugs. NPS (*cathinones). Therapeutics drugs.
$Amphetamine manufacturing component.

Table 3. Drugs reported and pattern of consumption by questionnaire method (n=69 participants, 1,061 questionnaires. 2 classifications: drugs common to hair method and others drugs only detected by questionnaire method).

	Drugs detected	Participants with drug reported (/69[♦]), N (%)	Questionnaires with drug reported (/1061[†])	Rare intake (<1/week)	Occasional intake (>1/week)	Regular intake (Daily)
Drugs common to the hair method	Cocaine	31 (45%)	129	94	27	8
	MDMA Ectasy	29 (42%)	102	90	8	4
	Ketamine Special K	15 (22%)	33	31	1	1
	Amphetamine Methamphetamine Speed Crystal	16 (23%)	35	28	6	1
	Mephedrone 4-MEC Cathinones MDPV NRJ3	16 (23%)	58	40	17	1
	Total conventional drug and/or NPS reported	39 (57%)	642	121	34	9
	Sildenafil (erectile drug)	34 (49%)	78	50	17	11
Others	GHB/GBL	25 (36%)	77	67	9	1
	LSD	6 (9%)	9	9	-	-
	Crack	0	0	0	0	0
	Heroin	0	0	0	0	0

Data are n (%).[♦]/69 participants. [†]/1061 questionnaires. Conventional drugs. NPS (*cathinones). Therapeutics drugs.

Table 4. Sociodemographic, economic, drugs use, sexual behaviours and psychiatric characteristics at baseline and during follow-up by the hair method (ANRS IPERGAY sub-study, n=69 participants, 1,061 questionnaires).

	Consumers n=53 participants n= 848 questionnaires	No consumers n=16 participants n= 213 questionnaires	*p*
At Baseline			
Age (years)	34.5 (28.3-40.9)	35.4 (27.6-40.4)	0.92
Sexual orientation			
MSM	51 (96%)	16 (100%)	1
Bisexual	2 (4%)	0 (0%)	
Circumcision	7 (13%)	1 (6%)	0.671
Education level (postsecondary¥)	35 (66%)	10 (62%)	0.77
Unemployed	9 (17%)	6 (15%)	0.1
Use of recreational drugs including GHB/GBL in past 12 months∞	26 (49%)	3 (19%)	**0.0433**
Use of recreational drugs excluding GHB/GBL in past 12 months∞	26 (49%)	2 (12%)	**0.0098**
In the context of Chemsex practice including GHB/GBL in past 12 months∞	6 (11%)	1 (6%)	1
During follow-up			
Use of recreational drugs including GHB/GBL∞	37 (70%)	4 (25%)	**0.0028**
Use of recreational drugs excluding GHB/GBL∞	36 (68%)	3 (19%)	**0.001**
In the context of Chemsex practice including GHB/GBL∞	22 (42%)	2 (13%)	**0.04**
At least one bacterial sexually transmitted disease‡	44 (83%)	7 (44%)	**0.0108**
At least one *Neisseria gonorrhoeae*	26 (49%)	5 (31%)	0.259
At least one *Chlamydia trachomatis*	30 (57%)	2 (13%)	**0.0033**
At least one syphilis	12 (23%)	1 (6%)	0.272
Psychiatric disorder	19 (36%)	3 (19%)	
Moderate	8 (15%)	3 (19%)	0.24
Severe	13 (25%)	1 (6%)	
Life threatening	3 (6%)	0	
Number of partners in past 2 months	8 (4-15)	5 (2-10)	**<0.001**
Number of sexual acts in past 4 weeks†	10 (4-15)	8 (3-15)	0.066
Sexual behaviours at most recent sexual encounter	N=795 questionnaires**	N=197 questionnaires**	
Type of partner			
Main partner	186 (23%)	88 (45%)	
Casual partner	500 (63%)	99 (50%)	**<0.001**
Sex party	68 (9%)	11 (6%)	
Sexual practices[a]			
Oral sex	114 (14%)	26 (13%)	
Insertive anal sex	270 (34%)	61 (31%)	0.264
Receptive anal sex	369 (46%)	109 (55%)	
High-risk HIV exposure: condomless anal sex[b]			
No	443 (56%)	139 (71%)	
Yes	196 (25%)	31 (16%)	**0.004**
No anal sex	115 (14%)	27 (14%)	
Hardcore sexual practices[c]			
No	614 (77%)	184 (93%)	
Yes	121 (15%)	12 (6%)	**<0.001**

Data are n(%), median (IQR), or n/N (%).¥Post baccalaureate certificate achieved at the end of high school. ∞Recreational drugs used reported by questionnaire including ecstasy, crack, cocaine, crystal (methamphetamine), speed (amphetamine), +/- GHB/GBL.*1 Missing answers to questions about most recent sexual encounter. †All types of sexual acts are included (insertive or receptive anal sex and oral sex).**Only follow-up questionnaires with a positive answer concerning partners in past 2 months or sexual acts in past 4 weeks. [a] 3 missing values, [b] 1 missing values [c] sadomasochistic practices, fisting, 21 missing values. ‡Included syphilis serological testing by rapid plasma regain confirmed with the use of a Treponema-specific assay ; *Neisseria gonorrhoeae* and *Chlamydia trachomatis* were detected by PCR with urine samples and throat and anal swabs.

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Publication n° 8

Hair testing for 3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl, carfentanil, acetylfentanyl and fentanyl by LC–MS/MS after unintentional overdose

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CASE REPORT

Hair testing for 3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl, carfentanil, acetylfentanyl and fentanyl by LC–MS/MS after unintentional overdose

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Abstract

Purpose To demonstrate the usefulness of hair testing to determine exposure pattern to fentanyls.

Methods A 43-year-old male was found unconscious with respiratory depression 15 min after snorting 3 mg of a powder labeled as butyrylfentanyl. He was discharged from hospital within 2 days without blood or urine testing. Two locks of hair were sampled 1 month (M1 A: 0–2 cm (overdose time frame); B: 2–4 cm; C: 4–6 cm) and 1 year (Y1: A: 0–2 cm; B: 2–4 cm) later to monitor his exposure to drugs of abuse by liquid chromatography–tandem mass spectrometry after liquid-liquid extraction.

Results Hair analysis at M1 showed a repetitive exposure to 3-fluorofentanyl (A/B/C: 150/80/60 pg/mg) with higher concentration in segment A reflecting the overdose period. The non-detection of butyrylfentanyl was consistent with the analysis of the recovered powder identified as 3-fluorofentanyl. Furanylfentanyl (40/20/15 pg/mg) and fentanyl (37/25/3 pg/mg) were also detected in hair. The second hair analysis at Y1 showed the use of three new fentanyls, with probably repetitive exposures to methoxyacetylfentanyl (A/B: 500/600 pg/mg), and single or few exposures to carfentanil (2.5/3 pg/mg) and acetyl fentanyl (1/1 pg/mg). A decreasing exposure to 3-fluorofentanyl (25/80 pg/mg), and increasing consumption of furanylfentanyl (310/500 pg/mg) and fentanyl (620/760 pg/mg) were also observed despite methadone treatment initiation. The patient claimed not consuming three out of the six detected fentanyls.

Conclusions We report single or repetitive exposure to several fentanyls using hair testing. To our knowledge, this is the first demonstration of 3-fluorofentanyl and methoxyacetylfentanyl in hair samples collected from an authentic abuser.

Keywords Authentic hair analysis · 3-Fluorofentanyl · Methoxyacetylfentanyl · Carfentanil · Repetitive exposure to several fentanyls · LC–MS/MS

Introduction

Over the last few years, new synthetic opioids (NSOs), primarily fentanyl derivatives are increasingly implicated in overdose deaths, and have caused a global health concern. Among the 70,237 drug-related deaths estimated in 2017 in the US, the sharpest increase occurred among those related to fentanyl analogs with more than 28,400 overdose deaths which represents 45.2% increase from 2016 to 2017 [1, 2]. In Europe, their use appears to be geographically localized, but the number of synthetic opioids has grown rapidly since the first substance was reported in 2009. In fact, around 50 NSOs have been reported to the EU Early Warning System with 11 being reported for the first time in 2018 [3]. From 2015 to early 2018, the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) has conducted eight joint investigations with Europol on fentanyls that have caused serious concern at European level. The two agencies investigated acetylfentanyl, acryloylfentanyl and furanylfentanyl, 4-fluoroisobutyrylfentanyl (4F-iBF), tetrahydrofuranylfentanyl (THF-F), carfentanil, methoxyacetylfentanyl and

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cyclopropylfentanyl. These substances have been involved in more than 250 deaths, many of which were attributed directly to these substances [4]. Reported overdose cases include people who unknowingly buy adulterated heroin, other illicit drugs or pain relievers [3, 5].

The risk of life-threatening poisoning or deaths by NSOs is being enhanced by the following reasons: most NSOs are particularly potent on μ-opioid receptors (10 up to 10,000 more potent than morphine) and can be toxic at very low dose due to severe respiratory depression [6]; high bioavailability using routes of administration that allow substances to rapidly reach the central nervous system such as injecting, snorting, and inhalation; ease of synthesis, purchase at low cost, and their adulteration especially with heroin facilitate the poisonings; they, in many cases, are undetectable by the standard screening methods [4]. A recent EMCDDA study has shown that not all laboratories have the capacity to detect the more uncommon substances [3]. Thus, the prevalence of these substances in opioid-related poisonings and deaths is most likely underreported.

In the actual context of opioids crisis, more reliable and sensitive methods dedicated to the identification and quantification of NSOs in biological and non-biological matrices are needed. Recently, these analytical methods often based on chromatography (liquid or gas) coupled with mass spectrometry (MS) detection were extensively reviewed [7, 8]. Even if targeted screening is highly sensitive and specific, they remain limited by the unavailability of reference standards [7, 9–12]. A new approach based on untargeted screening by liquid chromatography–high-resolution mass spectrometry (LC–HRMS) using a shared MS spectral database and without a need for reference standards has emerged in recent years [13–15].

To study the prevalence of fentanyl derivatives among other drugs of abuse, the EMCDDA has proposed five strategies as targeted data sources: wastewater or syringe residue analysis, emergency department or drug checking service data, and web surveys [3, 16, 17]. Self-reported use of drugs often includes reporting biases related to users' ignorance about the products for their consumption which often are different from the ones that they bought, especially in case of NSOs due to adulteration. Wastewater and syringe residue analysis strategies provide more informative data regarding drug use, but remain indirectly linked to the population of the consumers. Even if these approaches are not representative of the general population and present some limitations, they provide useful, timely and complementary data that offer valuable insights into drug use in Europe [5].

On the other hand, the analysis of biological specimens of drug users could be the most accurate and directly linked to their drug consumption. While blood and urine testing can provide useful information of recent exposure to drugs (few hours up to days after consumption), hair testing allows an extensive exposure pattern of past use, as it offers a wide detection window, up to months prior to sampling and is easy to collect. Given that hair is also a rot-proof material that does not decompose, it can be kept for a long period with no special storage conditions (ambient temperature) and retrospectively analyzed, as most substances are stable in the hair matrix. Lastly, unlike conventional matrices like urine, hair sample cannot be adulterated by dilution, for example, to produce negative results [18].

Recently, hair testing has gained an interesting role in harm reduction, which is often used as a therapeutic strategy to disclose how individuals who use drugs learning about exposure may affect their drug taking and their adherence to treatment [10].

However, hair analysis can be tricky, and some common pitfalls should be known to avoid wrong results and interpretation. The sample collection is the first step that should be considered, especially in case of segmental analysis. Given that hair growth differs in each region of the body, the collection of head hair, for example, has to be done in the vertex area behind the head where the growth rate is steady (~ 1 cm/month). Hair strand should also be oriented to distinguish between the proximal (recent) and distal side which can affect the interpretation of consumption trend (increase or decrease) [18]. From the analytical point of view, the analysis of hair samples may be more difficult than conventional matrices like urine, because it requires more preparation steps like decontamination or digestion where losses or degradation of drugs may affect the detection and increase variability of quantitative results. The choices of the right solvent, the extraction time and the analytical instrument are some of the important factors to consider for achieving unambiguous identification and accurate quantification [19].

The most serious pitfalls of hair analysis are in the interpretation of the results. While the incorporation of drugs in hair is dose related, it is well known that there is no good correlation between the dose of a drug taken and hair concentration because of interindividual variation. This can be due to differences in metabolism, frequency of use, purity of drugs, hair color, or the use of cosmetic products (dying, bleaching, etc.) among many factors that affect the incorporation of drugs into the hair matrix. It is, therefore, difficult to speculate on how much or how often an individual used drug. However, intraindividual correlation when analyzing multiple hair segments or different time frame could be used to monitor drug use pattern and detect changes in the frequency (decrease or increase) of use in the same subject [20].

Finally, while a positive result from the analysis of hair suggests that a person has used or been exposed to a drug (including external contamination), a negative hair result, however, does not categorically mean that the person did

not use drugs if they have been exposed to a drug infrequently or in low doses. Consequently, it is important that the limitations of the test should be highlighted when reporting the results [18].

Even if this approach remains scarce and requires some particular skills, it deserves to be considered as a direct and long-term indicator of drug exposure. Some prevalence and analytical data regarding new psychoactive substance (NPS) study by hair testing in high-risk populations are now available [21–27].

In this article, we report a case of a 43-year-old male hospitalized in intensive care unit (ICU) for 3-fluorofentanyl unintentional overdose from a mislabeled product and demonstrate the usefulness of hair to determine his exposure profile to other fentanyl analogs over 1 year.

Materials and methods

Chemicals and reagents

The reference standards of alfentanil, carfentanil, furanylfentanyl, furanylnorfentanyl, butyrylnorfentanyl, acetylnorfentanyl, norcarfetanil, norfentanyl, methylfentanyl, furanylnorfentanyl, methoxyacetylfentanyl, fentanyl-D_5, acetylfentanyl-D_5 and norfentanyl-D_5 were purchased from LGC Standards (Molsheim, France); sufentanyl, fentanyl, and butyrylfentanyl from Lipomed AG (Arlesheim, Switzerland); 3(meta)-fluorofentanyl, ocfentanil and acetylfentanyl from Cayman Chemical Company (Ann Arbor, MI, USA); U-47700 from Chiron AS (Trondheim, Norway); acetonitrile, dichloromethane, hexane, ethyl acetate, formic acid and methanol from Sigma-Aldrich (Paris, France) in MS or high-performance liquid chromatography (HPLC) grade; sodium carbonate and hydrogen carbonate from Prolabo (Paris, France). Ultra-pure water (18 MΩ) was obtained by ultrafiltration with a Q-Pod (Millipore Corp., Molsheim, France). Formate buffer containing 2 mM ammonium formate in 0.1% formic acid was prepared in ultra-pure water and stored after each analysis at +4°C away from light for a maximum of 1 week.

Calibration standards and quality controls

Eight calibration standards containing a mixture of screened fentanyls at 1, 2.5, 5, 10, 50, 100, 500, and 1000 pg/mg of hair were prepared by spiking stock solutions of reference standards with appropriate volume into a 20-mg drug-free hair powder. Quality controls (QCs) were prepared at concentrations of 5, 250 and 750 pg/mg by the same way. Drug-free human hair was obtained from healthy subjects who are not medically treated.

Hair sample preparation

Hair locks were obtained from the posterior vertex. Hair was washed once with warm water and decontaminated twice using dichloromethane (immersion for 2 min in each step). It was then ground into a fine and homogeneous powder using a ball mill (MM200; Fisher Scientific, Illkrich, France). Each 20 mg was incubated in 1 mL of phosphate buffer at pH 5.0 at 95 °C for 10 min, in the presence of an appropriate amount of deuterated internal standards (ISs: fentanyl-D_5, acetylfentanyl-D_5 and norfentanyl-D_5). After spiking the corresponding volume of calibration standard or QC working solution when necessary, liquid-liquid extraction was performed by 4 mL of a mixture of hexane/ethyl acetate (*v/v*, 1:1). After agitation and centrifugation for 20 min, the organic phase was recovered and evaporated to dryness. The residue was reconstituted in 80 µL of mobile phase and 10 µL was injected into the chromatographic system.

Liquid chromatography–tandem mass spectrometry system and conditions

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) system derived from a previously published method [22] and enriched by the addition of 17 NSOs was employed in the present study. Chromatography was performed on a Dionex Ultimate 3000 pump (ThermoFisher, Les Ulis, France) using a Hypersyl Gold PFP column (100 × 2.1 mm i.d., particle size 1.9 µm) preheated at 30 °C. The mobile phase was a gradient of acetonitrile (A) and 2 mM sodium formate buffer aqueous solution with 0.1% formic acid (B) starting from 20% (A) to 90% (A) in 10 min at a flow rate of 300 µL/min. The total run time was 12 min. Compounds were detected by a TSQ Endura triple-quadrupole mass spectrometer (ThermoFisher) equipped with an electrospray ionization source set in a positive mode with ion spray potential at + 3.5kV. Capillary temperature was set at 350°C. Nitrogen (Nitrox UHPLCMS 18, nitrogen generator; Domnick Hunter, Villefranche sur Saône, France) was employed as sheath gas at 35 arbitrary pressure unit. The argon gas collision-induced dissociation was used with a pressure of 1.5 mTorr. Data were collected in selected reaction monitoring (SRM) mode, with two *m/z* transitions per analyte. Data acquisition was performed using Xcalibur and LC-Quan softwares (both ThermoFisher).

Retention times, SRM transitions with corresponding collision energies for the 17 screened NSOs and their ISs are shown in Table 1.

Table 1 Retention times, selected reaction monitoring transitions and collision energies of the screened new synthetic opioids (NSOs) and their internal standards (ISs)

Compound	IS	RT (min)	Parent drug	Product ion (quantifier)		Product ion (qualifier)	
			m/z	*m/z*	CE (eV)	*m/z*	CE (eV)
Fentanyl-D_5 (F-D_5)	–	6.60	342.2	105.1	23	188.2	36
Acetylfentanyl-D_5 (AF-D_5)	–	5.50	328.2	188.1	22	105.1	35
Norfentanyl-D_5 (NF-D_5)	–	3.01	238.2	182.1	15	84.2	17
3-Fluorofentanyl (3-FF)	AF-D_5	7.16	355.5	188.2	22	105.2	34
3-Methylfentanyl	F-D_5	7.66	351.2	202.1	23	105.1	34
Acetylfentanyl	AF-D_5	5.50	323.2	188.0	18	105.17	32
Acetyl norfentanyl	AF-D_5	1.96	219.1	136.1	17	84.1	17
Alfentanil	AF-D_5	6.0	417.4	268.3	17	197.2	26
Butyrylfentanyl	AF-D_5	7.86	351.2	188.1	24	105.22	38
Butyryl norfentanyl	AF-D_5	3.96	247.1	84.1	18	55.1	18
Carfentanil (Ca-F)	F-D_5	7.60	395.4	335.3	18	240.1	21
Fentanyl (F)	F-D_5	6.60	337.0	105.1	23	188.2	36
Furanylfentanyl (Fu-F)	F-D_5	7.07	375.5	188.2	21	105.2	33
Furanyl norfentanyl	NF-D_5	3.5	271.1	188.1	16	84.1	16
Methoxyacetylfentanyl (MeO-AF)	AF-D_5	5.22	353.1	188.1	21	105.1	35
Norcarfentanil	F-D_5	3.63	291.1	259.1	10	231.2	14
Norfentanyl	F-D_5	3.04	233.1	177.1	14	55.1	33
Ocfentanil	F-D_5	5.89	371.2	188.1	23	105.3	37
Sufentanil	F-D_5	8.28	387.3	238.1	18	111.1	34
U-47700	F-D_5	6.46	329.2	172.9	27	144.9	46

RT retention times, *CE* collision energy

Method validation procedure

Selectivity and carry-over

Drug-free blank hair was analyzed to determine whether endogenous hair constituents interfere or not at the retention times and on the ion channels of NSOs. A replicate of blank sample was also analyzed immediately after the highest calibration standard to determine the carry-over.

Linearity

Six calibration curves were prepared with eight calibration standards ranging from 1 to 1000 pg/mg. Quantification was achieved by plotting the peak area ratios of NSOs to their respective ISs. Back-calculated concentrations of the calibration standards had to be within 85–115% of the nominal concentrations.

Limits of detection and quantification

The limit of detection (LOD) is the lowest concentration for each NSO that can be detected with a signal-to-noise ratio greater than 3. The limit of quantification (LOQ) was the lowest concentration of each NSO achievable with an accuracy of ± 20%.

Accuracy and precision

The accuracy (bias) and precision (coefficient of variation: CV) of the assay were determined at three QC levels (low 5; mid 250; high 750 pg/mg). For the intraday assay, six replicates of each QC were processed on the same day. For the interday assay, six replicates of each QC level were processed at three different days. The concentrations obtained were analyzed using analysis of variance (ANOVA), which separated the intraday and interday standard deviation and the corresponding CVs. An accuracy within the range 85–115% of the nominal values and a precision with a CV of ± 15% were required.

Matrix effect and overall method recovery

Matrix effect was defined as the ratio of the mean peak area obtained by analyzing six different blank hair matrices spiked after extraction with ISs and NSOs at two concentrations (100 and 500 pg/mg) to the mean peak area obtained in an aqueous solution at the same concentrations. Overall method recovery was defined as the ratio of the mean peak area obtained by analyzing six different blank matrices spiked before extraction with ISs and NSOs to the mean peak area obtained in an aqueous solution at the

same concentrations. The determination of method recovery remains optional for mass spectrometry techniques.

Application: case report

A 43-year-old man was hospitalized in the emergency department for opioid overdose. Over the week preceding the poisoning, he snorted 70 mg/day of a so-called "China White" powder. After 48 h without any reported consumption, he snorted 3 mg of another powder labeled as "butyrylfentanyl". Fifteen minutes later, he was found inanimate by his wife who alerted the emergency service. The first examination revealed unconscious state (Glasgow score: 3) and a respiratory depression. The patient also had a bilateral non-reactive tight miosis, cyanosis and bradypnea. He received injection of naloxone before arriving at the emergency department which allowed his recovery. He was discharged from the ICU within 2 days without any toxicological testing and was directed to the addiction clinic to follow a harm reduction program. This patient was a heroin addict and had already experienced different types of drugs, including NPS. Methadone substitution treatment was, therefore, initiated. The powder consumed just before the overdose was subjected to analysis. Two locks of hair were sampled 1 month (M1) (length: 6 cm) and 1 year (Y1) (4 cm) later to monitor his exposure to heroin, NSOs, and other drugs of abuse. Hair samples were cut into 2-cm segments: M1: A 0–2 cm, B 2–4 cm, C 4–6 cm; Y1: A 0–2 cm, B 2–4 cm. The segment A was the closest to the root (proximal) and reflects M1, the overdose time-frame. Each segment reflects a 2-month exposure period based on average hair growth rate of 1 cm/month [18]. Both hair samples were analyzed by the present LC–MS/MS method and by a previously published LC–HRMS method which used the mzCloud shared database containing 81 fentanyl analogs and several non-fentanyl NSOs (e.g., U-47700, U-49900, MT-45, etc.) by 27th June 2019 [15].

Results and discussion

Validation results for the analytical method

The SRM chromatograms for NSOs obtained in patient's hair are shown in Fig. 1. The method exhibited a good linearity (determination coefficient, $r^2 > 0.995$) with no interference from endogenous substances for detection of the analytes and ISs. The validation settings of the 6 detected NSOs are summarized in Table 2. Contamination by carry-over from a 1000 pg/mg QC to blank sample was not observed. The LODs and LOQs were 0.3 and 1 pg/mg for fentanyl, acetylfentanyl, and methoxyacetylfentanyl, and 0.8 and 2.5 pg/mg for 3-fluorofentanyl, carfentanil and furanylfentanyl, respectively. The CVs at the LOQs were $\leq 14.7\%$ for all compounds. Intra- and interday accuracy and precision were ≤ 8 and $\leq 14.7\%$, respectively. Recoveries ranged from 74 to 116% and matrix effect from 20 to 69%.

Analytical results for hair segment 5 months before and up to 1 month after the overdose

The analysis of the first lock of hair showed no exposure to butyrylfentanyl nor the presence of its main metabolite butyryl norfentanyl (LOD= 0.3 and 5 pg/mg, respectively, details not shown) during the last 6 months prior to sampling. This finding was consistent with the analysis of the recovered powder that contained 3-fluorofentanyl (purity 86%) rather than butyrylfentanyl. Segmental analysis demonstrated a repetitive exposure to 3-fluorofentanyl, confirming long-term use of this fentanyl analog by the patient (Table 3). In addition, slightly higher concentration of 3-fluorofentanyl in segment A with respect to segments B and C could be compatible with increased consumption before the overdose, or a consumption of high-purity product leading to patient's poisoning. These findings clearly demonstrated discrepancy that might exist between the product purchased as "butyrylfentanyl" on the Internet and the one actually delivered to the user. As the toxicity of delivered fentanyl analog might be much higher than the ordered ones, the consumption of the same dose can increase the risk of life-threatening overdose. This phenomenon is recurring and causing mass poisonings and even deaths [28–31]. Moreover, fentanyl analogs are sold as heroin or are adulterating heroin, other illicit opioids, or even pain relievers. Occasionally, they have also been used to make fake medicines and, less commonly, sold as cocaine [4].

Basic toxicological data regarding 3(meta)-fluorofentanyl are missing from the literature. Comparatively, 4 (para)-fluorofentanyl, one of the isomers has been scheduled as narcotic since 1989 under the United Nation's single convention on narcotic drugs of 1961 due to its toxicity and abuse potential. According to the Expert Committee on Drug Dependence (ECDD) of the World Health Organisation (WHO) who assessed multiple fentanyl derivatives in 1989, the potency of 4-fluorofentanyl is on a par with fentanyl [32]. Since 2016, 2 (ortho)-fluorofentanyl, another isomer of 3-fluorofentanyl was directly implicated in 16 fatalities in the US and two in Europe (Sweden and Norway). Consequently, it has been scheduled as narcotic (schedule I) in 2017 in the US and by the end of 2018 in Europe [33, 34]. Given the structural similarity among 3-fluorofentanyl, its two isomers and fentanyl itself, it is expected that 3-fluorofentanyl has high potency, dependence and abuse liability similar to that of fentanyl. Thus, the switch from

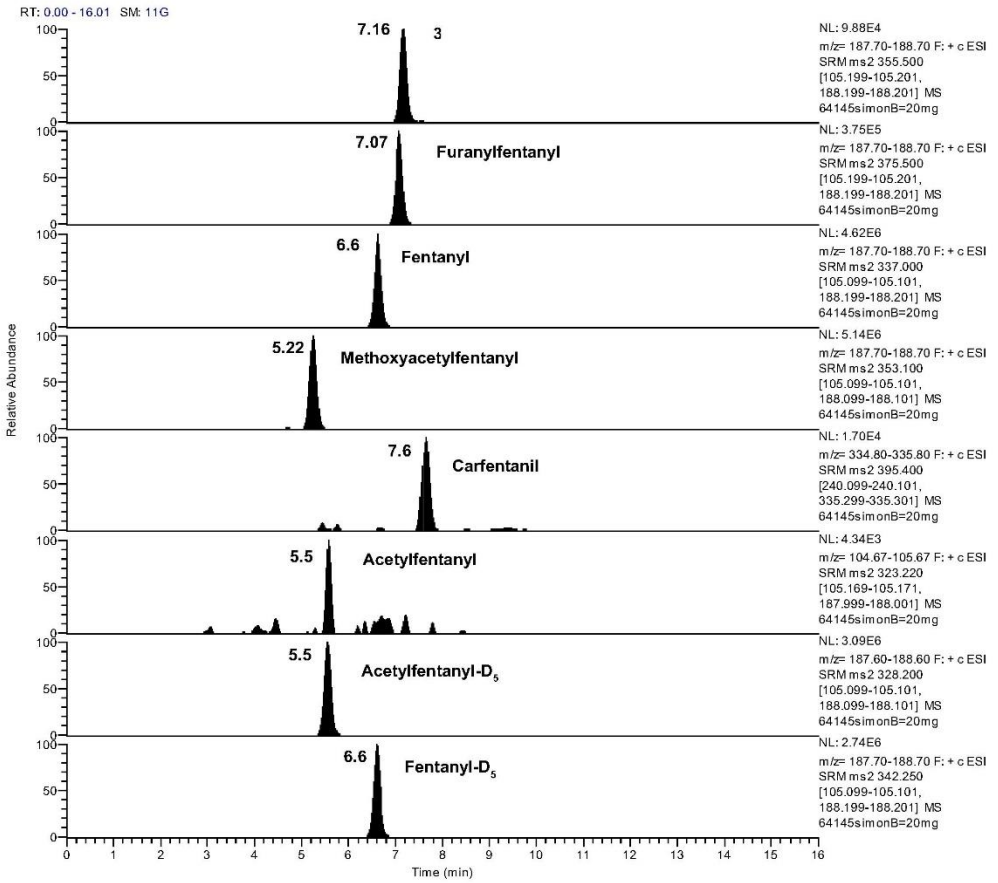


Fig. 1 Selected reaction monitoring chromatograms of an extracted hair segment A at Y1 showing the six detected fentanyls and their internal standards. Concentrations of drugs in pg/mg: 3-fluorofentanyl 25, furanylfentanyl 310, fentanyl 620, methoxyacetylfentanyl 500, carfentanil 2.5, acetylfentanyl 1

butyrylfentanyl to 3-fluorofentanyl in our patient seemed to significantly contribute to his poisoning as the few available preclinical studies suggested that butyrylfentanyl was about 30 times less potent than fentanyl itself [35]. The analysis of the second strand of hair 1 year later still showed an exposure to 3-fluorofentanyl in this patient but with about sixfold decrease with regard to the concentration initially observed (from 150 to 25 pg/mg). This finding might reflect a decrease in consumption due to its involvement in the precedent overdose or a consumption of a low-purity product.

The consumption of the so-called "China White" powder over a week prior to the poisoning could also have contributed to the opioid adverse effects, as the term "China White" was originally used to describe a highly refined and light-colored form of Southeast Asian heroin, but has also been used in contradictory ways to fentanyls (especially furanylfentanyl) sold as heroin since the 1980s of the last century [5]. This powder could correspond to heroin, furanylfentanyl, fentanyl or a mixture of these compounds as they were all found in patient's hair, with higher concentrations in segment A of M1 reflecting the overdose time-frame. The presence of and/or interaction with all these analogs may account for some of the reported effects.

Table 2 Validation settings of the detected NSOs

Compound	r^2	LOD	LOQ	CV (%)	Accuracy (% bias)					Precision CV (%)					Matrix effect (%)		Overall method recovery (%)	
					Intraday			Interday		Intraday			Interday					
					Low (n = 6)	Mid (n = 6)	High (n = 6)	Low (n = 18)	Mid (n = 18)	High (n = 18)	Low (n = 6)	Mid (n = 6)	High (n = 6)	Low (n = 18)	Mid (n = 18)	High (n = 18)	Low	High	Low	High
AF	0.9987	0.3	1.0	10.5	1.9	2.7	0.4	0.1	0.4	0.4	5.8	4.8	4.9	6.9	8.3	1.0	45	69	116	91
F	0.9992	0.3	1.0	8.6	4.4	3.6	0.7	4.5	3.5	0.7	4.7	5.0	6.2	9.1	8.6	0.6	20	33	110	84
MeO-AF	0.9978	0.3	1.0	8.7	6.0	7.7	6.5	6.0	5.2	6.4	3.9	6.3	7.4	10.0	13.0	7.5	43	67	117	91
3-FF	0.9972	0.8	2.5	14.7	4.7	2.7	3.7	1.0	2.0	1.2	5.8	11.5	8.0	13.7	6.3	13.0	34	48	109	82
Ca-F	0.9957	0.8	2.5	6.1	2.1	3.8	4.3	0.0	2.5	2.1	5.8	11.1	9.5	7.0	14.1	14.7	29	47	111	80
Fu-F	0.9979	0.8	2.5	7.6	7.9	7.6	3.2	8	7.7	3.2	4.1	7.0	11.1	12.4	13.3	12.2	29	43	97	74

Quality control (QC) levels for accuracy and precision: low 5 pg/mg, Mid 250 pg/mg, high 750 pg/mg

QC levels for matrix effect and overall method recovery: low 100 pg/mg, high 500 pg/mg

r^2 coefficient of determination, *LOD* limit of detection, *LOQ* limit of quantification, *CV* coefficient of variation, *AF* acetylfentanyl, *F* fentanyl, MeO-AF methoxyacetylfentanyl, *3-FF* 3-fluorofentanyl, *Ca-F* carfentanil, *Fu-F* furanylfentanyl

Analytical results for hair segments reflecting 9–12 months after the overdose

Furanylfentanyl and fentanyl were also found in the patient's hair, in much higher concentrations in the second strand (Y1), confirming long-term and polydrug abuse status of this patient, especially for fentanyls. In a recent study from a high-risk population of nightclub and festival attendees from the US, Salomone et al. [26] reported two positive hair samples for fentanyl at 2 and 6 pg/mg, and one for furanylfentanyl at 44 pg/mg out of 34 tested samples. Low rate of incorporation into the keratin matrix or the exposure to low quantities of the drug was suggested by the author. From the present case study, it seems that Salamone's findings would probably reflect occasional exposure to fentanyl and furanylfentanyl rather than low rate of incorporation into the keratin matrix because the concentrations in the second hair analysis clearly demonstrated high rate of incorporation of the two compounds into the hair. Moreover, Busardò et al. [27] have also reported high concentrations of fentanyl in hair (mean 2670 ± 184 pg/mg, n=2), confirming our findings.

The second hair analysis clearly revealed changes in fentanyl consumption by the patient over the year following his overdose because methoxyacetylfentanyl, carfentanil and acetylfentanyl appeared in the two segments of hair (Y1). The high concentrations of methoxyacetylfentanyl seemed to reflect repetitive exposure. Methoxyacetylfentanyl was first notified in late 2016 and then risk assessed by the EMCDDA and Europol by the end of 2017 due to relevant evidence of serious intoxications or fatalities. Two acute intoxications including one fatal outcome with confirmed exposure to methoxyacetylfentanyl were reported from users who bought products on the Internet supposed to be carfentanil or ketamine, respectively. Overall, at least 28 deaths were reported across Europe and the United States during 2017, largely in a home environment, which showed that in at least some of these cases the poisoning was so severe that the poisoned persons were unable to call for help [36]. Methoxyacetyl norfentanyl, a metabolite of methoxyacetylfentanyl was not screened in our method, but as reported by Busardò et al. [27], this metabolite is not a suitable biomarker of methoxyacetylfentanyl consumption, and was not detected when methoxyacetylfentanyl was taken, whether in blood or urine.

Acetylfentanyl and carfentanil were also found in patient's hair at very low concentrations. These concentrations probably reflect a single or few exposures, but because hair testing gives evidence of the cumulated incorporation of substances over time, it is difficult to speculate if these fentanyl analogs were taken alone or simultaneously, and if these analogs were present as an impurity of methoxyacetylfentanyl, furanylfentanyl or fentanyl [37]. As expected, respective metabolites of acetylfentanyl (acetyl norfentanyl) and carfentanil (norcarfentanil) were not detected (LOD = 1.5

Table 3 Results of hair analysis 1 month (M1) and 1 year (Y1) after the life-threatening poisoning in a patient

Compound	Hair concentrations (pg/mg) (M1)			Hair concentrations (pg/mg) (Y1)	
	Past exposure history ~ 2–5 months before the overdose		1 Month before, 1 month after overdose	~ 9–12 Months after the overdose	
	Segment C 4–6 cm	Segment B 2–4 cm	Segment A 0–2 cm	Segment B 2–4 cm	Segment A 0–2 cm
3(meta)-FF	60	80	150	80	25
Fu-F	15	20	40	500	310
F	3	25	37	760	620
AF	ND	ND	ND	1	1
Ca-F	ND	ND	ND	3	2.5
MeO-AF	ND	ND	ND	600	500
6-Acetylmorphine	550	980	1740	45[a]	
Methadone	ND	ND	12	3190[a]	
EDDP	ND	ND	5	30[a]	

For explanation of compound abbreviations, see Table 2

ND not detected, *EDDP* 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

[a]6-Acetylmorphine, methadone and EDDP were measured on cumulated A+B segments in Y1 due to insufficient sample

pg/mg for norcarfentanil and 3 pg/mg for acetyl norfentanyl; detailed data not shown) as the concentrations of parent drugs were very low in our case. In a previous study investigating exposure to fentanyl analogs through hair testing in 40 individuals in inpatient detoxification, Palamar et al. [37] have reported acetylfentanyl hair concentrations ranging from 2.1 to 3200 pg/mg (median: 26 pg/mg) showing that this fentanyl analog integrates well into the hair matrix. In a case of carfentanil related fatality, hair concentrations found in victim's hair ranged from 54 to 166 pg/mg, which probably reflected a repeated exposure to this fentanyl analog over the studied period of 9 months [38], but could also be due to contamination by sebum and postmortem fluids [39].

In the present case, the patient had probably unknowingly been exposed to methoxyacetylfentanyl, acetylfentanyl and carfentanil, because he claimed in different harm reduction sessions not consuming these analogs. This could come from mislabeling or adulterating with other fentanyl analog(s) that the patient used to order. In Palamar's previous study [37], 39 out of 40 individuals (98%) tested positive for fentanyl and 85% of participants tested positive for one or more fentanyl analogs, suggesting that exposure to more than one analog is common; 71.8% of the patients denied known use of these compounds [37].

Metabolites of fentanyls

Furanyl norfentanyl and norfentanyl, the respective metabolites of furanylfentanyl and fentanyl were not detected by the method described in Table 1 in the two analyzed samples (LODs = 1.5 and 15 pg/mg, respectively). Even if the LOD for norfentanyl was quite high in our method, our findings were consistent with previously published data. The mean parent- to- metabolite ratio for fentanyl/norfentanyl in Busardò's study was 32.5. Given that fentanyl concentrations in our case were low in M1, it is not surprising that norfentanyl was not detected. Moreover, previous studies showed that furanyl norfentanyl concentrations were much lower than that of furanylfentanyl, which does not make it a suitable biomarker of furanylfentanyl consumption [40]. Overall, as most metabolites are more hydrophilic than the parent drug, they are less likely to be integrated into the hair matrix.

In addition, the untargeted screening by LC–HRMS allowed the detection of 4-ANPP (4-anilino-*N*-phenethylpiperidine) in the two samples (detailed data not shown). 4-ANPP is both a precursor and a metabolite of several fentanyl analogs. It is a major metabolite of fentanyl [27], furanylfentanyl [41], methoxyacetylfentanyl [42] and a minor metabolite of acetylfentanyl [41]. However, in previous studies, reported 4-ANPP hair concentrations were lower than those of parent drugs, making it an inefficient and nonspecific marker of consumption [27]. Unfortunately, the quantification of this compound had not been performed in our study to confirm these findings, because the reference standard was not available in our laboratory.

Conclusions

As shown in a recent study in Canada, 75% of individuals reporting nonmedical opioid use and denying fentanyl use had their urine test positive for fentanyl [43]. Another study showed that, among patients denying known exposure to fentanyl and seeking opioid withdrawal management, two-thirds tested positive for fentanyl in urine [44]. Segmental hair analysis is especially useful for identification and monitoring the use of designer drugs, which often are different from ones that they bought; it enables to follow for months to nearly a year as we did. However, the number of cases for NSO identification and/or quantification using hair samples is still not many [45].

More data are needed to know to what extent the hair analysis can be effective in harm reduction. A large-scale adoption of this approach would allow a better understanding and response to NSO crisis at both health and regulatory levels. To our knowledge, this is the first report to describe the quantification of 3-fluorofentanyl and methoxyacetylfentanyl in hair samples collected from an authentic abuser.

Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval The analyses of toxic substances were requested by judicial authorities. About several tens of strands of scalp hair (length: 6 and 4 cm) were noninvasively collected from the patient with his informed consent.

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Drug-facilitated sexual assault (DFSA) involving 4-methylethcathinone (4-MEC),3,4-Methylenedioxypyrovalerone (MDPV), and doxylamine highlighted by hair analysis

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Drug-facilitated sexual assault (DFSA) involving 4-methylethcathinone (4-MEC), 3,4-Methylenedioxypyrovalerone (MDPV), and doxylamine highlighted by hair analysis

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Abstract

Recently, the emergence of new psychoactive substances (NPS) has led to their wide use among clubbers and men who have sex with men (MSM) for their stimulant effects. However, their use in drug-facilitated sexual assault (DFSA) has rarely been described. Herein we report a case of a 44-year-old man who was assaulted after a party. Due to late reporting of the offense, only hair (black) was sampled 15 days later and a segmental analysis was achieved to look for most DFSA agents and NPS. Twenty mg of each segment (A: 0–1 cm, B: 1–3 cm, and C: 3–5 cm) were incubated in phosphate buffer pH 5.0. After alkaline liquid extraction and chromatographic separation on 1.9 µm Hypersil GOLD PFP column, compounds were detected by a TSQ Vantage mass spectrometer with electrospray ionization in positive mode with multiple reaction monitoring (MRM) acquisition. 4-methylethcathinone (4-MEC), methylenedioxypyrovalerone (MDPV) and doxylamine were found in proximal segment at very low concentrations (3, 5, and 9 pg/mg, respectively) which is in agreement with a single exposure in the previous month corresponding to the alleged facts. These substances were not detected in segments B and C showing a lack of repetitive exposure before the alleged event. Thus, the results do not contradict the patient's claim of being assaulted. Doxylamine has already been encountered in such cases but no publications referring to 4-MEC or MDPV use have ever been documented. Our case reports the unusual administration of cathinones to achieve a sexual assault and stresses the interest of looking for designer drugs when dealing with DFSA cases.

KEYWORDS

cathinones, chemsex, drug-facilitated sexual assault (DFSA), hair, new psychoactive substances (NPS)

1 | INTRODUCTION

Drug-facilitated sexual assault (DFSA) has been defined as an offence in which the victim is subjected to non-consensual sexual acts, while they are incapacitated or unconscious due to the effects of drugs, and are therefore prevented from resisting or is unable to consent.[1] In the last few years, the emergence on the recreational market of so-called legal highs, or new psychoactive substances (NPS) highlights the fact that these drugs are legal, and do not fall under any legislative control, at least until they are placed on the list of the United Nation

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(UN) convention of illicit drugs.[2,3] Among NPS, cathinones are beta-keto-substituted analogs of amphetamine. Their effects include several psychedelic effects like a sense of wellbeing a profound insight and introspection, and increased appreciation of music and sociability.[4] Their use as psychostimulants and entactogens has widely been reported in sexual contexts, especially in men who have sex with men (MSM) in order to increase sexual performance and libido.[5,6] However, these drugs have rarely been encountered in drug facilitated crime (DFC) including DFSA.[7,8] This could be related to the fact that these substances do not induce anterograde memory loss, a desired symptom for a sexual assault. Here we present a case in which hair was used to highlight a DFSA involving 4-MEC and MDPV associated with doxylamine. This case illustrates the administration of cathinones beyond their recreational use to achieve a chemical submission probably for their disinhibiting effects and increased sexual arousal.

2 | EXPERIMENTAL

The liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was derived from a previously published method for 4-MEC and MDPV quantification in hair.[9] Doxylamine, GHB, GHB-d6, and alprazolam-d_5 were purchased from LGC Standards (Molsheim, France).

GHB was determined by GC-MS after hair hydrolysis at 56°C for 16 hours. Each 5-mm segment was then extracted by ethyl acetate (EA) before being derivatized with BSTFA (N,O-Bis(trimethylsilyl) vidéosurveillancen) and EA at 60°C for 20 minutes. 20 ng of GHB-d6 was used as an internal standard (IS) and 1 µL of the final residue was injected into the analytical system.

2.1 | Working solutions, calibration standard, and quality controls

Working solutions containing many DFSA agents (mainly benzodiazepines and sedatives substances) and NPS were prepared at 4 levels (0.001, 0.01, 0.1, and 1 mg/L) to compose a 10-point calibration curve ranging from 1 to 1000 pg/mg. Another stock solution was prepared by the same way to prepare 4 quality control (QC) levels: 5, 20, 250, and 750 pg/mg. All these solutions were stored at −20°C.

Working solutions for GHB were prepared at 0.1 and 1 mg/L to obtain 6 calibration standards (CS) at 100, 200, 500, 1000, 2000, and 5000 pg/mg. Two quality controls (QC) at 400 and 2500 pg/mg were prepared by the same way. GHB-d6 (IS) solution was prepared at 2 mg/L in MeOH.

3 | METHOD VALIDATION PROCEDURE

This method was validated in terms of selectivity, carryover, linearity, trueness, intra- and inter-day precision, lower limits of detection (LLOD) and quantitation (LLOQ), specificity (matrix effect), and recovery.

3.1 | Case report

A 44-year-old man had spent his evening in a nightclub. After consuming a drink, he experienced a blackout and flashes in which 2 men incited him to have sex, which he refused, but felt unable to oppose. Several hours later, he woke up naked in his bed and had memory flashes of sodomy. Some days later, he made a report at a police station and went to the Emergency Room (ER) where a preventive HIV treatment was initiated. Due to the late reporting of the alleged event, conventional drug testing in blood and urine was been performed. Fifteen days later, 2 strands of hair were taken from this patient and sent to our lab to investigate his presumed sexual assault. Based on an average hair growth of 1 cm per month, 1 proximal segment of 1 cm (corresponding to the alleged facts, segment A) and 2 other segments of 2 cm each (B and C) reflecting a total exposure period of 5 months were screened by LC–MS/MS. Most encountered DFSA drugs, pharmacologically capable of inducing amnesia, sedation, or impairment of judgment (benzodiazepines, z-drugs, neuroleptics, antihistaminic, atropine, scopolamine, ketamine, and other drugs of abuse) were screened. NPS including synthetics cathinones and cannabinoïds were also screened by the same method. GHB assay was carried out on a second strand by segmental analysis of 10, 5-mm segments using gas chromatography–mass spectrometry (GC–MS) validated method.

4 | RESULTS AND DISCUSSION

4.1 | Method validation

Doxylamine and GHB validation settings are summarized in Table 1. Data for 4-MEC and MDPV are already published.[9] No interferences were observed from constituents of drug-free human hair at the retention times and the ion channels of the detected compounds. Carry-over was less than 0.05% for each analyte when we injected a

TABLE 1 Validation settings for doxylamine and GHB measurement in hair. (NA): not applied

Validation Settings		Doxylamine (LC-MS/MS)	GHB (GC-MS)
Linearity (pg/mg)		5–1000	100–5000
R^2		>0.99	>0.99
LOD (pg/mg)		1	50
LOQ (pg/mg)		5	100
Matrix Effect (%)		<5	<5
Recovery (%)		74	NA
Accuracy (trueness and precision)			
QC1 (pg/mg)		**5**	**400**
	Intra-day CV (%)	11	3
	Inter-day CV (%)	7	3
	Trueness (%)	100	104
QC 2 (pg/mg)		**20**	**2500**
	Intra-day CV (%)	8	1
	Inter-day CV (%)	10	2
	Trueness (%)	103	101
QC 3 (pg/mg)		**250**	
	Intra-day CV (%)	7	
	Inter-day CV (%)	8	
	Trueness (%)	106	
QC 4 (pg/mg)		**750**	
	Intra-day CV (%)	5	
	Inter-day CV (%)	5	
	Trueness (%)	108	

blank sample after the highest CS (1000 pg/mg). The calibration curves were linear from 1 to 1000 pg/mg for 4-MEC and MDPV and from 5 to 1000 pg/mg for doxylamine. They exhibited a good correlation coefficient (R^2 > 0.99). The weighting factor was set at 1/x. The LLOD and LLOQ were 0.5 and 1 pg/mg for both MDPV and 4-MEC, 1 and 5 pg/mg for doxylamine, respectively. All CVs were < 15%. The data indicated satisfactory criteria regarding method accuracy (trueness and precision). Matrix effects were insignificant (<5%) and the overall recoveries were acceptable for all compounds. QCs were stable when stored 24 hours at +4°C (no significant difference has been observed for processed samples when compared to the initial value (data not shown).

4.2 | Application: Case report

Physiological GHB concentrations were measured in all segments ranging between 0.2 and 0.7 ng/mg, which discards the involvement of this drug in this presumed DFSA, as recently shown by Busardo et al.[10] Five compounds – 4-MEC, MDPV, doxylamine, tramadol, and mepivacaine – were found by LC–MS/MS. 4-MEC, MDPV and doxylamine were detected in the first segment (A, 0–1 cm) at very low concentrations of 3, 5 m and 9 pg/mg, respectively (Figure 1), while not being detected in the 2 following segments (Figure 2). Since segment A corresponds with the alleged facts, the results offer evidence of a single exposure of this patient to these drugs. The absence of these substances in the 2 remaining segments confirms a lack of prior exposure in the last 5 months, and objectify the possibility of chemical submission to achieve a sexual assault.

Doxylamine has already been encountered in many DFSA cases.[11-13] In France, a prevalence study has shown that doxylamine was the fourth most used compound in DFSA, just behind clonazepam, zolpidem, and bromazepam.[14] In a set of data based on a 10-year experience of interpreting DFC cases using hair analysis, Kintz has reported doxylamine concentration of 17 pg/mg after a single intake of 30 mg (2 tablets) to achieve a sexual assault.[15] Duffort et al. have also reported a case of DFSA in a young woman assaulted after a party. Doxylamine was identified at 40 pg/mg in the segment reflecting the alleged event.[16]

In the hair of 4 subjects receiving a single 15-mg dose and collected 4 weeks later, doxylamine was detected in the proximal segment at 18–52 pg/mg. After daily 15-mg doses for at least 4 months, the hair concentrations of 3 subjects under doxylamine therapy were 840–2050 pg/mg.[17]

Therefore, our result of 9 pg/mg of doxylamine is consistent with a single exposure to this drug.

Regarding 4-MEC and MDPV, no publication referring to their identification in the case of DFSA has been reported so far. One case of robbery highlighted the use of MDPV where blood concentration was 52 ng/mL.[7] The data involving other NPS in DFSA are also scarce. Kiara et al... has reported 6 methylone-positive cases in urine.[8] In regular consumers, cathinone concentrations found in hair were

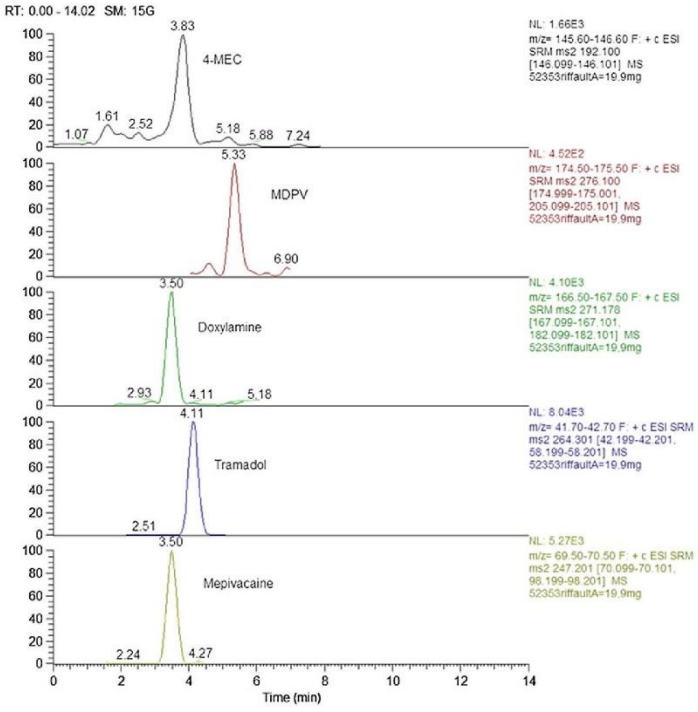


FIGURE 1 Chromatogram of the extracted segment A. A, 4-MEC; B, MDPV; C, doxylamine; D, tramadol; and E, mepivacaine [Colour figure can be viewed at wileyonlinelibrary.com]

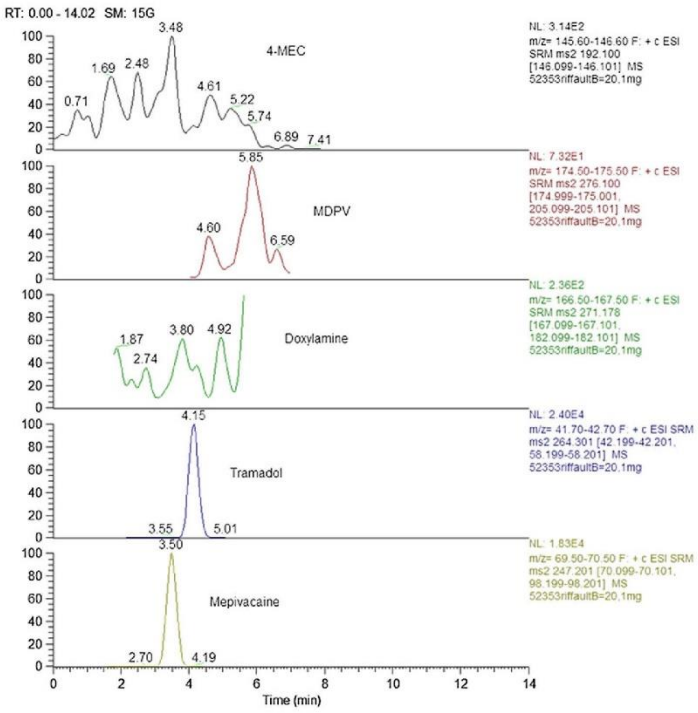


FIGURE 2 Chromatogram of the extracted segment B. A, 4-MEC; B, MDPV; C, doxylamine; D, tramadol; and E, mepivacaine [Colour figure can be viewed at wileyonlinelibrary.com]

usually high. Alvarez et al described a case of a 30-year-old man who consumed cathinones for 6 months and was admitted to the hospital for delirium and tachycardia after absorption of 10 g of 4-MEC and 5 g of MDPV in 1 week. The concentrations of 4-MEC and MDPV found in his hair were 30 ng/mg and 1 ng/mg, respectively.[9]

In our case, the concentrations of 4-MEC and MDPV were very low, and probably corresponded to single exposure. Moreover, the association of a strong stimulant (MDPV), an entactogen (4-MEC) and a central nervous system (CNS) depressant with memory loss liability (doxylamine) appears to be an effective combination to commit sexual assault and does not contradict the patient's claim about his presumed aggression. In fact, desired effects reported by users of synthetic cathinones include increased energy, empathy, openness, and libido.[18] This practice is widely documented as part of chemsex, where men have sex with men in a context of drug injection (slam) or use.[5,6,19] In that situation, the use of drugs is thought to be consensual from the 2 partners to enhance sexual stamina and libido. However, cathinone use for sexual assault has rarely been documented. This case shows a new way of NPS use beyond their recreational consumption.

Tramadol and mepivacaine had also been detected in all segments, ranging from 90 to 1840 pg/mg, and from 20 to 100 pg/mg, respectively (validation data not shown). These results reflect a repetitive exposure to tramadol[11,15] and mepivacaine between the second and third month (segment B) prior to sampling, as shown in Figure 3. Low

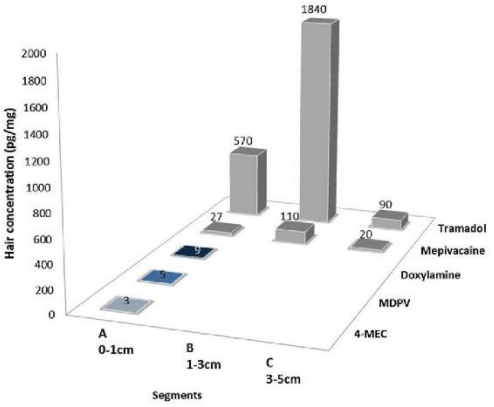


FIGURE 3 Patient exposure profile over 5 months [Colour figure can be viewed at wileyonlinelibrary.com]

concentrations found in segments A and C do not exclude the possibility of contamination by migration of these drugs from segment B, as the concentrations in this later were relatively high. For mepivacaine, we did not find any references describing hair concentrations. Thus, the concentrations we found are still hard to interpret, even though

the results appear to be in favor of a repetitive exposure to this drug. Mepivacaine is often used as an anesthetic in dental care or in surgery. Unfortunately, the dosage used in this patient was not recorded in his medical file.

However, the presence of tramadol and mepivacaine in all segments allow validating that the absence of 4-MEC, MDPV, and doxylamine from segments B and C was not related to a failure in the extraction process.

5 | CONCLUSION

This case reports the administration of cathinones as sexual assault agents, beyond their recreational use. Hair analysis has become an essential complement to conventional matrices like blood or urine to elucidate DFSA. Finally, this case stresses the need to look for designer drugs when dealing with DFSA cases.

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2. Discussion

La première étude observationnelle concerne l'évaluation de la consommation des NPS et des drogues classiques en Ile-de-France dans différentes populations d'usagers de drogues admis principalement dans les services des hôpitaux de Paris entre 2012 et 2017. Au total, 480 patients adultes (280 hommes et 200 femmes, âge ≥ 15 ans) ont été inclus. Ils ont été répartis en 4 groupes : *1)* patients potentiellement intoxiqués ; *2)* patients suivi en addictologie ; *3)* patients admis pour d'autres explorations cliniques ; *4)* patients vus ou autopsiés dans les services de médecine légale.

Cent quarante et un patients étaient positifs à au moins un NPS. La prévalence dans cet échantillon d'étude est relativement élevée (29%) par rapport à celles déjà décrites dans la littérature (0,3 à 37%) [80]. Cette prévalence reste considérable quand on la compare aussi à la prévalence des drogues classiques évaluée dans la même population (amphétamines 32% ; cocaïne 38% ; opioïdes licites et illicites 52%). Ces informations sont primordiales car elles donnent un premier aperçu du niveau de consommation de ces nouvelles drogues de synthèse, et il est fort probable que ce chiffre soit sous-estimé du fait du nombre important de NPS surveillés et de l'impossibilité de les rechercher d'une façon exhaustive et systématiquement. Il est aussi important de noter que cette prévalence était légèrement plus élevée chez les patients admis en réanimation (13%) où la piste de consommation de NPS n'a pas été évoquée systématiquement, que chez les patients suivis en addictologie pour ce type d'usage (11%). De ce fait, une attention particulière doit être portée aux NPS dans les demandes de criblage large en toxicologie d'urgence même s'ils ne sont pas directement incriminés dans l'intoxication présumée. En toxicologie médicolégale, l'implication des NPS était faible dans notre échantillon (prévalence : 1,5%). Ceci semble être lié à un échantillon non représentatif incluant souvent des recherches de cause de la mort où la piste toxique était pratiquement écartée (expertise toxicologique de routine négative), ou des cas de soumissions chimiques chez des victimes présumées n'ayant pas le profil d'usagers de drogues.

S'agissant des profils des consommateurs, la tendance de consommation de NPS dans notre étude était deux fois plus élevée chez les hommes que chez les femmes, ce que confirment la plupart des enquêtes réalisées [9,75]. Les utilisateurs de NPS étaient souvent jeunes (médiane d'âge : 33 ans) comparés aux utilisateurs de cocaïne (36 ans), d'amphétamines (38 ans) ou d'opioïdes (40 ans), et nous notons une utilisation inquiétante de ces produits parmi les jeunes parisiens de moins de 20 ans (11 % dans notre étude, moyenne européenne déclarée : 4%).

Plus de la moitié des utilisateurs de NPS (54%) avaient consommé plus d'un NPS sur une période de deux mois, et les avaient souvent associés à la cocaïne (66%), aux amphétamines (65%) ou aux opioïdes (64%). Cette population peut correspondre aux sous-groupes des ***expérimentateurs connaisseurs*** (***psychonautes***) ou aux ***habitués de l'espace festif*** (gay ou techno), déjà à l'aise avec la consommation de drogues classiques, et qui sont en quête de nouvelles expériences de consommation. Néanmoins, 32% des utilisateurs de NPS identifiés n'avaient consommés aucune drogue classique parmi celles recherchées, et pourraient correspondre au groupe des « ***novices » ou d'usagers occasionnels,*** qui sont les sujets à risque de surdosages ou de dépendance, car rappelons-le, ce sont souvent des jeunes qui se servent d'Internet pour réaliser leurs achats et sont donc peu visibles par les systèmes de soins ou de RdR. Ces consommateurs seraient aussi invisibles par l'analyse toxicologique si seul un criblage de routine, n'incluant pas les NPS, avait été réalisé.
Vingt-sept NPS ont été identifiés durant cette étude appartenant à plusieurs familles chimiques, avec une tendance à la hausse du nombre de NPS détectés entre 2012 (9%) et 2017 (28%). L'identification a culminé en 2014 où plus de la moitié des cas analysés (28/55) étaient positifs aux NPS. Cette observation dont l'origine reste inexpliquée est en parfaite cohérence avec l'enquête de l'OFDT évaluant le nombre de NPS recensé cette année-là [6]. Après 2015, le nombre de cas de NPS a diminué mais reste tout de même considérable. Cette diminution était probablement liée aux différentes mesures de contrôle prises par les gouvernements, incluant un surveillance plus strict d'approvisionnement en précurseurs de synthèse des NPS, dont certains étaient en vente libre [81].

La famille des cathinones est la plus fréquemment rencontrée dans notre étude, une observation partagée par les autres études. Cependant, des différences résident dans la nature des composés identifiés, différente d'un pays à l'autre à cause des législations imposées qui conditionnent le type de produit circulant sur chaque marché local. La 4-MEC et la méphédrone (4-MMC) sont les cathinones les plus détectées, suivies par la methylone et la MDPV. Cette observation est cohérente avec l'étude réalisée à Paris par Néfau et al., en 2012 montrant une tendance à la hausse de l'utilisation de la 4-MEC par injection [78]. Cependant, la prévalence élevée de la méphédrone malgré son interdiction peut être expliquée par l'incapacité de l'outil analytique que nous avons utilisé (chromatographie liquide) à la distinguer de son isomère, la 3-MMC, un autre NPS apparu plus tard sur le marché, et qui aurait (d'après les récits des expérimentateurs) un effet plus puissant que celui de la méphédrone. La distinction entre ces deux isomères nécessite l'emploi d'une colonne de séparation spécifique, ou une technique de

chromatographie gazeuse, ce qui n'était pas le cas dans notre étude. De ce fait, les cas positifs à la méphédrone dans notre étude peuvent correspondre à une prise seule ou simultanée de la 3-MMC.

Contrairement aux cathinones, très fréquemment rencontrées dans les études de prévalence utilisant l'analyse capillaire comme approche, les CS, le plus grand groupe de NPS saisis et surveillés en Europe, d'après le rapport de l'OEDT de 2018, n'ont été détectés dans notre étude qu'à travers un seul cas d'intoxication confirmée au JWH-122 (Cf. partie I). Dans les autres études, la prévalence variait de 1,29 à 7,82 % [65,82,83]. Cette faible identification des CS pourrait s'expliquer en partie par leur utilisation à de très faibles doses (du fait de leur effet pharmacologique très puissant), ce qui réduit leur détectabilité dans les cheveux, ou de par leur métabolisme rapide conduisant à une plus faible intégration capillaire du composé parent. Elle peut également refléter une meilleure surveillance de cette classe de NPS, ou tout simplement une préférence des usagers parisiens, orientés vers les produits stimulants et entactogènes plutôt que les dépresseurs de type CS, facilement remplaçables par le cannabis.

Nous avons inclus deux autres molécules dans la liste des NPS recherchés, bien que ce ne sont pas des produits nouveaux. Il s'agit de la kétamine (KET) et du dextrométhorphane (DXM) qui ont vu leur utilisation comme dissociatifs augmenter en milieu récréatif. La kétamine était la molécule la plus identifiée dans notre étude (104/141, 73%), dont 10 cas correspondaient à un traitement au cours de l'hospitalisation. Afin d'éviter ce biais lié à l'utilisation thérapeutique, nous avons associé les cas de kétamine à celle de la cocaïne, car cette association (connue sous le nom CK pour « Cocaine-Ketamine ») est très utilisée par les usagers de NPS et a déjà été décrite [84]. En effet, plus de 70% des usagers de la kétamine ont consommé de la cocaïne. Il en est de même pour le DXM, un antitussif, qui a été associé à d'autres drogues dans 70 % des cas, montrant son usage détourné à des fins récréatives en tant que tel, ou en remplacement des comprimés de la MDMA (ecstasy).

Le dosage quantitatif des NPS dans les cheveux a montré une disparité des concentrations retrouvées suggérant l'existence de sous-groupes de consommateurs occasionnels (concentrations généralement de l'ordre de pg/mg cheveux) et chroniques (concentrations de l'ordre de ng/mg cheveux). Cette analyse quantitative a permis aussi de décrire les concentrations capillaires de plusieurs composés jamais rapportées dans la littérature (3-fluorofentanyl, n-ethylpentylone, pentedrone, mexedrone…etc.). Ces données difficiles à

interpréter à travers le peu de cas que nous avons décrits vont certainement permettre une meilleure interprétation des niveaux d'exposition lors de futures expertises.

A travers ce travail, nous avons dressé le profil des consommateurs de NPS en région parisienne en cernant mieux leurs caractéristiques sociodémographiques, le type et le nombre de produits consommés, et les tendances quantitatives de consommation à l'aide d'une approche objective représentée par l'analyse des cheveux. Ce profil est bien illustré à travers le case-report que nous avons décrit (publications n° 8) et qui concerne l'usage de plusieurs NPS chez un homme de 43 ans, consommateur régulier de drogues classiques, devenu expérimentateur des dérivés de fentanyl et d'autres NPS comme le montre clairement le suivi réalisé par analyse de ses cheveux à un mois puis à une année suivant son intoxication sévère au 3-fluorofentanyl. Ces analyses montraient clairement comment cet ancien héroïnomane sous traitement de substitution à la méthadone a changé ses pratiques de consommation en remplaçant progressivement l'héroïne par les NOS, moins couteux d'après ses dires lors des différentes sessions de RdR auxquelles il a participé. Plusieurs NPS appartenant à différentes classes chimiques (5 dérivés de fentanyl, 2-CE, kétamine, DXM) ont été identifiés et quantifiés dans ses cheveux dont les concentrations reflétaient des expositions occasionnelles pour certains tels que le carfentanil ou l'acetylfentanyl (laissant évoquer la piste de la prise unique, de la contamination externe ou de l'adultération de l'héroïne), et régulières pour d'autres (ex : furanylfentanyl, methoxyacetylfentanyl). Des discordances entre les produits annoncés (ex : butyrylfentanyl) par le patient et ceux réellement retrouvés (3-fluorofentanyl, methoxyacetylfentanyl) confirme que plusieurs usagers de ces NPS les consomment à leur insu car souvent, les produits livrés ne correspondent pas à ceux que les consommateurs pensent avoir achetés, ce qui constitue un risque supplémentaire de surdosage et/ou de dépendance par des produits plus puissants et addictogènes. Cette exploration par les cheveux nous a aussi permis de confirmer son statut de polyconsommateur de drogues classiques (cocaïne, MDMA, méthamphétamine, amphétamine), et son usage des inhibiteurs de la 5-phosphodiestérase de type sildénafil et tadalafil semble évoquer des pratiques de chemsex.

La deuxième étude de prévalence menée au cours de ces travaux de thèse avait comme objectif d'évaluer qualitativement et quantitativement la consommation de NPS et des drogues classiques dans un groupe de HSH bénéficiant d'un traitement antirétroviral de prophylaxie (PrEP) contre le VIH associant l'emtricitabine et le ténofovir disoproxil (Truvada®) dans le cadre d'un essai multicentrique « IPERGAY » de l'ANRS. Le but était de cerner le profil de ses utilisateurs et identifier les produits consommés afin de leur proposer des programmes de

soins et de RdR adaptés, et réduire ainsi les risques liés à l'usage de drogues et les pratiques à risques (ex : rapports sexuels non protégés). 69 volontaires ont été inclus dans la présente étude. Deux approches d'évaluation ont été comparées : 1) l'analyse des cheveux et 2) l'auto-questionnaire en ligne au début de l'étude, puis chaque deux mois pendant toutes la durée de l'étude. Les résultats issus des questionnaires dressent un tableau de ses participants, plutôt jeunes (âge médian : 34,7 ans), ayant un niveau d'éducation élevé (65%) et un travail (78 %). Ils avaient jusqu'à 15 partenaires sexuels par période de deux mois, avec une fréquence des rapports sexuels allant jusqu'à 16 par mois et comportant souvent des pratiques à risques (64%) et du chemsex (27%). Au cours de cette étude, nous avons analysés par LC-MS/MS et LC-HRMS 219 segments correspondant à 137 prélèvements de cheveux.

Au regard des résultats issus de la première étude de prévalence de NPS que nous avons réalisée à Paris et ceux des études rapportées dans la littérature (prévalence entre 0,3 et 37%) au sein de différents groupes d'usagers, la prévalence de NPS chez les HSH est plus élevée (39%), et témoigne du risque plus élevé pris par ce groupe de consommateurs. De même, les niveaux de consommation de drogues classiques (particulièrement la cocaïne) en association des NPS est plus élevée. En effet, les NPS étaient respectivement associés à la cocaïne, MDMA ou kétamine dans 93%, 74 % et 70% des cas, contre 66%, 65% et 67% dans la première étude parisienne. Cependant, le type de NPS consommés semble moins diversifié chez les HSH. Ainsi, 15 NPS ont été détectés dans ce groupe contre 27 dans l'étude précédente. Ce nombre de NPS presque deux fois moins élevé que dans les autres groupes montre une consommation plus restreinte des produits et n'a pas mis en évidence l'existence significative d'un sous-groupe d'expérimentateur au sein des HSH.

La famille des cathinones reste la plus employée dans les deux études, notamment chez les HSH où les effets recherchés (stimulation, sociabilité, accentuation de la libido) correspondent parfaitement aux effets de certaines cathinones utilisées (ex : 4-MEC, 3-MMC, MDPV).

Cette étude montre l'utilité de l'analyse des cheveux et sa supériorité aux enquêtes déclaratives. En effets, l'analyse capillaire a permis d'identifier plus de NPS et drogues classiques que ceux déclarés dans les auto-questionnaires (77% versus 57%, $p < 0,05$). Ce constat est le même quand on compare ces résultats à ceux des enquêtes déclaratives précédemment publiées où le taux de déclaration variait entre 18 et 77%. Les biais multiples inclus dans les déclarations des usagers comme leur méconnaissance des produits consommés réellement, la possibilité d'oubli

par effet amnésique de certains produits (ex : GHB/GBL) peuvent expliquer ce décalage dans les résultats observés entre les deux approches.

Même si cette étude présente un biais de recrutement (HSH volontaires avec des pratiques à risques confirmées), elle montre que la consommation de drogues dans ce groupe est un facteur de risque réel qu'il faut prendre en compte car une association positive et significative a été mise en évidence entre la consommation de drogues et le nombre de partenaires sexuels, les rapports non protégés, les pratiques intenses et surtout le nombre d'IST déclarées durant l'étude. De plus, l'utilisation de NPS dans un contexte de chemsex peut être détournée pour des fins de soumission chimique d'usagers novices et moins habitués fréquentant l'espace gay tel que nous l'avons mis en évidence à travers le 2e case report (***publication n° 9***), et montre à quel point l'usage volontaire ou involontaire de ces nouvelles substances peut être dangereux.

PARTIE III : Etude du métabolisme des NPS

1. Rationnel de l'étude

Peu de données existent concernant les propriétés pharmacologiques des NPS dont le type et le nombre sont en constante évolution. L'étude des différentes voies métaboliques peut permettre de comprendre le devenir des NPS dans l'organisme et proposer ainsi des réponses efficaces quant aux traitements des intoxications (ex : dialyse) ou leur identification dans les différentes matrices biologiques. Généralement, la recherche du composé consommé (molécule mère ou composé parent) est la méthode la plus employée. Cependant, certains de ces composés possèdent des demi-vies courtes, rendant leur détection difficile. L'une des alternatives est de rechercher leurs métabolites ayant une élimination plus longue et permettant ainsi d'objectiver des intoxications ou des prises de NPS à distance de leur consommation. Pour l'analyse capillaire, la recherche des métabolites peut s'avérer très utile pour exclure une potentielle contamination externe des cheveux par la manipulation des produits ou par l'environnement. Cette étude métabolique permet également l'exploration des intéractions pharmacocinétiques entre NPS et d'autres drogues ou médicaments, et permet d'expliquer certains effets indésirables rencontrés au cours des intoxications. Pour des raisons d'éthique, les études cliniques sur les NPS ne sont pas autorisées et plusieurs modèles in vivo ou in vitro sont utilisés à ces fins. Parmi eux, les HLM sont les mieux établis et les plus fréquemment utilisés en toxicologie de par leur faible coût, leur bonne conservation (-80°C pendant des années) et leur utilisation simple [54].

La difficulté d'identification de nouveaux métabolites réside dans le choix du modèle d'étude (*in vivo ou in vitro*), mais surtout dans le développement d'outils analytiques fiables et performants permettant d'identifier de la façon la plus exhaustive possible tous les composés issus du métabolisme. Ces outils analytiques sont basés sur deux principaux axes : *1)* le développement d'une méthode de détection sensible et spécifique du composé étudié ; et *2)* le retraitement adéquat des données brutes acquises (souvent très fournies) afin d'identifier les spectres des métabolites potentiels et proposer des structures adéquates de ces derniers.

A travers nos travaux analytiques (partie I), nous avons décrits et discuté notre approche de criblage non ciblé par LC-HRMS. Cette méthode sera donc utilisée pour l'acquisition des données brutes (raw data) issues de l'incubation de 9 NPS avec les HLM, ou des échantillons biologiques de patients ayant consommé ces drogues.

A noter que le choix des NPS à étudier a été réalisé sur la base des cas rencontrés au cours de nos différentes études où nous avons pu collecter après accord des participants différents milieux biologiques (sang, urine, cheveux), ou dans le but d'apporter de données scientifiques nouvelles pour certains NPS.

Cette dernière partie (III) des travaux de thèse sera donc consacrée :

- Au développement et à la validation d'un outil (désigné « workflow ») de retraitement des données brutes, inspiré des approches métabolomiques ;
- A l'application de ce workflow à l'étude in vitro et in vivo du métabolisme de 9 NPS appartenant à la famille des hallucinogènes et anesthésiques dissociatifs (O-PCE, 3-HO-PCE, 3-HO-PCP, 3-MeO-PCP, 4-MeO-PCP, 4-HO-MET) ou à la famille des sédatifs antalgiques (3-fluorofentanyl, furanylfentanyl, methoxyacetylfentanyl) ;
- A la création d'une bibliothèque de spectres haute résolution des métabolites identifiés ;
- A la proposition d'un schéma métaboliques pour certains NPS étudiés avec identification des structures correspondantes ;
- A la comparaison des résultats obtenus aux données de littérature existantes.

Un exemple complet décrivant toutes ces étapes sera illustré à travers l'étude métabolique de la deschloro-N-ethyl-ketamine (O-PCE) (***publication n°10***).

2. Méthode

Cette partie sera consacrée uniquement à la description et l'optimisation du workflow métabolique. Tous les détails techniques relatifs à la méthode analytique par LC-HRMS et l'incubation in vitro avec les HLM sont abordés dans la partie matériel et méthode de la **publication n°10** présentée ci-après

a) Description du workflow de retraitement des données HRMS

Le workflow métabolique a été créé à l'aide du logiciel Compound Discoverer 3.1 (ThermoFisher, USA). Il s'agit d'un algorithme qui se présente sous forme de cluster de plusieurs filtres (ou nœuds) reliés entre eux (***figure 8***). Chaque nœud contient des paramètres spécifiques nécessaire au retraitement des données, que nous avons optimisé tout au long de cette étude.

Le workflow se divise en deux axes :

- Axe du « ***retraitement ciblé*** » (nœuds avec l'icône 🎯) dans lequel le NPS à étudier est indiqué spécifiquement (sa structure comportant sa masse exacte est téléchargée sous format .mol) pour permettre d'identifier ses métabolites potentiels et d'indiquer leurs structures.
- Axe du « ***retraitement non ciblé*** » (nœuds sans l'icône 🎯) dans lequel la recherche est effectuée sur la librairie « mzCloud » afin de savoir si le NPS étudié (dont la nature est inconnue), ses métabolites potentiels ou d'autres composés ayant des structures similaires (même classe chimique) ont déjà été identifiés et incrémentés dans cette librairie.

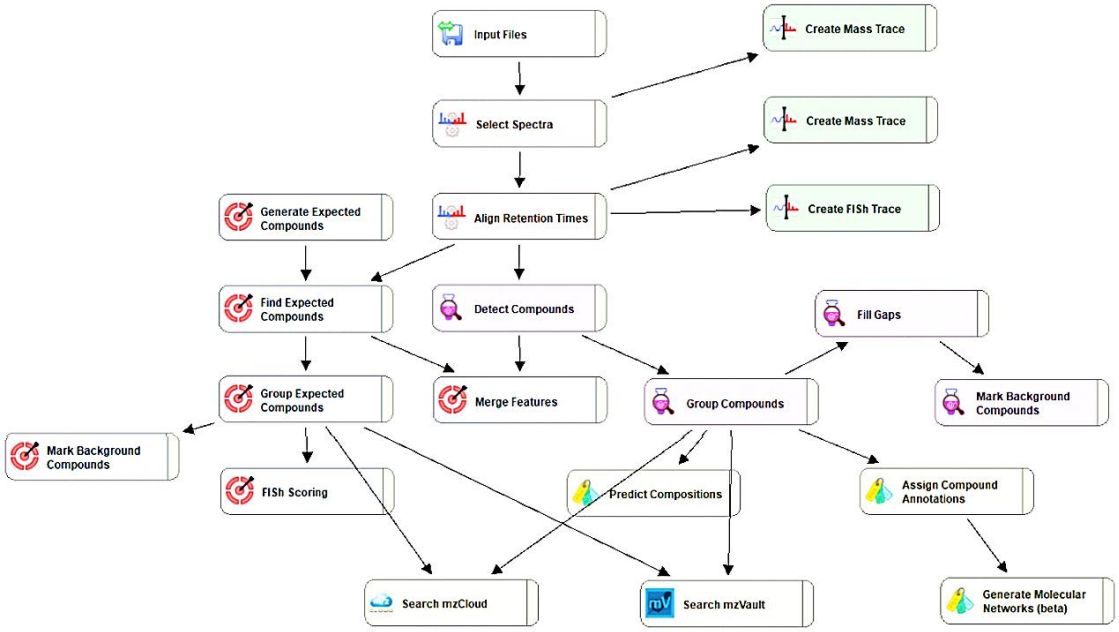


Figure 8: Workflow dédié à l'étude métabolique des NPS.

Les fonctions des principaux nœuds peuvent être sommairement décrites ainsi :

- « **Select spectra** » : permet de filtrer les spectres de masse ;
- « **Align retention time** » permet de compenser les différences légères de temps de rétention observées à chaque injection (analyse) ;
- « **Generate expected compounds** » permet de prédire des fragments à partir de la molécule mère indiquée et des réactions de métabolisme préalablement renseignées, « **Find expected compounds** » : recherche les fragments générés par le nœud précédent.
- « **Fill gaps** » est un filtre qui recherche des pics chromatographiques détectés par le nœud « Detect compounds » dans un des fichiers (échantillon) mais manquants dans un autre.
- « **Mark background compounds** » : met en évidence les composés également présents dans le blanc (contrôle négatif) afin de les soustraire de l'analyse.
- « **Fish Scoring** » : fournit un score de confirmation du composé détecté et permet d'annoter les structures possibles des fragments générés.
- « **Merge features** » combine les composés similaires (générés par des fragmentations répétitives) listés par « **Find expected compounds** » et « **Detect compounds** » grâce aux temps de rétention et les valeurs de m/z exactes.
- « **Predict composition** » propose une formule brute des fragments.

- « **Assign compound annotation** » a servi à sélectionner les données adéquates pour le nom, la formule et la structure à partir de différentes sources.
- « **Search mzCloud** » et « **Search MzVault** » permettent de comparer les fragments obtenus à la librairie mzCloud ou à une librairie de spectres interne MzVault introduite par l'utilisateur (dans notre cas celle des métabolites identifiés en cours de création).

b) Fonctionnement

Après analyse par LC-HRMS des incubats d'HLM avec le NPS étudié, les données brutes (.raw) sont récupérées et chargées dans le logiciel Compound Discoverer. A l'aide des paramètres préalablement optimisés pour chaque nœud du workflow, l'algorithme métabolique retraite les données et assigne tous les pics ayant déclenché une acquisition en mode ddMS2. L'étape « Fish scoring » permet de proposer une structure pour chaque pic avec les réactions ayant conduit à sa formation.

Une étape manuelle et très longue consiste ensuite à scruter scrupuleusement toutes les lignes générées afin d'identifier les métabolites les plus probables en fonction de leur poids moléculaires, formules brutes, spectres ddMS2 et leurs intensités (**figure 9**).

Les métabolites obtenus dans les HLM sont ensuite confrontés à ceux issus de l'analyse de milieux biologiques de patients ayant consommé la même drogue et de contrôles négatifs (blancs). Enfin, les métabolites validés (présents dans les HLM et milieux biologiques et absents des blancs) sont alors nommés et une proposition d'un schéma métabolique global regroupant le NPS étudié ainsi que ses potentiels métabolites est réalisée à l'aide d'un 2^{e} logiciel (Mass Frontier 8.0) selon les règles de base en pharmacocinétique et en chimie organique.

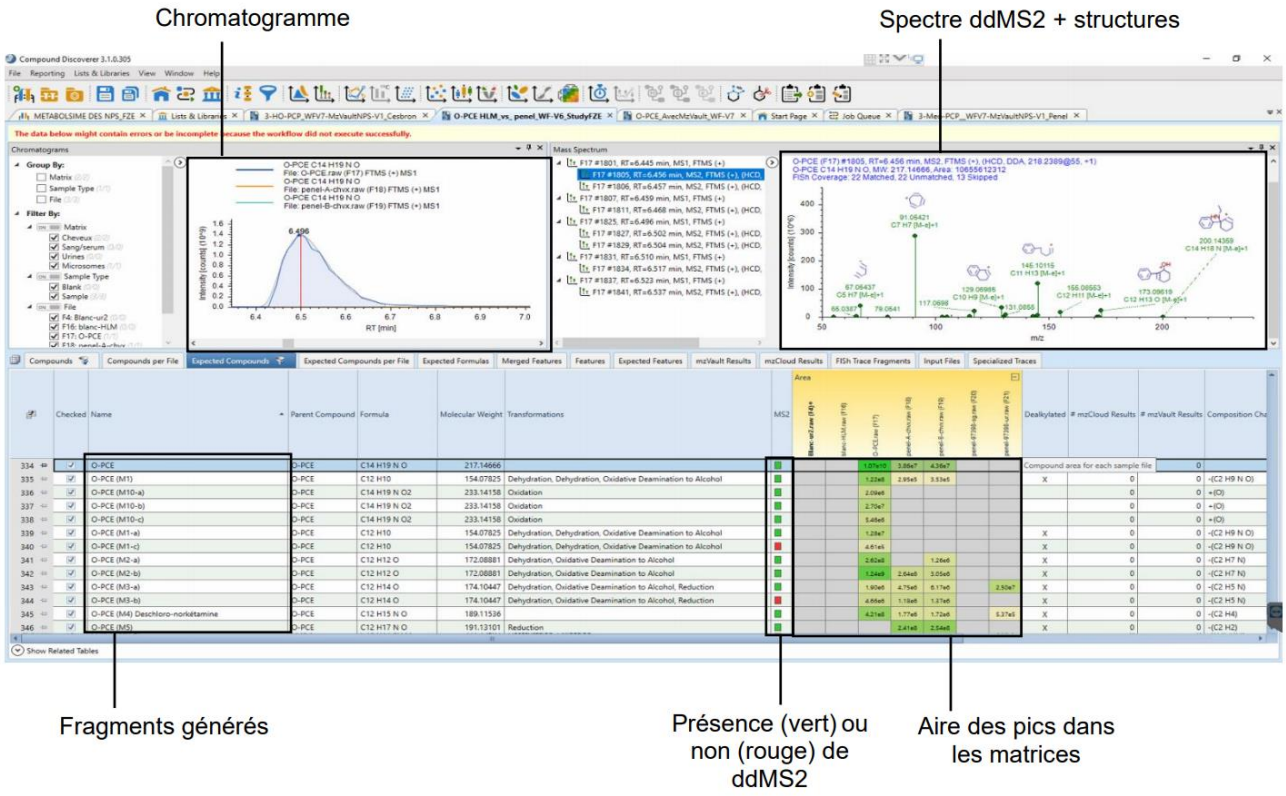


Figure 9: Exemple d'un traitement des données brutes issues de l'analyse de l'O-PCE par LC-HRMS

c) Création de la librairie de spectres des métabolites (MzVault)

Une fois les métabolites d'intérêt identifiés et validés, les données spectrales acquises en haute résolution sont ajoutées à une librairie nouvellement créée et baptisée « NPS_Métabolites » en utilisant l'interface MzVault intégrée au logiciel Compound Discoverer.

Cette librairie qui sera enrichie continuellement a été intégrée au workflow métabolique (sous forme d'un nœud) dans sa dernière version pour permettre d'identifier ultérieurement les métabolites préalablement incrémentés.

3. Résultats

A. Résultats généraux issus de la validation du workflow par analyse des 9 NPS étudiés

Quatre versions du workflow comportant chacune des optimisations des paramètres de retraitement des données étaient nécessaires avant d'aboutir au workflow le plus optimal dont les paramètres sont présentés dans le tableau 2.

Tableau 2: paramètres optimaux du workflow de retraitement des données métaboliques

Nœud	Paramètre	Valeur
Select spectra	Intervalle de masse de l'ion précurseur	[100-1000] Da
Align retention time	Décalage maximum de Rt	0.5 min
Create Fish Trace	Composé Tolérance de la mass Polarité du scan Mode de fragmentation	Ex : O-PCE 10 ppm + Data indépendant
Generate expected compounds	Composé Bio-transformations Ionisation	Ex: O-PCE Phase I/ Phase II Positive [M-H]+
Find expected compounds	Mass tolerance Intensité minimale du pic	5 ppm 10 000
Group expected compounds Group compounds	Tolérance du Rt	0.3 min
Mark background compounds	Ratio du signal sur bruit de fond (S/N)	3
Fish Scoring	Seuil S/N masse minimale m/z d'un fragment Tolérance de masse pour les spectres HRMS Tolérance de masse pour les spectres de basse résolution	3 50 5 ppm 0 ppm
Merge features	Tolérance du Rt (largeur pic)	0.5 min
Detect compounds	Tolérance de la masse Seuil d'intensité Ionisation Largeur maximale de pic	5 ppm 3 Positive [M-H]+ 0.5
Fill gaps	Tolérance de masse Seuil S/N	5 ppm 1.5
Predict compositions	Tolérance de masse Nombre d'éléments attendus utilisés pour la prédiction Nombre maximal de composés candidats Tolérance d'intensité Seuil d'intensité Seuil S/N Pourcentage minimal pour match de spectre	5 ppm CH / $C_{90}H_{190}Br_3Cl_4N_{10}O_{18}P_3S_5$ 10 30% 0.1% 3 30%
Assign compounds annotations	Tolérance de masse Data source #1 Data source #2	5 ppm MzCloud Search Predicted compositions
Search MzCloud	Classe de composé Tolérance de la masse du précurseur Recherche de l'identité Recherche de similarité Seuil du facteur de correspondance	Médicaments, drogues, produits dopants, 10 ppm High Chem High Res Similarity forward 60
Search MzVault	Librairie de métabolites créé Algorithme de recherche Seuil du facteur de correspondance	NPS_V1 High Chem High Res 50

L'application de ce workflow aux 9 NPS a permis d'identifier plusieurs métabolites, certains communs à ceux déjà rapportés dans la littérature tandis que d'autres sont décrits pour la première fois. Le nombre de métabolites identifiés in vitro et in vivo pour les 9 NPS étudiés sont présentés dans le tableau 3 et sont comparés aux données de la littérature disponibles.

Tableau 3: NPS étudiés, nombre total et par matrice des métabolites identifiés in vitro (HLM) et in vivo (plasma, urine, cheveux) comparé aux données de la littérature

	Nombre de métabolites identifiés							
NPS	HLM	Plasma	Urine	Cheveux	Total	Littérature	Référence	
O-PCE	17	N/A	10	11	23	Non décrit	N/A	N/A
3-HO-PCE	12	N/A	4	9	19	Non décrit	N/A	N/A
3-HO-PCP	15	N/A	7	4	17	4	Davidsen et al., (2020)	[85]
3-MeO-PCP	22	5	8	10	29	5	Ameline et al., (2019)	[86]
4-MeO-PCP	21	3	6	8	26	Non décrit	N/A	N/A
4-HO-MET	7	11	24	28	38	15	Bruni et al., (2018)	[87]
3-FF	15	N/A	7	14	22	Non décrit	N/A	N/A
Fu-F	26	N/A	11	15	34	17	Richeval et al., (2017)	[57]
						15	Goggin et al., (2017)	[88]
						17	Labutin et al., (2017)	[89]
						14	Watanabe et al., (2018)	[58]
MeO-AF	19	N/A	12	3	20	10	Mardal et al., (2018)	[90]
						21	Nordmeier et al., (2019)	[91]

N/A: non disponible ou non-applicable ; Fu-F : Furanylfentanyl ; MeOAF : Methoxyacetylfentanyl ; 3-FF : 3-Fluorofentanyl

Du fait des données de retraitement très fournies de par la sensibilité de notre méthode d'analyse LC-HRMS et du workflow de retraitement développé, une centaine de signaux pouvant correspondre à de potentiels métabolites ont été détectés. L'étude approfondie du profil métabolique est une procédure très longue conditionnée par une analyse manuelle minutieuse de chaque signal formellement identifié (existence de spectre de masse ddMS2, masse exacte et formule et identifiés) avant de permettre d'élaborer un profil métabolique complet avec les structures et la voie de synthèse de chaque composé.

Au moment de la rédaction du présent manuscrit, l'étude est achevée pour l'O-PCE (comme nous allons le voir à travers la publication n° 10), elle est en cours de finalisation pour le 3-FF et la 3-HO-PCE, deux NPS dont le métabolisme n'a jamais été décrit auparavant (calcul des ratios métaboliques et schémas de fragmentation en cours d'élaboration). Pour l'ensemble des autres NPS, l'analyse est en cours. Nous exposons ci-après les principaux résultats que nous avons obtenus pour l'ensemble des NPS (excepté l'O-PCE qui sera développée plus loin), chacun devant faire l'objet d'une publication :

- Dix-sept métabolites de la 3-HO-PCP ont été identifiés dont 15 in vitro (HLM). Trois des 4 métabolites déjà identifiés très récemment par Davidsen et al., [85] ont été identifiés par le présent workflow. Parmi nos résultats, 7 métabolites ont été identifiés dans les urines d'un consommateur, et 4 ont été confirmés dans ses cheveux. Les réactions d'oxydation et de N-désalkylation prédominent les réactions de métabolisation de phase I, quant à la phase II, on observe majoritairement des réactions d'O-glucuronoconjugaison.
- Pour le 3-fluorofentanyl, nos analyses ont permis de mettre en évidence 22 métabolites potentiels, avec la confirmation de 7 d'entre eux dans l'urine et 14 dans les cheveux. Les réactions principales de phase I correspondent à la désamination et désfluorination oxydatives, et une O-glucuronoconjugaison pour la phase II.
- Ameline et al., [86] a identifié cinq métabolites de la 3-MeO-PCP, tous ont été confirmés dans notre étude parmi les 29 métabolites que nous avons identifiés. Cinq métabolites sont retrouvés dans le plasma, huit dans l'urine et 10 dans la matrice capillaire d'un usager de cette substance.
- Vingt-six métabolites jamais décrits ont été mis en évidence pour la 4-MeO-PCP, dont certains sont partagés avec son isomère de position, la 3-MeO-PCP. Les principales voies métaboliques de phase I sont l'oxydation et la désamination oxydative, celles de phase II sont dominées par l'O-glucuronoconjugaison.
- Bruni et al., [87] a décrit 15 métabolites de la 4-HO-MET, dont 9 étant des isomères. Nous avons détecté formellement 5 des 15 métabolites identifiés parmi les 38 signaux détectés et en cours d'interprétation (pouvant correspondre à de potentiels métabolites), dont 11 dans le plasma, 24 dans les urines et 28 dans les cheveux d'un usager de cette substance.
- Le furanylfentanyl est la molécule la plus documentée en termes de métabolisme parmi celles que nous avons étudiées. Jusqu'à 17 métabolites ont été identifiés dans les différentes études. Nous avons pu identifier 34 métabolites, 9 métabolites sont communs avec ceux retrouvés par Richeval et al., [57], 7 avec ceux de Watanabe et al., [88], 6 avec ceux identifiés par Labutin et al., [89] et 4 en communs avec ceux de Goggin et al., [88]. Notre étude a permis de mettre en évidence 21 métabolites dont 9 isomères du furanylfentanyl non-identifiés à ce jour. Nous avons confirmé la présence de 11 métabolites dans l'urine, et 15 dans les cheveux d'un usager de ce NPS. Les principales biotransformations observées correspondent à l'oxydation, nitro-réduction et désamination oxydative pour la phase I, et des réactions de O-glucuronoconjugaison pour la phase II.
- Enfin, Nordmeier et al., [91] a décrit 21 métabolites du methoxyacetylfentanyl, chacun d'entre eux est détecté dans notre étude, de même pour les 10 métabolites décrits par Mardal et al., [90]. De plus, nous avons mis en évidence trois métabolites non décrits dans ces deux études. 12 métabolites sont confirmés dans l'urine et 3 le sont dans la matrice capillaire.

B. Exemple de l'approche complète de l'étude métabolique appliquée à la Deschloro-N-ethyl-ketamine (O-PCE)

Publication n° 10

Metabolic Profiling of Deschloro-N-ethyl-ketamine (O-PCE) and identification of new target metabolites in urine and hair using human liver microsomes and high-resolution accurate mass spectrometry

Islam Amine Larabi, Fanny Zerizer, Alice Ameline, Isabelle Etting, Pascal Kintz, Jean Claude Alvarez

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Metabolic Profiling of Deschloro-N-ethyl-ketamine (O-PCE) and identification of new target metabolites in urine and hair using human liver microsomes and high-resolution accurate mass spectrometry

Running head: Deschloro-N-ethyl-ketamine (O-PCE) metabolism

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Abstract

The aim of this study was to identify new markers of O-PCE, a ketamine analog that has been involved in acute intoxications with sever outcomes including death and whose metabolism has never been studied before. In vitro study after 2h incubation with pooled human liver microsomes (HLMs) cross-checked by the analysis of urine and hair from a 43 years-old O-PCE user (male) were performed by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Acquired Data were processed by an advanced metabolic workflow with Compound Discoverer® software and a full metabolic profile of O-PCE was proposed. Seventeen metabolites were identified in HLMs, ten were confirmed in biological specimens. While O-PCE was no longer detected in urine, 10 metabolites were identified allowing to increase its detection window. Deoxo-deschloro-norketamine-*N*-glucuronides (42% of metabolites), 1-phenyl-2-cyclohexenol (20%), dehydro-deschloro-norketamine-*O*-glucuronide (19%), and 3-hydroxy-PCE (11%) were the most abundant metabolites in urine. In hair, dehydro-deschloro-norketamine (77%) was the major detected compound with a relevant metabolite to parent drug ratio (= 6). We recommend dehydro-deschloro-norketamine-*O*-glucuronides, 1-phenyl-2-cyclohexenol, deoxo-deschloro-norketamine-*N*-glucuronide and 3-hydroxy-PCE as target metabolites to increase the detection window of O-PCE in urine. In hair, dehydro-deschloro-norketamine seems the best marker for long-term monitoring of O-PCE exposure, as it integrates well into the hair matrix.

Keywords

O-PCE, deschloro-N-ethyl-ketamine, human liver microsomes, high resolution mass spectrometry, new psychoactive substances, hair, data processing, workflow

Introduction

Hallucinogens are a diverse group of naturally occurring or synthetic drugs that produce altered perception of reality known as "Synaesthesia" observed with *classic hallucinogens* (e.g. LSD, dimethyltryptamine, 5-MeO-DMT…etc.), also referred to as "psychedelics", or a feeling of detachment and dissociation from the self and the environment encountered with *dissociative anaesthetics* (e.g. ketamine, phencyclidine, methoxetamine…etc). Classic hallucinogens act as serotonin receptor agonists while dissociative anaesthetics possess both stimulant and dissociative properties by inhibiting the reuptake of dopamine, norepinephrine and serotonin, and modulating effects at the N-methyl-D-aspartate (NMDA) receptor in the brain [1]. New psychoactive substances (NPS) are a range of drugs that mimic established illicit drugs of abuse, such as cannabis, MDMA and ketamine. In 2017, hallucinogens accounted for 18% of the 492 NPS reported to the UNODC (United Nations Office On Drugs and Crime) [1].

In the last decade, recreational use of ketamine has gained popularity, especially in Asia (India, China), and part of Europe (Netherlands, Belgium, France). Among the 47 countries reporting ketamine seizures to UNODC over the period 2001–2017, 21 were in Europe and 16 in Asia [1,2]. By slight chemical modification on ketamine scaffold, a wide variety of compounds with similar or even more pronounced effects have been clandestinely synthesized. Among these, deschloro-N-ethyl-ketamine, known to as "Eticyclidone", "2-oxo-PCE" or "O-PCE" has reappeared in 2016 for the first time in France, after early studies on its anaesthetic effect conducted by Stevens in 1966 to overcome phencyclidine (PCP) adverse effects [3,4]. O-PCE is an aryl-amino-cyclohexan-2-one derivative that share with ketamine and methoxetamine (MXE) the core structure of 2-phenyl-2-aminocyclohexanone. Ketamine has a chloro substituent at the 2-position of the phenyl ring and a methyl substituent on the amine group, O-

PCE has an ethyl substituent on the amine group only, and MXE has a similar structure than O-PCE with an additional methoxy group at the 3-position of the phenyl ring (Fig.1) [5].

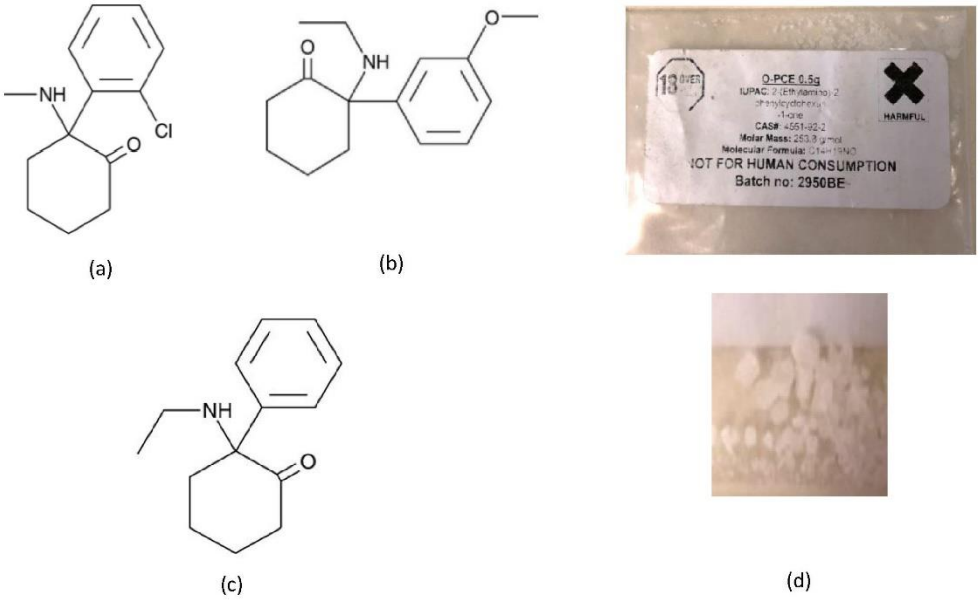


Fig.1. Structure of Ketamine (a) and its derivatives methoxetamine (MXE) (b), and O-PCE (c) as a crystalline solid (d) labelled "not for human consumption" and used in the present study.

Whilst extensive pharmacological and toxicological data are available for ketamine, and to some extent for MXE, only few overdose cases with analytically confirmed acute O-PCE toxicity have been reported recently [6,7]. The main clinical symptoms associated with O-PCE use included impaired consciousness (84%), confusion (60%), abnormal behaviour (44%), hypertension (80%), tachycardia (40%) and convulsions (16%). The toxicity of O-PCE was reported to be more severe than ketamine as it seemed more potent at low dose [6]. Moreover, O-PCE can be used alone, or in mixture with ketamine or other drugs of abuse (methamphetamine, MDMA, pentylone), which could lead to more toxicity as reported by Cheng et al., [5]. In this last study, O-PCE and its metabolite deschloro-norketamine were detected in blood of four 4 DUID (Driving Under The Influence) cases in the range of 80 – 310

ng/mL and 40 – 90 ng/mL, respectively [5]. Knowledge about the metabolic pathways allows method development targeting the most relevant markers of drug intake, including parent drug and/or metabolites. It also explores drug-drug interaction, which may explain some of the reported effects. For ethical reasons, studies in humans are not possible to perform. Hence, in vivo animal experiments or in vitro approaches are employed as an alternative [8]. While studies using animals require specific facilities and scientific background, in vitro metabolism models seem more convenient in most forensic laboratories equipped with mass spectrometry and/or nuclear magnetic resonance (NMR) based instruments. Human liver microsomes (HLMs) are among the most well established and frequently used in vitro tools in forensic and clinical pharmacology and toxicology [9–15]. This model allows to investigate the formation of phase I and some phase II metabolites (e.g. glucuronides), and to elucidate their chemical structure, and the kinetic of formation, mostly by using high resolution mass spectrometry [16].

To our knowledge, there are currently no data regarding O-PCE metabolism, thus the aim of this study was to elucidate its metabolic profile using HLMs and to identify new target metabolites that can be used as markers for its detection in man. The proposed metabolic pathways were validated by analysing urine and hair specimens from a O-PCE user by high-resolution accurate mass spectrometry (HRMS).

Experimental procedures

Chemicals and reagents

Alamethicin (*Trichoderma viride)*, tris hydrochloride (HCl), magnesium chloride anhydrous, uridine 5'-diphosphoglucuronic acid tris (UDPGA), glucose-6-phosphate dehydrogenase (G6PDH), D-Glucose 6-phosphate (G6P), reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH, 98%) were provided by Sigma-Aldrich (France).

A pool of human liver microsomes (HLMs) at a concentration of 25 mg of microsomal protein/mL obtained from 17 donors (7 female and 10 male) aged 31-78 years was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and stored at -80°C until use.

Acetonitrile (ACN), chloroform, isopropranol, ethyl acetate, formic acid, hexane and methanol (MeOH) were purchased from Sigma Aldrich (Paris, France) in MS or high-performance liquid chromatography (HPLC) grades; sodium carbonate and hydrogen carbonate were from Prolabo (Paris, France). Ultra-pure water (18 MΩ) was obtained by ultrafiltration with a Q-Pod (Millipore Corp., Molsheim, France). Formate buffer containing 2 mM ammonium formate in 0.1% formic acid was prepared in ultra-pure water and stored at +4°C away from light for a maximum of one week. Methanolic solutions of O-PCE were prepared from a crystalline solid (purity: 99%) at 1 mg/mL and 0.1 mg/mL, and stored at -20°C.

Nuclear Magnetic Resonance (NMR) analysis

The purity of O-PCE purchased from the Internet by a drug user and recovered during harm reduction session was assessed by NMR analysis, according to a previously published procedure [17]. Briefly, an aliquot of 10-30 mg of material is dissolved in deuterated methanol and analyzed. Proton 1H NMR, carbon 13C, and DEPT carbon 13 NMR were performed. Two-dimensional (2D) NMR were also performed: Correlated Spectroscopy (COZY), Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Link Correlation (HMBC). NMR spectra were recorded with Brüker Avance spectrometers operating at 300 MHz and equipped with BBFO probe.

In vitro incubation with human liver microsomes

The procedure derived from previous publications [13,15]. 100 µL of HLM solution (25 mg/L) were activated by 250 µL of alamethicin (50µM) on crushed ice after adding 150 µL of Tris-HCl (100mM)-$MgCl_2$ (10mM) buffer solution (pH 7.4). Fifty microlitres of this mixture were added to a vial containing 50 µL of O-PCE solution (394 µM) previously dried. 50 µL of a cofactor solution (5 mM UDPGA, 3.2 mM NADPH, 8.5 mM G6P and 0.5 U/mL G6PD) prepared in the same buffer (Tris-HCl-$MgCl_2$) were added. The enzymatic reaction was monitored for 2 hours at 37 °C, and was quenched with 200 µL of MeOH. Samples were

centrifuged at 4 °C for 14 min at 14000 rpm and 10 µL of the supernatant were injected onto the chromatographic system.

A zero point (blank HLM) consisting of HLM incubates without addition of substrate (O-PCE) was analysed simultaneously to subtract background signal related to matrix effect.

Liquid chromatography-high resolution mass spectrometry (LC–HRMS) analysis

Sample preparation

Urine/hair

200 µL of non-hydrolysed urine or 20 mg of decontaminated hair underwent liquid-liquid extraction using 4 mL of hexane/ethyl acetate (1/1, v/v). After agitation and centrifugation for 20 min, the organic phase was recovered and the aqueous phase underwent a second extraction by 4 mL of chloroform/isopropanol (80/20, v/v). The two organic phases were merged and evaporated to dryness. The residue was reconstituted in 80 µL of mobile phase and 10 µL were injected onto the chromatographic system.

Instrument conditions

The LC-HRMS procedure has already been published [18]. Briefly, chromatographic separation in gradient mode at a flow rate of 500 µL/min was performed in 20 min on an Ultimate 3000 pump (Thermo Fisher, Les Ulis, France) using a phenyl-hexyl column (PH, 100x2.1 mm i.d., 2.6 µm particle size, Thermo, Waltham, USA) maintained at 40°C. Compounds were detected using an Orbitrap accurate mass spectrometer (Q- Exactive, Thermo, Waltham, USA) equipped with a heated electrospray ionization (HESI) probe operating in positive-ion mode. Data were acquired in Data Dependent Acquisition (DDA) mode. MS cycles were composed of 1 Full MS scan and up to 5 data-dependent MS/MS scan (Full MS/ddMS2). The 5 ions with the most intense signal detected in the full MS experiment scan (intensity threshold, $1.0x10^5$) triggered a specific MS/MS spectrum. An exclusion list allowing to achieve the lowest limit of identification (LOI) was built from raw files containing solely the mobile phase. Full MS settings were: resolution, 70000 FWHM; scan range, m/z 125–650; automatic gain control

(AGC) target, $1.0x10^6$; and maximum injection time (MIT), 10 ms. ddMS2 settings were: resolution, 17500 FWHM; AGC target, $1.0 x10^5$; MIT, 100 ms; Isolation mass window, 1 m/z, normalized collision energy (NCE), 55 %; dynamic exclusion time, 9s. The mass spectrometer was daily calibrated over a mass range 50–2000 m/z. Data were acquired with Xcalibur software (v.4.0, Thermo, Waltham, USA)

Data processing and metabolites' rates calculation

Raw data were processed with Compound Discoverer 3.1 software (Thermo, Waltham, USA), and metabolic profiles were generated using MassFrontier 8.0 software (Thermo, Waltham, USA). Fragmentation patterns of O-PCE were studied taking into consideration those of ketamine and methoxetamine [9,10,19].

The identification of compounds was performed by a specific metabolic workflow including phase I (e.g. oxidation, reduction, hydrolysis…etc.) and phase II (e.g. glucuronide conjugation, sulfate conjugation…etc.) reactions. After uploading O-PCE chemical structure (in .mol format), raw data of HLM incubate was processed after background subtraction. A prediction of potential metabolites, transformation pathways and corresponding structures were given according to exact mass, elemental composition, isotopic pattern, and ddMS2 spectra.

In HLMs, metabolites 'rates (%) were expressed as the ratio of peak area of a metabolite to total peak area of all metabolites, excluding O-PCE peak area (corresponding to the remaining fraction after 2h incubation). However, in biological samples (urine, hair), O-PCE peak area whenever present was taken into account in the calculation of total peak area as it represented the fraction of unchanged O-PCE excreted in urine or as a parent drug integrated into the hair shaft (Table 1).

Application to authentic samples

Urine and hair samples were taken simultaneously along with a sample of crystalline solid labelled as O-PCE from a known O-PCE user (43 years, male) after giving informed consent

during harm reduction session, few days after his last O-PCE intake (by snorting). Due to this long delay, urine and hair were sampled, the later was most likely to reveal O-PCE exposure as it offers a wide detection window. Two segments of hair A (proximal, 0-2cm) and B (2-4 cm) reflecting an average exposure period of 4 months based on hair growth of 1cm/month [20] were analyzed. Crystalline solid was also recovered and analysed by the procedure described above. Raw data generated by LC-HRMS were processed by the metabolic workflow to cross-check the metabolites previously identified in HLM incubate. Free urine and hair samples were analyzed and processed simultaneously to subtract background signal. Validated metabolites from HLMs and authentic samples were included to the final metabolic pathway scheme.

Results and discussion

Identification of deschloro-N-ethyl-ketamine (O-PCE) by NMR

Data of 1H, 13C and 2D NMR (COZY, HSQC and HMBC) formally confirmed the structure of 2-(ethylamino)-2-phenylcyclohexanone (C14H19NO), known as deschloro-N-ethyl-ketamine or O-PCE. The product was pure at 99%, with impurities $\leq 1\%$. The carbon detected at 207.17 ppm (a) confirmed the presence of ketone group in the aliphatic cycle. The carbon in the position 5 was characteristic of an alpha carbon of an amine. 1H NMR and 13C NMR O-PCE spectra are presented in supplemental data Fig.1 (a, b).

O-PCE ddMS2 fragmentation

O-PCE eluted at 6.5 min ([MH^+], m/z 218.15379) and was fragmented by neutral loss of water (m/z 200.14359) or ethylamine group (m/z 173,09596) followed by secondary neutral losses: dehydration (m/z 155.08543), carbon monoxyde (m/z 145.10118), ethanone (129.06985), propanone (117.06985), butanone (105.0696), pentanone (91.05421). Further fragmentations involved a cleavage between alicyclic and cyclohexanone rings leading to a loss of N-ethyl-2-oxo- cyclohexylamine (79.05441) group, or by alicyclic ring degradation generating N-ethyl-

1-methyl-2-oxo- cyclohexylamine group (67.05437). O-PCE ddMS2 spectrum and assigned fragmentation patterns are given in Fig.2.

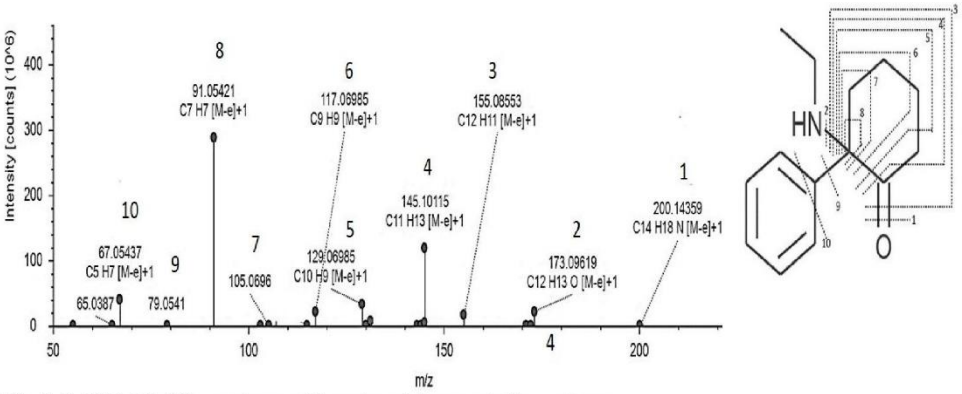


Fig.2. O-PCE ddMS2 spectrum with assigned fragmentation patterns.

O-PCE metabolites in HLMs

After 2 h incubation with HLMs, seventeen metabolites were identified, counting for 17% of total peak area (TPA), 83% being that of unchanged O-PCE that did not undergo biotransformation. Among these, 10 features with the same exact mass and different retention indices corresponded to potential isomers. Three major metabolites were detected: phenylcyclohexan-2,5-dienol (M2a/b isomers) and deschloro-norketamine (M4), represented 86% of total peak area of metabolites.

O-PCE and detected metabolites, with proposed names, biotransformation reaction, exact mass, formula, composition change with added/lost group(s) and their rates in different matrices (HLM, urine, hair) with corresponding retention times are given in Table 1. Metabolites and isomers are sorted in ascending order of m/z and retention times, respectively. ddMS2 spectra with fragmentation patterns of metabolites sorted according to m/z were provided in supplemental data Fig. 2. ddMS2 spectra of isomers were presented once with the most prevalent (most stable) structure. ddMS2 of M3b and M10a were not provided as their signal in full MS were not sufficient to trigger a ddMS2 experiment. A proposed metabolic

pathway combining all metabolites of O-PCE identified in HLMs and authentic samples are presented in Fig. 3

Extracted ion chromatograms of O-PCE and its related metabolites are given in Fig.4.

O-PCE phase I metabolism involved three main metabolic pathways: N-dealkylation, oxidation and reduction. Further predominant reactions implied oxidative deamination from reduced metabolites. Phase II metabolism reactions were characterized by *O- or N*-glucuronidation of hydroxylated and dealkylated metabolites, respectively.

Overall, O-PCE metabolism seemed similar to that of methoxetamine, as described by Menzies et al., [10].

1. N-dealkylation

N-deethylation of O-PCE to the primary amine led to the formation of deschloro-norketamine, which was the second most prevalent metabolite detected in HLMs, the first being phenylcyclohexa-2,5-dienol (M2b). Deschloro-norketamine represented 19% of TPA, and was the sole metabolite of O-PCE known so far [5].

N-deethylation was also observed as a result of the biotransformation of 2 or 3-hydroxy-PCE (M9a/b isomers) and hydroxy or phenol-O-PCE (M11a/b/c isomers) leading to the formation of dehydro-deschloro-norketamine (M5) and hydroxy or phenol-deschloro-norketamine (M7), respectively. Dehydro-deschloro-norketamine was not detected in HLMs incubates while M7 was of minor relevance (<1% TPA).

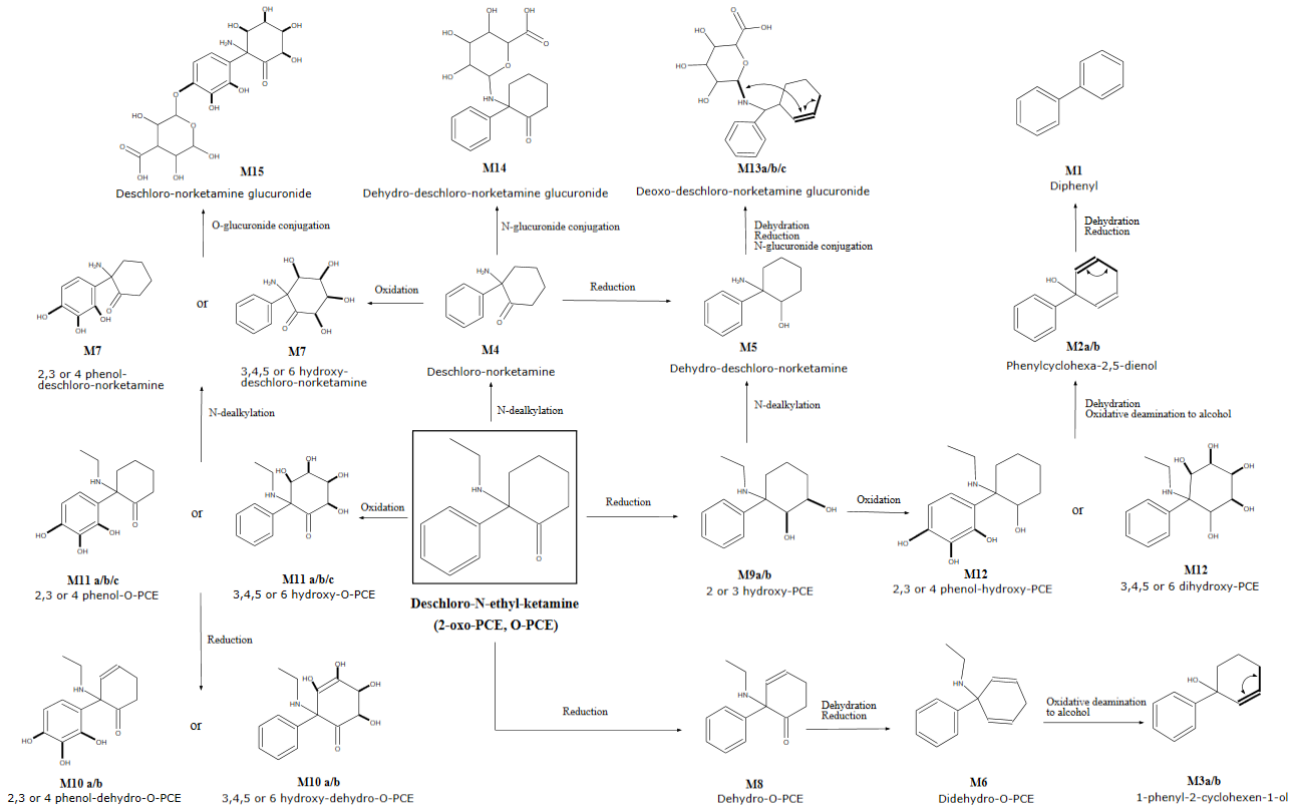


Fig.3. Proposed metabolic pathways of O-PCE in man

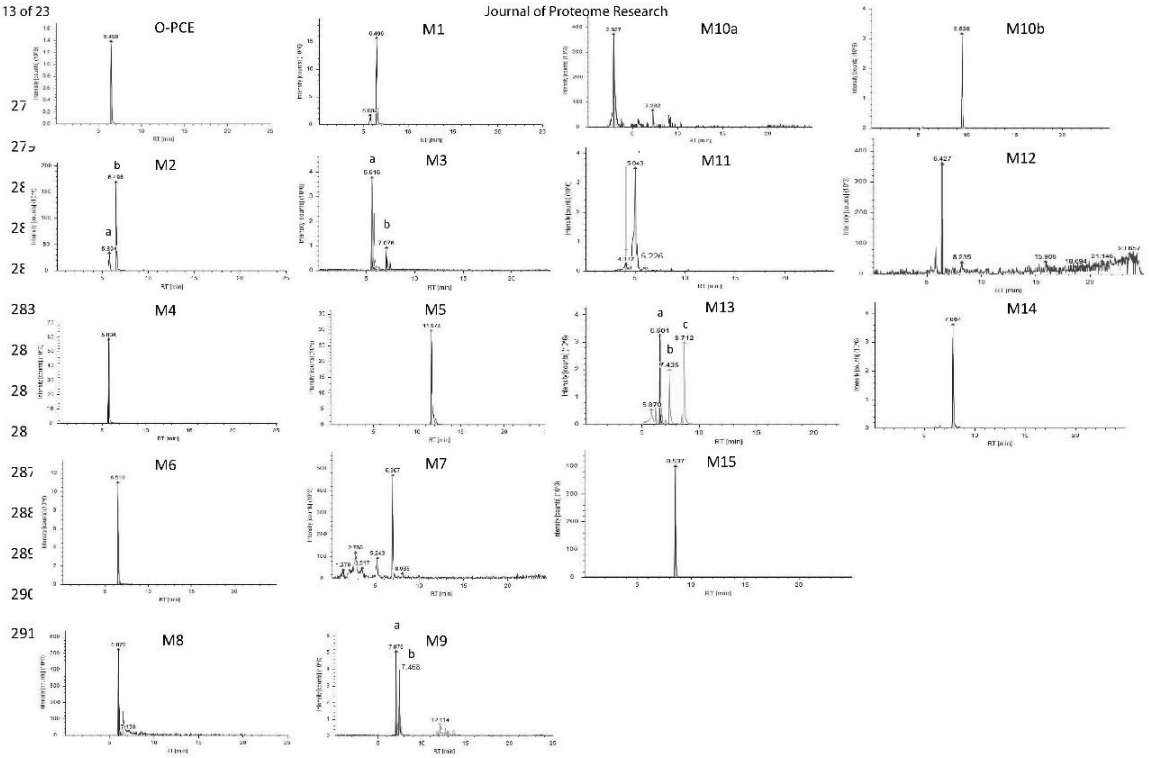


Fig.4. Combined extracted ion chromatograms of O-PCE and its metabolites detected in HLMs and authentic samples

2. Reduction

Different reactions involving the reduction of O-PCE or one of its metabolites were observed. Reduction of the ketone group on O-PCE scaffold generated hydroxy-PCE. This reaction was indicated by the +2.0156 Da (+2H) shift observed from parent drug. The mass difference was observed for fragments 173.09622, 155.0855, 131.0491 and 67.05437, but not for fragment 105.06959 (representing the benzene ring), which confirmed the reduction on the aliphatic ring (Supplemental Fig.2). Two peaks corresponding to 2 isomers (Rt 7.06 and 7.46 min) were detected, in accordance with the hydroxy-group position at carbon 2 or 3 of the cyclohexane ring. These hydroxylated metabolites counted for up to 2% of TPA.

The second possible site of reduction of O-PCE corresponded to carbon 5-6 of the cyclohexanone ring, generating dehydro-O-PCE. This minor metabolite (0.2%) underwent dehydration on the ketone site and further reduction of the aliphatic ring to form didehydro-O-PCE.

Secondary reductions involving several metabolites included the reduction of deschloro-norketamine to dehydro-deschloro-norketamine, hydroxy-O-PCE to hydroxy-dehydro-O-PCE (with both phenolic or alcoholic configurations), or the reduction of M2a/b to diphenyl (M1). Reduction pathways were similar to those of methoxetamine (10), and represented up to 12% of metabolites' signal in HLMs.

3. Oxidative deamination

Unlike ketamine or MXE for which oxidative deamination has not been reported so far [9,19], this reaction seemed among the most relevant biotransformation observed in HLMs.

The most relevant reaction corresponded to the oxidative deamination of hydroxy-PCE (M12) leading to the formation of the two isomers M2a or M2b according to the position of the double bound on carbon C1-6 or C5-6 of the cyclohexanone ring. M2b accounted for 56% of TPA. Minor oxidative deamination of didehydro-O-PCE led to the formation of two other possible isomers of 1-phenyl-2-cyclohexen-1-ol according to the position of the double bound at carbon 2-3 (M3a) or 3-4 (M3b). Didehydro-O-PCE was considered as an intermediate metabolite as only its final products (M3a/b) were detected in authentic samples.

4. Glucuronidation

Among five identified glucuronides, only deschloro-norketamine-glucuronide (M15) was detected in HLMs. The reaction corresponded to *O*-glucuronidation with two configurations (phenol or alcohol) and seven possible binding sites of the glucuronic acid according to the hydroxylation site of deschloronorketamine as explained above. This minor metabolite (0.1%) was not detected in human samples, probably due to its short elimination half-life and/or late urine sampling. Another explanation could be the subcellular localization of UDP-glucuronlytransferases enzymes (UGT) in the internal membrane of the endoplasmic reticulum, limiting in vivo the access of substrates. This phenomenon known as "latency" contributes to difficulties in extrapolating in vivo effects of UGT enzymes from in vitro experiments using isolated tissue microsomes [21].

O-PCE metabolic profile in urine and hair samples

Among the 17 metabolites identified in HLMs, 10 were detected in urine or hair. Six further metabolites were identified in urine and/or hair while absent from HLMs incubates (Table 1)

Table 1. Proposed O-PCE metabolites, names, biotransformation, exact mass, formula, composition change from parent drug with lost or added groups, peak areas in HLMs, urine and hair samples and retention times.

ID	Name	Bio-transformation	Exact mass m/z	Formula	Composition change	Lost/Added group	Peak areas (Counts)								Rt (min)
							HLM	%(*)	Urine	%	Hair A (0-2cm)	%(**)	Hair B (2-4cm)	%(**)	
O-PCE	Deschloro-N-ethyl-ketamine	N/A	218,15379	$C_{14}H_{19}NO$	N/A	N/A	1,066E+10	83	ND	N/A	3,863E+07	12.5	4,355E+07	13	6,50
M1	Diphenyl	Reduction	155,08543	$C_{12}H_{10}$	(-) C_2H_7N (-) H_2O	Ethylamine Water	1,222E+08	5	ND	N/A	2,952E+05	0.1	3,527E+05	0.1	6,50
M2a	Phenylcyclohexa-2,5-dienol	Oxidative deamination to alcohol	173,09596	$C_{12}H_{13}O$	(-) C_2H_7N	Ethylamine	2,619E+08	12	ND	N/A	ND	N/A	1,259E+06	0.5	5,79
M2b							1,241E+09	55	ND	N/A	2,636E+06	0.8	3,047E+06	1	6,50
M3a	1-phenyl-2-cyclohexen-1-ol	Oxidative deamination to alcohol, hydrogenation	175,11182	$C_{12}H_{14}O$	(-) C_2H_7N (−) H_2	Ethylamine Dihydrogen	1,900E+06	0.1	2,502E−07	20	4,747E+06	1.5	6,172E+06	1.9	5,61
M3b							4,655E+06	0.2	ND	N/A	1,178E+06	0.4	1,372E+06	0.4	7,06
M4	Deschloro-norketamine	N-dealkylation	190,12227	$C_{13}H_{15}NO$	(-) C_2H_5	Ethyl	4,211E+08	19	5,371E−05	0.5	1,765E+06	0.6	1,724E+06	0.5	5,79
M5	Dehydro-deschloro-norketamine	Reduction N-dealkylation	192,13766	$C_{13}H_{17}NO$	(-) C_2H_5 (−) H_2	Ethyl Dihydrogen	ND	N/A	ND	N/A	2,405E+08	77.5	2,539E+08	77	11,67
M6	Didehydro-O-PCE	Dehydration	200,14334	$C_{14}H_{17}N$	(-) H_2O	Water	8,371E+07	4	ND	N/A	ND	N/A	ND	N/A	6,50
M7	Hydroxy-deschloro-norketamine	Oxidation	206,11763	$C_{13}H_{15}NO_2$	(-) C_2H_5 (+) O	Ethyl Oxygen	3,010E+06	0.2	5,935E−05	0.5	ND	N/A	ND	N/A	6,96
M8	Dehydro-O-PCE	Reduction	216,13820	$C_{14}H_{17}NO$	(-) H_2	Dihydrogen	3,652E+06	0.2	ND	N/A	1,403E+06	0.5	1,498E+06	0.5	6,04
M9a	2-hydroxy-PCE	Reduction	220,16939	$C_{14}H_{21}NO$	(−) H_2	Dihydrogen	2,256E+07	1	4,549E−06	3.5	7,384E+06	2.4	6,651E+06	2	7,06
M9b	3-hydroxy-PCE						1,775E+07	0.8	1,424E−07	11	8,388E+06	2.7	8,852E+06	2.8	7,46
M10a	2,3 or 4-phenol-dehydro-O-PCE or 3,4,5 or 6 hydroxy-dehydro-O-PCE	Desaturation, Oxidation	232,13313	$C_{14}H_{17}NO_2$	(-) H_2 (+) O	Dihydrogen Oxygen	ND	N/A	4,596E−06	3.5	ND	N/A	ND	N/A	2,95
M10b							1,793E+07	0.8	ND	N/A	ND	N/A	ND	N/A	9,54
M11a	2,3 or 4 phenol-O-PCE or 3,4,5 or 6 hydroxy-O-PCE	Oxidation	234,14874	$C_{14}H_{19}NO_2$	(+) O	Oxygen	2,092E+06	0.1	ND	N/A	ND	N/A	ND	N/A	4,10
M11b							2,696E+07	1.2	ND	N/A	ND	N/A	ND	N/A	5,02
M11c							5,456E+06	0.2	ND	N/A	ND	N/A	ND	N/A	5,22
M12	2,3 or 4 phenol-hydroxy-PCE or 3,4,5 or 6 dihydroxy-PCE	Oxidation	236,16440	$C_{14}H_{21}NO_2$	(−)H_2O	Water	1.347E+06	0.1	ND	N/A	ND	N/A	ND	N/A	6,41
M13a	Deoxo-deschloro-norketamine	N-glucuronide	350,15924	$C_{18}H_{23}NO_6$	(−) $C_4H_4O_5$	Glucuronide	ND	N/A	2.110E−07	16.5	3.218E+06	1	1.062E+06	0.3	6,61

M13b	glucuronide	conjugation					ND	N/A	1.272E−07	10	ND	N/A	ND	N/A	7,42
M13c							ND	N/A	1.986E−07	15.5	ND	N/A	ND	N/A	8,71
M14	Dehydro-deschloro-norketamine glucuronide	O-glucuronide conjugation	366,15408	$C_{18}H_{23}NO_7$	(−) $C_4H_4O_6$	Glucuronide	ND	N/A	2.422E−07	19	ND	N/A	ND	N/A	7,85
M15	Deschloro-norketamine glucuronide	O-glucuronide conjugation	382,14951	$C_{18}H_{23}NO_7$	(−) $C_4H_4O_7$	Glucuronide	2.156E+06	0.1	ND	N/A	ND	N/A	ND	N/A	8,53

Footnotes

N/A not applicable, ND not detected, Rt retention time, HLM human liver microsomes.

Metabolites were sorted in ascending order of m/z, isomers were sorted in ascending order of Rt

(*) Percentage of metabolites was expressed as the ratio of peak area of a metabolite to total peak area of all metabolites, excluding that of O-PCE (remaining after HLM incubation).

(**) O-PCE peak area in hair was included in the calculation of total peak area (=100%) as parent drug and metabolites integrate hair in the same way.

In urine, 10 metabolites were identified while O-PCE was no longer detected. 1phenyl-2cyclohexen-1-ol was the most relevant phase I metabolite (20%), followed by 3-OH-PCE (11%), 2-OH-PCE and OH-dehydro-PCE (3.5 % each).

While deschloro-norketamine was of minor relevance in urine (0.5%), its glucuronide, dehydro-deschloro-norketamine-*O*-glucuronide counted for 19% of TPA. Interestingly, the opposite finding has been observed in HLMs, where deschloro-norketamine was present (19%), but not its glucuronide. This could suggest that deschlor-norketamine glucuronidation occurred in extrahepatic tissues as some UGT isoforms were not present in the liver (e.g. UGT1A7, UGT1A8, UGT1A10), and could explain why this glucuronide was missing in HLMs [21].

The three isomers of deoxo-deschloro-norketamine-*N*-glucuronide (M13a/b/c) obtained from dehydro-deschloro-norketamine (M5) were also among the most abundant metabolites excreted in urine (16.5, 10, and 15.5%, respectively). N-glucuronidation seems specific to O-PCE metabolism when compared to ketamine and MXE [9,10,19]. The absence of these glucuronides in HLMs incubates was also probably related to extrahepatic glucuronidation.

In hair, O-PCE and 11 metabolites were identified in both segments (except M2a detected only in segment B). Dehydro-deschloro-norketamine (M5) produced by a dehydrogenation of deschloronorketamine (M4) was the most abundant metabolite accounting for 77% of TPA, while deschloronorketamine was of minor relevance (0.5%). By comparison to ketamine, it is well known that N-demethylation and dehydrogenation of ketamine (K) to the respective metabolites norketamine (NK) and dehydronorketamine (DHNK) are the major metabolic pathways. However, norketamine was the major metabolite in hair as reported by Favretto et al., while the presence of DHNK was excluded [22]. Interestingly, deschloronorketamine/O-PCE ratio found in our study (mean = 0.042, n=2) was very similar to that of NK/K (mean=0.041, n= 2) reported by Favretto et al. Nevertheless, in our study dehydro-deschloro-norketamine

was present at high level with dehydro-deschloro-norketamine/O-PCE ratio of 6, which showed its good integration into keratin, even though metabolites are less likely to integrate into hair than parent drug in general, due to their high hydrophilicity (20). The absence of dehydro-deschloro-norketamine in urine could be the consequence of its total transformation to deoxo-deschloro-norketamine glucuronides (M13 a/b/c) as they were of major relevance in urine (42% TPA).

Limitation of the study

Since time and amount of ingestion of O-PCE were unknown, the results of urine analysis do not reflect the general excretion profile of O-PCE in human. Another limitation was the number of authentic sample (n=1) used to validate the results of in vitro study. Consequently, further data are needed to confirm our findings.

Conclusion

The present study showed an extensive metabolic profile of O-PCE in HLMs and authentic samples similar to that of methoxetamine and ketamine. While phenylcyclohexa-2,5-dienol seemed presumably the most abundant metabolite in HLMs, two phase I (1-phenyl-2-cyclohexen-1-ol and 3-hydroxy-PCE) and two phase II metabolites (dehydro-deschloro-norketamine glucuronide and deoxo-deschloro-norketamine glucuronide) were the most abundant in urine, when O-PCE was no longer detected. Therefore, we recommend these metabolites as target markers to increase the detection window of O-PCE in urine.

Hair appears to be a good alternative in drug metabolism study as an exploratory matrix, especially when urine collection is taken late after presumed consumption or was not sampled. It appears from our study that dehydro-deschloro-norketamine is of great interest for long-term monitoring of O-PCE exposure, as it integrates well into hair and had a relevant metabolite to parent drug ratio.

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4. Discussion

Nous avons développé, optimisé et validé une méthode de retraitement des données issues de l'analyse par spectrométrie de masse haute résolution (LC-HRMS) afin de l'adapter à l'étude du métabolisme des NPS. L'optimisation du workflow a été réalisée à travers l'ajustement de plusieurs paramètres tels que la fenêtre des temps de rétention des composés, l'ajout d'un nœud (fill-gap) permettant de rechercher les métabolites potentiels ayant des intensités faibles du moment où ils ont été détectés au moins une fois dans une précédente analyse, ou enfin la création d'une bibliothèque de spectres haute résolution des métabolites (NPS_Métabolites) afin de les identifier lors de futures analyses.

Au total, l'association du modèle d'étude in vitro sur HLM à l'analyse des échantillons biologiques provenant d'usagers de ces drogues a permis grâce à ce workflow métabolique l'identification de 228 métabolites pour les 9 NPS étudiés. Parmi eux, 90 métabolites (39%) correspondant à 4 NPS (O-PCE, 3-HO-PCE, 3-fluorofentanyl et 4 MeO-PCP) n'ont jamais été décrits auparavant. L'association de plusieurs matrices biologiques incluant le sang, l'urine et les cheveux a permis de maximiser la probabilité d'identifier ces métabolites et à reconstituer le profil métabolique de ces NPS. Le sang souvent prélevé tardivement par rapport au moment de la dernière prise du produit n'a été analysé que pour 3/9 NPS ce qui a permis d'identifier seulement 20% de l'ensemble des métabolites. Dans l'urine, 89/228 (39%) des métabolites ont été identifiés malgré un prélèvement tardif, ce qui montre clairement l'intérêt de cette matrice de par une fenêtre de détection plus longue que celle du sang et une accumulation de plusieurs métabolites éliminés longuement par cette voie. En effet, l'étude du métabolisme de l'O-PCE a permis d'identifier 4 métabolites pertinents à rechercher dans les urines (1-phényl-2-cyclohexén-1-ol, 3-hydroxy-PCE, déhydro-deschloro-norkétamine glucuronide et déoxo-deschloro-norkétamine glucuronide). Ces métabolites permettraient de prolonger la fenêtre de détection d'une consommation d'O-PCE de par leurs demi-vies d'élimination plus longues qui nous ont permis de les détecter lorsque que l'O-PCE était absente de l'échantillon urinaire analysé.

L'un des constats intéressant que nous avons pu faire durant cette étude est le nombre considérable de métabolites identifiés dans les cheveux. Ainsi, 102/228 (45%) ont été détectés dans la matrice capillaire ce qui représente la plus grande part des métabolites identifiés, toute matrice biologique confondue. Ceci est en partie lié à la fenêtre de détection des composés plus longue dans les cheveux par rapport aux matrices conventionnelles.

Même s'il est scientifiquement admis que l'intégration des molécules dans les cheveux est proportionnelle à leur caractère lipophile entrainant une moins bonne intégration des métabolites par rapport aux composés parents, nos résultats semblent montrer un intérêt de cette matrice dans les explorations métaboliques notamment quand les matrices classiques sont prélevées tardivement ou n'ont pas été échantillonnés. L'exemple de l'O-PCE révèle même la présence d'un métabolite (déhydro-deschloro-norkétamine) s'intégrant beaucoup mieux (intensité plus élevée) dans la kératine des cheveux que l'O-PCE elle-même, faisant de ce métabolite un biomarqueur d'intérêt majeur pour la surveillance des expositions à long terme à ce NPS. Cette observation laisse aussi évoquer d'autres mécanismes possibles d'intégration des métabolites polaires dans les cheveux, autres que leurs propretés physicochimiques, qui pourraient expliquer pourquoi ces métabolites sont plus abondants que les produits dont ils sont issus.

Par ailleurs, la présence de certains métabolites in vitro et leur absence des échantillons biologiques (ex : hydroxy ou phénol-O-PCE) peuvent s'expliquer par une demi-vie courte n'ayant pas permis leur identification, d'autant plus que les échantillons sanguins et urinaires ont été souvent prélevés tardivement. De même, des métabolites peuvent être présents dans les échantillons biologiques (ex : déhydro-deschloro-norkétamine-O-glucuronide) et absents in vitro dans les microsomes. Cela pourrait suggérer un métabolisme extra hépatique, notamment pour les glucuronides où certaines isoformes de l'UDP-glucuronlytransférases (UGT) (enzymes catalysant leur formation) telles que l'UGT1A7, UGT1A8, UGT1A10 sont absentes du foie. Une autre explication pourrait être la localisation subcellulaire des enzymes comme l'UGT dans la membrane interne du réticulum endoplasmique, limitant in vivo l'accès aux substrats. Ce phénomène de «latence» contribue aux difficultés d'extrapolation des résultats obtenus in vitro aux résultats observés in vivo [53]. Ces observations montrent certaines limites du modèle utilisant les HLM qui ne permet pas d'explorer tout le spectre des métabolites générés in vivo. Cela souligne aussi la nécessité d'employer d'autres modèles (in vitro ou in vivo), mais surtout de valider les résultats expérimentaux par l'analyse des échantillons biologiques authentiques.

La comparaison de nos résultats à ceux déjà rapportés dans la littérature pour certains NPS nous a permis de valider notre workflow métabolique, et enrichir les données métaboliques de ces NPS par l'identification de nouveaux métabolites. A noter que les disparités observées dans les résultats de ces études sont directement liées à l'absence de consensus sur la méthodologie

à utiliser pour ce type d'exploration métabolique, et à la performance différente des outils analytiques employés dans chacune d'entre elles.

Enfin, l'une des limites de notre étude est la représentativité des échantillons collectés et le délai souvent inconnu entre la prise présumée de drogues et les prélèvements réalisés qui ne représentent pas le profil d'excrétion général des NPS étudiés.

CONCLUSION GENERALE

Ce travail a permis d'étudier les nouveaux produits de synthèse sous différents aspects liés à leur consommation, leur détection et leur métabolisme.

La première partie nous a permis de développer à travers l'utilisation de techniques innovantes telles que la chimiluminescence sur biopuces ou la spectrométrie de masse deux approches complémentaires, permettant le criblage ciblé et non ciblé, ainsi que la quantification des NPS dans différentes matrices biologiques (sang, urine, cheveux). Ces méthodes ont permis aussi de mettre à jour la liste des molécules recherchées par le laboratoire en incluant la famille des cannabinoïdes de synthèse et des nouveaux opioïdes de synthèse, de plus en plus rencontrées dans des cas d'intoxications ou de pharmacodépendance.

Grace à ce développement analytique, plusieurs études observationnelles incluant un nombre significatif d'échantillon ont pu être initiées et réalisées afin d'étudier la prévalence des NPS dans différentes populations d'usagers à haut risque (overdose, pharmacodépendance, chemsex…etc.). Ces études montrent clairement une prévalence élevée de ces produits (29 à 39%), qui restent souvent méconnus par les cliniciens et non recherchés systématiquement par la plupart des laboratoires. Cette tendance de consommation, similaire à celle des drogues classiques (ex : cocaïne ou amphétamines) incite à une réflexion générale sur le plan législatif et scientifique pour inclure leur recherche systématique dans les différentes expertises cliniques ou médico-judiciaires. Grace à ces études, les différents profils de consommateurs (caractéristiques sociodémographiques, tendance de consommation, pratiques à risque…etc.) ont pu être cernés afin que des programmes efficaces de soins et de réduction des risques puissent leur être proposés.

La troisième partie de ce travail consacrée à une approche plus fondamentale basée sur l'étude du métabolisme des NPS a permis de développer un outil analytique de retraitement des données efficace permettant la recherche de nouveaux biomarqueurs d'exposition à ces produits. Cette étude métabolique a pu apporter de nouvelles données scientifiques quant au métabolisme de certains NPS jamais décrits dans la littérature.

Enfin, ce travail a montré clairement l'intérêt des cheveux comme matrice alternative dans les études métaboliques ou de prévalence qui confirme leur rôle essentiel en recherche fondamentale et clinique.

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Titre : Nouveaux produits de synthèse : analyse, consommation et métabolisme ; Applications cliniques et médicolégales

Mots clés : Nouveaux Produits de Synthèse (NPS) ; Prévalence ; Spectrométrie de masse, Microsomes du foie humain (HLM) ; Métabolisme ; Cheveux

Résumé : L'objectif de ce travail a été de développer deux approches analytiques dédiées à l'analyse toxicologique des nouveaux produits de synthèse (NPS) dans différentes matrices biologiques (sang, urine et cheveux). La première est basée sur le criblage non ciblé par chimiluminescence sur biopuces et chromatographie liquide couplée à la spectrométrie de masse haute résolution (LC-HRMS) et la deuxième correspond à un criblage ciblé par spectrométrie de masse en tandem (LC-MS/MS). Ces deux approches ont ensuite été appliquées dans des études observationnelles pour évaluer la consommation de NPS dans des populations à risques de surdosage, de pharmacodépendance ou de soumission chimique dans un contexte clinique ou médico-judiciaire.

La dernière partie a été consacrée au développement d'un nouvel outil analytique de traitement des données issues de la LC-HRMS qui a permis d'étudier le métabolisme de 9 NPS in vitro sur des cultures de microsomes du foie humain (HLM) et in vivo sur des échantillons biologiques d'usagers de ces drogues. Cette dernière approche a permis la création d'une bibliothèque de spectres de haute résolution composée de 228 métabolites dont certains ont été proposés comme marqueurs pertinents d'exposition aux NPS dont ils sont issus. Ce travail a été concrétisé par la rédaction de 10 publications scientifiques et a permis d'initier plusieurs collaborations pluridisciplinaires.

Title : New psychoactive substances: Analysis, prevalence and metabolism; Clinical and forensic applications

Keywords : New Psychoactive Substances (NPS); Prevalence; Mass spectrometry; Human liver microsomes (HLM); Metabolism; Hair

Abstract : The aim of the present work was to develop two analytical approaches dedicated to the analysis of new psychoactive substances in different biological matrices (blood, urine and hair). The first approach is based on untargeted screening by both biochip array technology chemiluminescence assay and liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) and the second corresponds to a targeted screening by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). These two approaches were then applied in observational studies to assess the consumption of NPS in high risk populations (overdose, drug abuse, drug facilitated crimes) in clinical and forensic settings.

The last part of the work was devoted to the development of a new analytical tool for LC-HRMS data processing which made it possible to study the metabolism of 9 NPS *In vitro* on human liver microsomes (HLM) and *In vivo* in biological samples from drug users. This approach has enabled the creation of HRMS spectral library containing 228 metabolites, some of which have been proposed as relevant markers of NPS exposure.
This work has resulted on 10 scientific publications and allowed to initiate many multidisciplinary collaborations.