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Aspects analytiques, cliniques et médico-judicaires des nouvelles substances psychoactives

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Abréviations

ANSM : Agence nationale de sécurité du médicament et des produits de santé

CEIP-A: Centre d'évaluation et d'information sur ma pharmacodépendance, Addictovigilance

DRAMES : Décès en relation avec l'abus de médicament et de substances

EMCDDA: The European monitoring center for drugs and drugs addiction

GC : Chromatographie gazeuse

HLM : Human liver microsomes (Microsome hépatiques humains)

HSH: Homme(s) ayant des rapports sexuels avec un (des) autre(s) homme(s)

HSS : Haute résistance de particules de la silice

ILS : Infraction de la législation sur les stupéfiants

IML : Institut de médecine légale

LC : Chromatographie liquide

m/z : Masse sur charge

mL : Millilitre

mM : Millimolaire

MRM : Multriple reaction monitoring (suivi des réactions multiples)

MS/MS : Spectrométrie de masse en tandem

ng : Nano gramme

NPS: New psychoactives substances ou Nouvelles substances psychoactives

OEDT : Observatoire européen des drogues et des toxicomanies

ONUDC: Office des Nations Unies contre la drogue et le crime

RC: Research chemical

RF : Fréquence de radiation

RMN ^{1}H : Résonance magnétique du proton

RMN : Résonance magnétique nucléaire

SFTA : Société française de toxicologie analytique

SM : Spectrométrie de masse

TQD : Détecteur triple quadripolaire

TR : Temps de rétention

UE : Union européenne

UHPLC : Chromatographie liquide ultra haute performance

Introduction

Les nouvelles substances psychoactives, généralement nommées par l'acronyme anglais NPS (New Psychoactives Substances), désignent un éventail hétérogène de substances synthétiques, modifiables à l'infini, qui imitent les effets des stupéfiants et médicaments usuels. L'augmentation planétaire de ces substances est un véritable fléau. Depuis une dizaine d'année, le nombre de NPS n'a cessé d'augmenter avec 119 pays et territoires qui ont signalés la circulation d'une ou plusieurs NPS entre 2008 et fin 2018. Fin 2018, le système d'alerte des Nations Unies contre la drogue a comptabilisé 899 nouvelles substances psychoactives.

Face à ce phénomène, l'EMCDDA (The European Monitoring Centre for Drugs and Drug Addiction), basé au Portugal a lancé une alerte dans son rapport annuel de 2016 afin de sensibiliser les laboratoires d'analyses toxicologiques à la nécessité de disposer d'une méthode analytique permettant d'identifier un maximum de NPS.

A mon arrivée à l'Institut de médecine légale de Strasbourg en 2015, aucune méthode dédiée à l'identification des NPS n'était disponible. Ma première année de thèse a donc pleinement été consacrée au développement d'une méthode dédiée à l'identification de ces substances. Après plusieurs mois d'optimisation, cette méthode a été utilisée en routine et a permis de mettre en évidence plusieurs cas d'intoxication et de décès liés aux NPS. Les caractéristiques de cette méthode sont présentées dans cette thèse.

A l'heure actuelle, la librairie de spectre comporte 114 NPS, et est mise à jour régulièrement lorsque de nouvelles substances apparaissent sur le marché. Il est impératif de se tenir informé des nouvelles substances synthétisées et commercialisées.

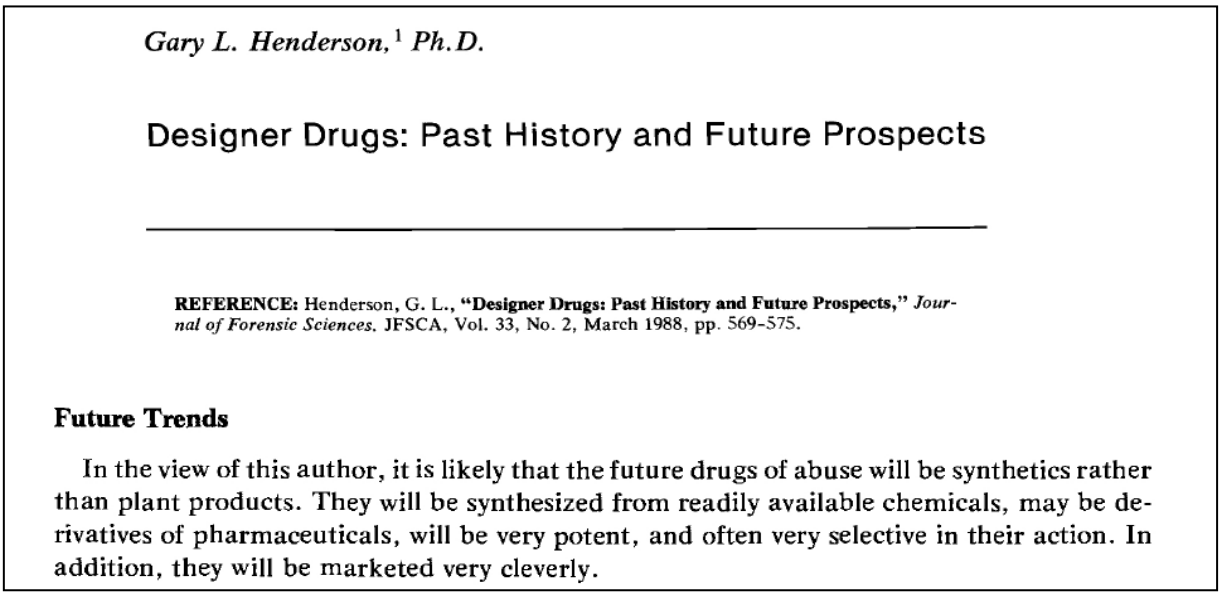
Les cas observés lors de ces trois dernières années sont présentés dans cette thèse sous forme d'articles publiés ou en cours d'évaluation.

Partie 1 : Généralités

Les nouvelles substances psychoactives – Définition

Tout commence dès 1988…

A cette date, le pharmacologue G. L. Henderson rédige une publication intitulée « Designer drugs: past history and future prospects » dans laquelle il prédit plusieurs choses concernant les futures drogues proposées aux consommateurs *[Henderson, 1988]*. D'après lui, ces futures drogues seront synthétiques et non plus végétales, puissantes, sélectives, et commercialisées avec ingéniosité. Les analogues non commercialisés de certains médicaments rentrent également dans cette catégorie (Figure 1). Alors même qu'Internet n'existait pas, G.L. Henderson avait déjà tout dit …



Gary L. Henderson,[1] *Ph.D.*

Designer Drugs: Past History and Future Prospects

REFERENCE: Henderson, G. L., **"Designer Drugs: Past History and Future Prospects,"** *Journal of Forensic Sciences*, JFSCA, Vol. 33, No. 2, March 1988, pp. 569-575.

Future Trends

In the view of this author, it is likely that the future drugs of abuse will be synthetics rather than plant products. They will be synthesized from readily available chemicals, may be derivatives of pharmaceuticals, will be very potent, and often very selective in their action. In addition, they will be marketed very cleverly.

Figure 1: Publication du pharmacologue G. L. Henderson, rédigé en 1988.

Les nouvelles substances psychoactives désignent un éventail hétérogène de substances synthétiques, modifiables à l'infini, qui imitent les effets des stupéfiants et médicaments usuels, tels que l'ecstasy, l'amphétamine, le cannabis, les benzodiazépines ou encore les opiacés. Ces substances sont définies par l'Office des Nations Unies contre la drogue et le crime (ONUDC) comme toute substance consommée, pure ou en préparation, à des fins abusives, n'étant contrôlée ni par la convention unique des Nations Unies sur les stupéfiants de 1961 (la Convention de Vienne), ni par celle sur les psychotropes de 1971, et qui peut représenter une menace pour la santé publique *[UNODC, 2018]*.

Les termes utilisés pour désigner ces produits sont variés : *research chemical (RC), legal highs (euphorisants légaux), herbal highs (euphorisants végétaux), party pills, sel de bains, encens, etc.*

Ces nouvelles substances sont synthétisées dans le but d'obtenir les mêmes effets que les stupéfiants et médicaments usuels, tout en contournant la loi. En modifiant la structure chimique de base d'un

composé usuel, la nouvelle molécule appartient encore à la même famille chimique, ou tout au moins se lie sur le même récepteur ou la même enzyme, mais n'est plus identifiable comme tel d'un point de vue légal au moment de sa mise sur le marché. Plusieurs nouvelles structures chimiques, trois dérivées d'une benzodiazépine (le diazépam) et trois dérivés de l'amphétamine, sont présentées Figure 2. Malgré la dangerosité qu'elles représentent, certaines nouvelles substances ne sont ni interdites, ni réglementées.

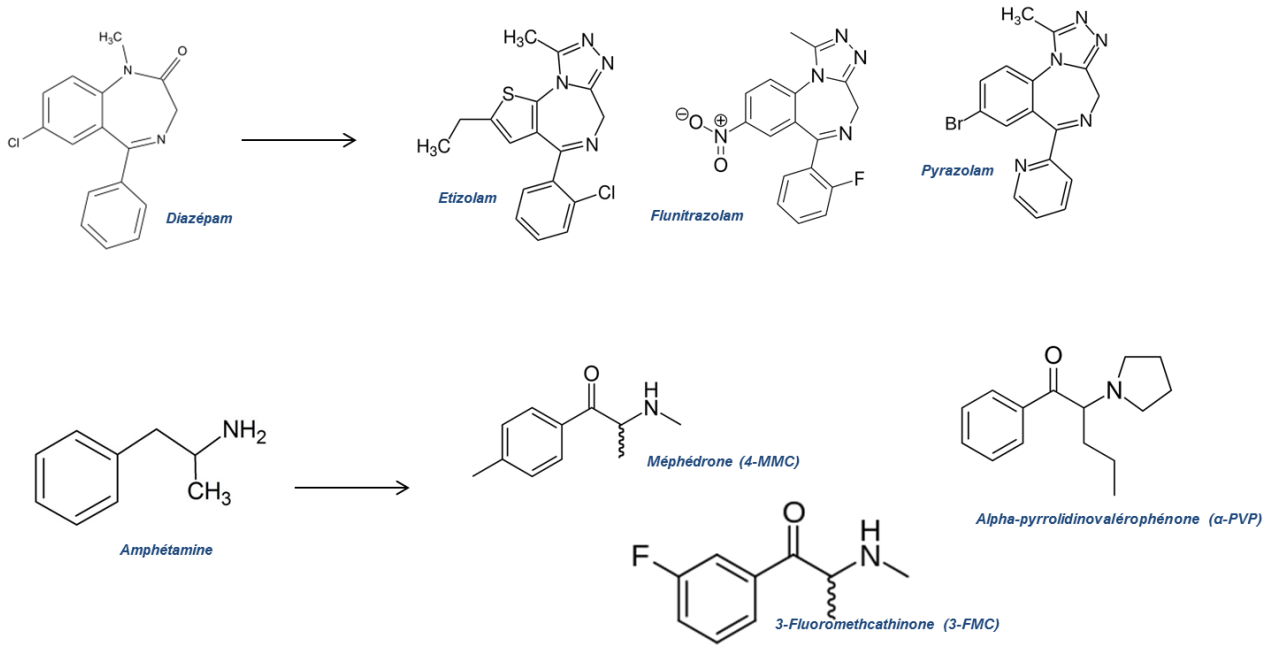


Figure 2: Trois structures de benzodiazépines de synthèse (l'étizolam, le flunitrazolam et le pyrazolam), dérivées du diazépam (Valium®) et trois structures de cathinones (la méphédrone, la 3-fluoromethcathinone et l'alpha-pyrrolidinovalérophénone) dérivées de l'amphétamine.

Les NPS sont généralement vendues sous forme de poudre, de comprimé ou de buvard. Leur présentation n'a souvent aucun rapport avec le nom qu'elle porte, par exemple « sels de bains », « engrais » ou « encens ». Ces noms servent à dissimuler leurs réels usages. Ces substances peuvent être achetées sur Internet, mais également dans la rue auprès de revendeurs et de trafiquants.

Sur Internet, la majorité des NPS est proposée entre 8 à 20 euros le gramme ou les 10 comprimés et ce prix peut diminuer lorsque la quantité commandée est importante *[Lahaie E, 2013]*. Dans la rue, les prix peuvent être jusqu'à 3 fois plus élevés.

Ces produits sont proposés sous différentes appellations, soit par leur nom chimique, soit par un nom commercial qui ne reflète pas leur composition. Certains sites ciblent majoritairement les

jeunes consommateurs qui sont à la recherche de nouvelles expériences et de découvertes. Ces acheteurs sont attirés par des produits colorés, au design original, vendus dans des emballages attractifs et aux noms décalés. Pour les consommateurs plus expérimentés, il existe des sites plus sobres sur lesquels les produits sont vendus sous leur nom chimique, sans surplus commercial. Les produits sont généralement livrés sous 48h par voie postale, comme une lettre classique, après paiement par carte bancaire, PayPal ou encore en bitcoins. Les inscriptions « laboratory reagent only » ou « not approved for human consumption » sont souvent lisibles sur les emballages. Ces mentions permettent aux sites Internet de s'affranchir d'éventuelles poursuites juridiques à la suite d'une mauvaise utilisation des produits.

Les NPS identifiées en Europe sont principalement produites en Chine et, dans une moindre mesure, en Inde et dans les pays d'Europe de l'Est. Selon l'Observatoire européen des drogues et des toxicomanies (OEDT), au sein de l'Union européenne (UE), seuls la Pologne et les Pays-Bas avaient signalé la production, en faible quantité, de NPS sur leur territoire en 2012. Les substances pénètrent en Europe via les pays de l'UE n'ayant pas encore statué sur leur situation juridique et elles sont ensuite redistribuées vers les autres pays. Les matières premières sont alors mélangées, éventuellement conditionnées dans des laboratoires ou de simples zones de transformation. Les sites de vente sur Internet sont situés dans des pays différents des lieux d'expédition de la marchandise, et majoritairement animés par des personnes basées en Grande Bretagne et au Danemark. Ce système de vente brouille la traçabilité des circuits de production et de distribution *[Martinez M, 2013]*.

Les NPS peuvent être classées selon leurs effets psychoactifs. D'après le Centre d'Addictovigilance de Paris, il existe 7 classes de NPS (Figure 3):

- Les substances **stimulantes**, telles que certaines cathinones (alpha-pyrrolidinovalérophénone) et les phénidates (méthyl-, éthylphénidate), qui augmentent l'activité du système nerveux central et facilitent les rapports sociaux, la confiance en soi et l'euphorie.
- Les substances **entactogènes**, qui augmentent la capacité d'empathie, le désir de contact avec autrui et facilite l'expression. Parmi ses substances, on distingue de nombreuses cathinones, comme la méphédrone (4-méthylmethcathinone, ou 4-MMC).
- Les substances **hallucinogènes**, aussi appelées psychédéliques, sont des perturbatrices du système nerveux central et induisent l'altération des perceptions, de la cohérence et de la régularisation de l'humeur. On distingue le diéthyllysergamide (LSD), les tryptamines et les N-Methyl-Benzyl-Oxy (NBOMe).

- Les substances **dissociatives**, telles que la kétamine et ses dérivés, ainsi que les dérivés de la phéncyclidine, comme la 3-methoxyphencyclidine (3-MeO-PCP), ont des effets dépresseurs sur le système nerveux central. Ces effets sont marqués par une sensation de partage artificiel entre l'esprit et le corps (dé-corporation), un état de rêve et d'hallucinations. Elles peuvent entrainer également une dépression respiratoire, des problèmes cognitifs, de l'amnésie et un coma.
- Les **cannabinoïdes** ou spices (JWH-018, AB-PINACA, SGT-151), sont des analogues du cannabis et ont les mêmes effets que le Δ9-tétrahydrocannabinol (THC), c'est-à-dire un effet de relaxation, d'apaisement et de calme.
- Les **dépresseurs**, tels que les benzodiazépines de synthèse (diclazépam, flubromazépam, …), sont des substances qui entrainent une relaxation, une réduction de l'anxiété, une somnolence et un ralentissement de la respiration.
- **Les opioïdes**, dérivés de la morphine, de l'héroïne et du fentanyl (U-47700, furanylfentanyl, …), qui augmentent la sensation de bien-être et d'invincibilité. Un usage régulier peut entrainer une addiction et une toxicité élevée.

La Figure 3 permet de visualiser la classification des NPS, comparée à celle des stupéfiants usuels.

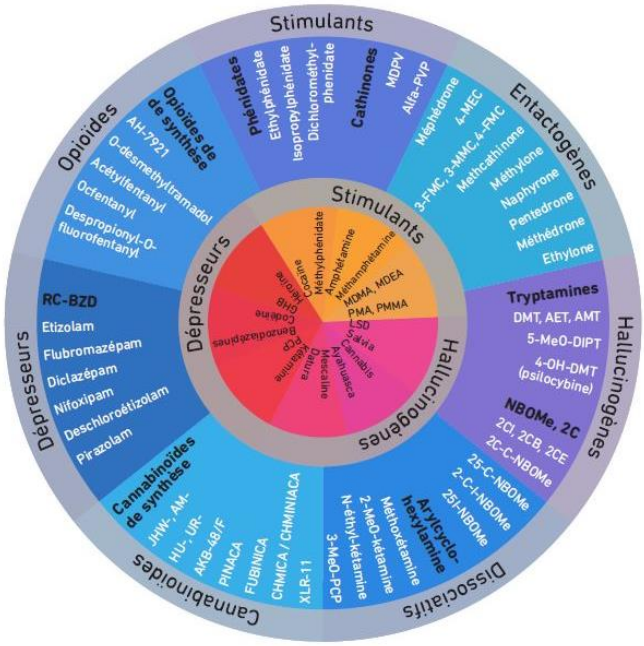


Figure 3: Classement des NPS, proposé par le Centre d'Addictovigilance de Paris, intitulée " Nouvelle ère des drogues".
Cercle intérieur : substances psychoactives classiques
Cercle extérieur : nouveaux produits de synthèse

État des lieux des NPS

L'émergence généralisée des NPS, qui a attiré l'attention de la communauté médicale vers 2009, n'a aucun précédent. En quelques années, ce phénomène est devenu planétaire, avec 119 pays et territoires qui ont signalés la circulation d'un ou plusieurs NPS entre 2008 et fin 2018 (Figure 4).

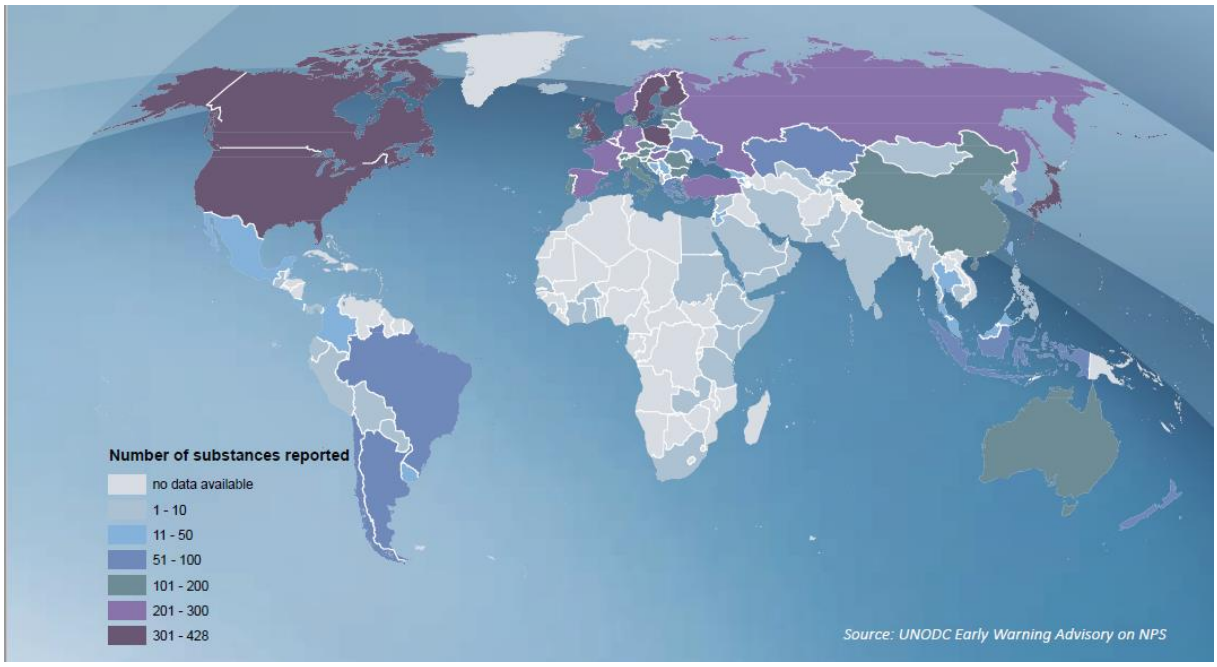


Figure 4 : Répartition mondiale du nombre de NPS signalisés, fin 2018.

En France, les premiers signalements de NPS sont apparus en 2008, avec l'apparition d'un cannabinoïde de synthèse « spice » (JWH-018) et de la méphédrone (4-MMC), qui a défrayée la chronique à la suite d'un décès en 2010. En quelques années, le nombre de NPS sur le marché a explosé. En 2012, 72 nouvelles substances psychoactives ont été officiellement notifiées par le système d'alerte précoce de l'UNODC, soit plus d'une nouvelle substance par semaine *[EMCDDA, 2012]*. C'était plus que les années précédentes, avec 49 nouvelles substances en 2011, 41 en 2010, et 24 notifiées en 2009 *[EMCDDA, 2017]*. Fin 2018, le système d'alerte a comptabilisé 899 nouvelles substances psychoactives (Figure 5) *[UNODC, 2019]*.

Malgré une connaissance assez avancée concernant les différents types de NPS les plus consommées, les informations relatives aux usagers restent très limitées. Leurs profils, pratiques et motivations sont mal connus. En effet, le mode de diffusion des NPS, principalement par Internet et

voie postale, permet l'éparpillement et la discrétion des consommateurs. Seules les études ciblant des populations spécifiques permettent d'apporter des informations sur leurs pratiques.

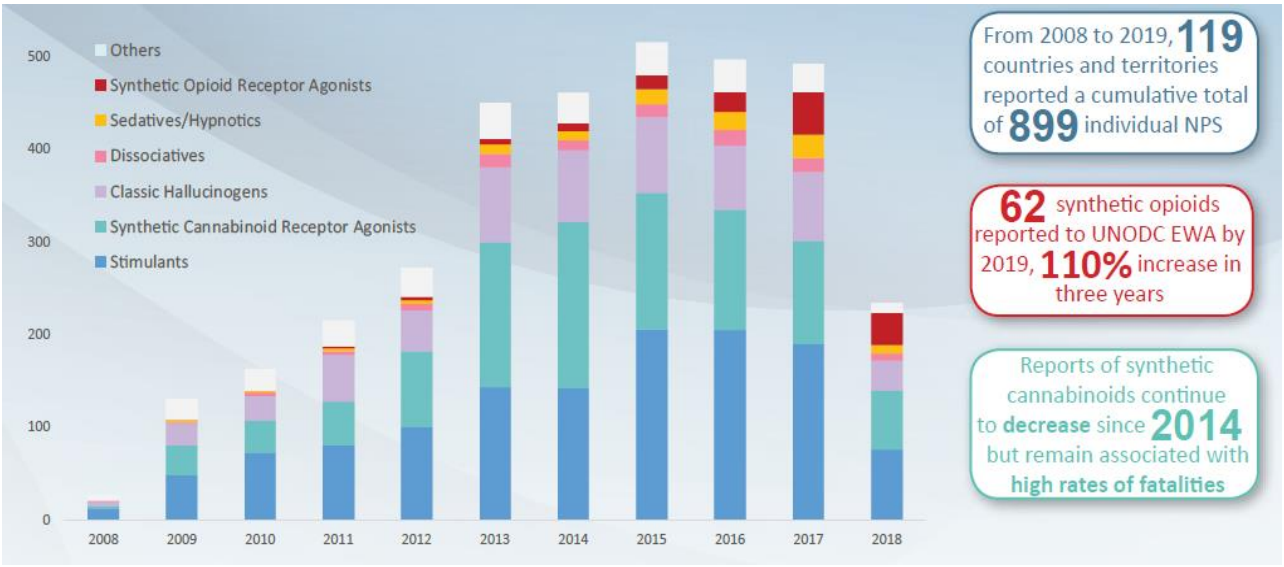


Figure 5: Nombre de NPS identifiés par famille, entre 2008 et 2018, au niveau mondial

A titre d'exemple, la consommation de NPS semble se généraliser parmi les hommes ayant des relations sexuelles avec les hommes (HSH), notamment lors de « Chemsex ». Ce terme provient de la culture gay anglo-saxonne et désigne le fait de consommer des produits psychotropes pendant des activités sexuelles pour les rendre plus intenses, plus longues et faciliter la désinhibition des partenaires. Par exemple, une étude française a été réalisée sur cette population, reposant sur le déclaratif des participants *[Alvarez, 2018].* L'objectif était d'établir le profil de consommation de NPS par analyse des cheveux et poils. Soixante-neuf volontaires ont été inclus dans cette étude. Quinze NPS ont été mis en évidence chez 27 sujets (39 % des volontaires). Par ordre de fréquence, il a été retrouvé la méphédrone (4-méthylmethcathinone, ou 4-MMC), la 4-methylethcathinone (4-MEC), l'éthylphénidate, la méthylone, la méthoxétamine, la méthiopropamine, la paraméthoxymethamphétamine (PMMA), la méthylènedioxypyrovalérone (MDPV), la métamfépramone, la 5F-PB22, la diphénidine, la phendimétrazine, la phentermine, la N-methyl-2-AI et la diméthylone. Aucun cas de consommation de NPS seul n'a été identifié, toujours en association avec de la cocaïne, de la 3,4-méthylènedioxy méthamphétamine (MDMA) ou de la kétamine. D'après les résultats de cette étude, les cathinones sont les substances les plus souvent identifiées dans cette population.Plusieurs cas d'intoxications ont été rapportés ces dernières années en France. La majorité de ces intoxications sont concomitantes à la consommation de cathinones, tels que l'alpha-pyrrolidinovalérophénone (α-PVP), la 3-méthylmetcathinone (3-MMC), la méphédrone (4-MMC) et la

méthylènedioxypyrovalérone (MDPV), seule ou en association *[Daveluy, 2017; Dumestre-Toulet, 2016; Dumestre-Toulet, 2017; Ferec, 2016; Nachon-Phanithavong, 2017]*, mais également de cannabinoïde de synthèse (l'AB-FUBINACA) *[Allibe, 2017]* et de phénéthylamines (2,5-dimethoxy-4-chloroamphetamine ou DOC, et NBOMe) *[Abbara, 2016 ; Boucher, 2015 ; Ihadadene, 2017 ; Pizzoglio, 2018].* Des cas d'intoxications à la suite de polyconsommation de NPS sont également décrits, tels que des associations de méthoxétamine et d'α-PVP *[Tournebize, 2018]*, ou de méthiopropamine, de méthoxphénidine, de diphénidine (DPD), de diphényl-2-pyrrolidynil méthanol (D2PM) et de 5-méthoxy-N,N-diallytryptamine (DALT) *[Ferec, 2015]*.

Dans certains cas, les intoxications sont fatales. Plusieurs cas de décès, impliquant une ou plusieurs nouvelles substances psychoactives, ont été rapportés au cours de congrès nationaux de la SFTA (Société Française de Toxicologie Analytique) ces dernières années. On retrouve, ici aussi, majoritairement des cathinones (4-MEC, 3-MMC et α-PVP) *[Allard, 2017 ; Bottinelli, 2016 ; Frezil, 2015]* mais également des phénéthylamines (25I-NBOMe et éthylphénidate) [Bodeau, 2017 ; Lelong, 2018], une arylakylamine (5-APB) *[Dumestre-Toulet, 2015]*, et une benzodiazépine de synthèse (diclazépam) *[El Balkhi, 2015]*.

Le nombre de NPS ne cesse d'augmenter et représente ainsi un risque d'autant plus important pour la santé publique. Pour une grande majorité des NPS, très peu de données sont disponibles dans la littérature scientifique. Pour certaines, ces données sont inexistantes.
Les risques liés à l'usage des NPS sont ceux de tout usage de produits psychoactifs mais à cause de leur nouveauté et de leur statut légal particulier, elles présentent certaines menaces spécifiques.

- Contrairement aux stupéfiants usuels, tels que l'héroïne ou la cocaïne, les autorités sanitaires et le personnel médical n'ont pas de recul sur ces produits. Leurs effets cliniques à moyen et à long terme sont inconnus.
- Du fait de leur renouvellement constant, les spécialistes n'ont pas suffisamment de temps pour étudier les nouvelles substances présentes sur le marché et ainsi ne peuvent pas informer les autorités sanitaires de leurs dangers.
- Certaines NPS ne sont pas réglementées et des études ont montré que, très souvent, il n'y avait pas de correspondance entre les étiquettes des produits et les ingrédients actifs contenus dans les sachets achetés sur Internet. Cela démontre clairement le péril de consommer ces substances pour lesquelles l'identité et la pureté font défaut.

Face à ce phénomène en pleine expansion, l'EMCDDA (The European Monitoring Centre for Drugs and Drug Addiction) a lancé une alerte dans son rapport annuel de 2016 *[EMCDD, 2016]*. Le nombre de NPS ne cessant d'augmenter, l'EMCDDA a sensibilisé les laboratoires d'analyses toxicologiques sur

la nécessité de disposer d'une méthode analytique permettant d'identifier un maximum de NPS. Dans ce sens, des experts en toxicologie médico-judiciaire de la SFTA se sont réunis en 2017 afin d'établir des recommandations pour la réalisation d'investigations dans le cas de décès impliquant des NPS. L'objectif de ces recommandations était de proposer une approche raisonnable dans la mise en place d'une stratégie analytique nationale dans le cadre de la détermination de la cause de la mort impliquant des NPS. Plusieurs points ont été fixés, dont la liste minimale de NPS à rechercher, les milieux biologiques à analyser, les éventuels métabolites à identifier, le tout, complété par une discussion sur la stabilité des analytes *[Ameline et le groupe de travail de la SFTA, 2018].*

Tel qu'indiqué précédemment, à mon arrivée à l'Institut de médecine légale de Strasbourg en 2015, aucune méthode dédiée à l'identification des NPS n'était disponible. Ainsi, il est possible que plusieurs cas d'intoxications n'aient pas été mis en évidence, puisque ces substances n'étaient pas recherchées. Pour résoudre ce problème, la première année de ma thèse a été consacrée au développement d'une procédure analytique capable d'identifier les NPS et surtout leurs métabolites, et ainsi d'améliorer l'évaluation de la prévalence de consommation en particulier dans le Grand Est.
Pour enrichir la librairie de spectres MS, il a été nécessaire de recueillir le plus grand nombre possible de standards et de produits de saisie, en collaborant avec le Centre Antipoison de Strasbourg, la brigade des stupéfiants, l'Observatoire Français des Drogues et des Toxicomanies (OFDT) et certains laboratoires de toxicologie français et étrangers. Ce développement a demandé plusieurs mois d'optimisation afin d'obtenir une méthode efficace et sensible. A l'heure actuelle, la librairie de spectres comporte 114 NPS, et elle est mise à jour régulièrement pour être utilisée en routine au laboratoire.
Le laboratoire reçoit quotidiennement des prélèvements hospitaliers et judiciaires. Les échantillons hospitaliers sont analysés en respectant les demandes faites par les cliniciens (voir Partie 2), et une expertise toxicologique de référence est réalisée pour les échantillons médico-légaux (voir Partie 3). Lorsque ces analyses ne suffissent pas pour déterminer la cause de l'intoxication ou du décès, des investigations complémentaires sont réalisées en utilisant divers outils analytiques (voir Partie 4).

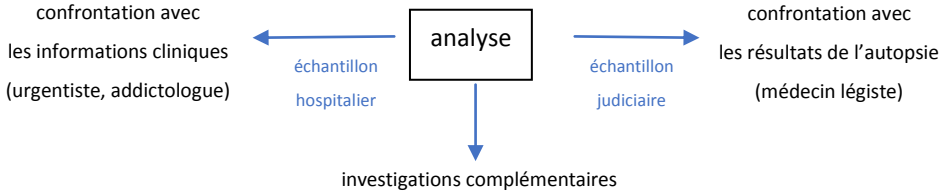


Figure 6: Principe des investigations analytiques appliqué à cette thèse

Développement d'une méthode analytique dédiée aux NPS

La technologie utilisée pour l'identification des NPS est la chromatographie liquide ultra haute performance couplée à la spectrométrie de masse en tandem (UHPLC-MS/MS).

La chromatographie en phase liquide est une technique d'analyse qualitative et quantitative. La séparation s'opère suivant les interactions entre les analytes entrainés par la phase mobile, et la phase stationnaire.

La spectrométrie de masse est une technique physique d'analyse permettant de détecter et d'identifier des molécules d'intérêt par mesure de leur masse, et de caractériser leur structure chimique. Son principe réside dans la séparation de molécules chargées (ions) en fonction de leur rapport masse/charge *(m/z)*.

Protocole d'extraction

L'extraction mise en place est une extraction de type liquide-liquide. A 1 mL de matrice biologique (sang périphérique, sang cardiaque, urines hydrolysées par la β-glucuronidase, contenu gastrique dilué à 1/10ème voire plus, humeur vitrée et bile hydrolysée) sont ajoutés 20 ng de MDMA d_5 utilisé comme standard interne, 1 mL tampon borate saturé à pH 9,5 et 5 mL d'un mélange de solvants d'extractions composé d'éther, de dichlorométhane, d'hexane et d'alcool isoamylique (50:30:20:0.5). Après 15 minutes d'agitation et de centrifugation (15 minutes, 3000 rpm), la phase organique supérieure est récupérée et évaporée à sec sous azote. Le résidu obtenu est reconstitué dans 30 µL de tampon formate d'ammonium à 5 mM ajusté à pH 3.

Pour l'analyse de cheveux, une étape préliminaire de décontamination au dichlorométhane (2 fois 5 mL) est réalisée. Les cheveux sont ensuite séchés puis coupés en petits segments de taille inférieure au millimètre. Ils sont ensuite incubés dans 1 mL de tampon borate saturé à pH 9,5 en présence de 2 ng de MDMA d_5 pendant 15 heures. Par la suite, 5 mL de solvant d'extraction, composé d'éther, de dichlorométhane, d'hexane et d'alcool isoamylique (50:30:20:0.5) sont ajoutés. La suite de l'extraction est identique à celle des autres matrices biologiques.

Le reliquat est injecté dans le système de chromatographie liquide muni d'une colonne Acquity™ UHPLC HSS C_{18} (150 x 2 mm, 1,8 µm) (Waters), thermostatée à 50°C. La séparation des composés est réalisée à l'aide d'un gradient tampon formate d'ammonium 5 mM ajusté à pH 3 / acétonitrile + 0,1 % d'acide formique à un débit de 0,4 mL/min. Le temps d'analyse est de 15 min (tableau 1).

Temps (min)	Tampon formate d'ammonium (%)	Acétonitrile + 0.1 % HCOOH (%)
0	87	13
0,50	87	13
10,00	50	50
10,75	5	95
12,25	5	95
12,50	87	13
15,00	87	13

Tableau 1: Gradient d'analyse UHPLC

Le spectromètre de masse utilisé pour la détection est un Xévo ® TQD, dont les caractéristiques sont les suivantes : mode d'ionisation : ES^+ ; température de la source : 150 °C; température de désolvatation : 600 °C; débit de gaz de désolvatation : 1000 L/Hr ; gaz de collision : argon. L'acquisition des données est réalisée en mode MRM (Multiple Reaction Monitoring) et optimisée pour chaque molécule. Après infusion manuelle des substances pures, deux transitions spécifiques sont définies (une transition de quantification et une transition de confirmation). Il s'agit généralement des deux transitions les plus abondantes ou des plus significatives. La liste des molécules recherchées est présentée dans le Tableau 2 (a et b).

Cette méthode est utilisée en première intention comme criblage des NPS. Lorsque qu'une molécule est identifiée avec certitude (temps de rétention et rapport des ions identiques à ceux de la substance pure), une méthode spécifique est alors développée. Une mini-validation est réalisée incluant la répétabilité, la justesse, la linéarité et l'effet matrice. A titre d'exemple, une mini-validation a été faite pour la 3-MeO-PCP et publiée *[Kintz, 2019]*.

Une fois cette méthode développée, des cas d'intoxications et de décès impliquant des NPS ont rapidement été mis en évidence. Ces cas ont donné lieu à des publications nationales et internationales.

Nom	Tr (min)	Transition 1	Transition 2
1,3-Chlorophenyl-piperazine (mCPP)	4,2	198,9 > 117,9	198,9 > 90,8
25B-NBOMe	8,2	381,8 > 120,9	381,8 > 90,9
25C-NBOMe	8,0	336,0 > 120,9	336,0 > 90,9
25D-NBOMe	8,1	316,1 > 120,9	316,1 > 90,9
25E-NBOMe	8,8	330,0 > 120,9	330,0 > 90,9
25H-NBOMe	6,8	302,1 > 120,9	302,1 > 90,9
25I-NBOMe	8,9	428,9 > 120,9	428,9 > 90,9
25N-NBOMe	6,9	347,1 > 120,9	347,1 > 90,9
25P-NBOMe	10,0	344,1 > 120,9	344,1 > 90,9
2-Benzylamino-(3,4-méthylènedioxyphényl)propanone (BMDP)	4,4	284,0 > 90,9	284,0 > 266,1
2-Fluorométhamphétamine	2,8	167,9 > 136,9	167,9 > 108,9
2-oxo-3-hydroxy-LSD	5,0	356,1 > 237,0	356,1 > 74,0
3-(1,3-Benzodioxol-5-yl)butanamide (BDB)	3,0	193,9 > 134,9	193,9 > 146,9
3,4-Methylenedioxy-alpha-pyrrolidinobutiophenone (MDPBP)	2,1	262,7 > 161,7	262,7 > 112,2
3,4-Methylènedioxyamphetamine (MDA)	2,2	180,1 > 163,1	180,1 > 133,0
3,4-Methylènedioxy-N-ethylamphetamine (MDEA)	3,0	208,1 > 163,2	208,1 > 135,1
3,4-Methylènedioxy-N-methamphetamine d5 (MDMA-d5)	2,5	198,9 > 164,9	198,9 > 106,9
3,4-Methylènedioxy-N-methamphetamine (MDMA)	2,6	194,1 > 163,0	194,1 > 105,0
3,4-Methylenedioxypyrovalerone (MDPV)	4,5	275,8 > 125,9	275,8 > 134,8
3-Fluorofentanyl	6,8	355,1 > 105,0	355,1 > 134,0
3-Fluorophenmetrazine (3-FPM)	3,2	195,9 > 114,8	195,9 > 108,9
3-Methoxyphencyclidine (3-MeO-PCP)	6,5	274,0 > 188,8	274,0 > 120,8
3-Methylmethcathinone (3-MMC)	2,9	178,1 > 143,9	178,1 > 91,1
3-Trifluoromethylphenylpiperazine (TFMPP)	5,5	230,9 > 43,8	230,9 > 118,2
4-Bromo-2,5-dimethoxyphenethylamine (2C-B)	4,6	261,9 > 229,7	261,9 > 90,7
4-Chloroethcathinone (4-CEC)	3,5	211,9 > 193,9	211,9 > 158,9
4-Ethylmethcathinone (4-EMC)	2,0	192,0 > 144,1	192,0 > 104,9
4-Fluoro-alpha-pyrrolidinohexiophenon (4-F-PHP)	5,3	264,1 > 108,8	264,1 > 122,9
4-Fluoro-alpha-pyrrolidinovalerophenone (4-F-PVP)	5,6	266,0 > 124,8	266,0 > 138,8
4-Fluoroamphétamine	2,7	153,8 > 82,7	153,8 > 40,7
4-Fluorométhamphétamine	3,0	167,8 > 108,8	167,8 > 82,8
4-Fluoromethcathinone	3,0	181,9 > 148,9	181,9 > 102,8
4-Methoxyphencyclidine (4-MeO-PCP)	6,5	274,0 > 188,8	274,0 > 120,8
4-methyl-AM-2201 (MAM-2201)	1,8	375,2 > 111,0	375,2 > 136,8
4-methyl-AM-2201 d5 (MAM-2201 d5)	1,8	379,2 > 115,0	379,2 > 141,8
4-Methylethcathinone (4-MEC)	3,6	192,0 > 144,1	192,0 > 130,1
4-Méthylthioamphétamine (4-MTA)	3,8	181,9 > 116,8	181,9 > 90,7
5-(2-Aminopropyl)-benzofuran (5-APB)	1,4	177,0 > 90,8	177,0 > 84,9
5-(2-Methylaminopropyl)-benzofuran (5-MAPB)	3,9	190,0 > 158,9	190,0 > 130,9
5-iodo-2-aminoindane (5-IAI)	7,0	259,8 > 115,9	259,8 > 88,9
AB-PINACA	4,0	331,5 > 215,5	331,5 > 286,5
Acetylfentanyl	5,1	323,1 > 105,0	323,1 > 78,9
Acide ritalinique	2,8	220,0 > 83,9	220,0 > 55,8
AH-7921	6,4	329,1 > 172,9	329,1 > 284,1
Alfentanil	6,1	417,2 > 268,2	417,2 > 197,0
Alpha-Methylfentanyl	6,4	351,2 > 202,2	351,2 > 119,1
Alpha-pyrrolidinohexiophenone (Alpha-PHP)	4,9	245,9 > 90,8	245,9 > 104,7
Alpha-pyrrolidinopentiophenone (Alpha-PPP)	2,2	203,9 > 90,8	203,9 > 104,7
Alpha-pyrrolidinovalerophenone (Alpha-PVP)	4,3	231,8 > 90,8	231,8 > 104,7
Amfépramone	2,8	206,0 > 132,9	206,0 > 104,9
Amphetamine	2,2	136,1 > 119,1	136,1 > 91,1
Atropine	3,1	290,1 > 124,0	290,1 > 93,0
Benzodioxolyl-N-methylbutanamine (MBDB)	3,4	209,2 > 135,5	209,2 > 177,8
Benzylpiperazine (BZP)	1,4	176,9 > 90,8	176,9 > 84,8
Buphedrone	2,6	177,8 > 90,8	177,8 > 130,9
Buthylone	7,0	221,9 > 174,0	221,9 > 145,9

Tableau 2: Liste des molécules recherchées (nom, temps de rétention et deux transitions spécifiques)

Nom	Tr (min)	Transition 1	Transition 2
Butyrylfentanyl	7,2	351,1 > 105,0	351,1 > 188,1
Carfentanil	7,0	395,2 > 112,9	395,2 > 105,0
Cathine	4,0	153,8 > 118,1	153,8 > 118,0
Clonazolam	7,6	353,9 > 102,8	353,9 > 279,9
Cumyl-pegaclone (SGT-151)	2,1	373,1 > 255,0	373,1 > 119,0
Deschloroetizolam	8,4	308,9 > 254,9	308,9 < 280,0
Desomorphine	2,2	272,0 > 215,0	272,0 > 43,9
Dextromethorphane (DXM)	6,3	271,9 > 215,4	271,9 > 146,9
Diazepam d5	10,4	291,9 > 197,8	291,9 > 155,8
Dibutylone	2,6	236,0 > 148,8	236,0 > 85,8
Diclazepam	10,9	320,9 > 89,2	320,9 > 156,4
Diethyllysergamine (LSD)	5,1	324,2 > 223,1	324,2 > 208,1
Diethyllysergamine d3 (LSD d3)	5,1	327,1 > 225,9	327,1 > 207,9
Dimethyltryptamine	2,7	189,0 > 57,9	189,0 > 116,9
Diphenidine	6,5	266,1 > 181,0	266,1 > 102,9
Ephédrine	1,8	166,1 > 117,3	166,1 > 132,9
Ethcathinone	5,0	177,9 > 130,0	199,7 > 104,9
Ethylone	2,4	221,9 > 173,9	221,9 > 145,9
Ethylphenidate	5,2	247,9 > 83,8	247,9 > 55,8
Etizolam	9,2	342,9 > 288,9	342,9 > 313,9
Fentanyl	6,2	337,1 > 105,0	337,1 > 132,0
Fentanyl d3	6,2	340,2 > 108,1	340,2 > 135,7
Flubromazepam	9,3	334,9 > 266,1	334,9 > 185,9
Fluclotizolam	8,3	332,8 > 297,9	332,8 > 278,9
Flunitrazolam	7,1	337,9 > 263,9	337,9 > 102,8
Furanylfentanyl	6,4	375,1 > 105,0	375,1 > 188,1
JWH-210 d9	3,0	379,4 > 183,0	379,4 > 223,0
Kétamine	3,2	239,9 > 126,7	239,9 > 88,8
Meclonazepam	9,3	327,9 > 285,9	327,9 > 291,9
Mephedrone (4-MMC)	2,9	178,1 > 143,9	178,1 > 91,1
Mescaline	5,5	332,1 > 120,9	332,1 > 90,6
Metamfépramone	2,0	177,9 > 104,8	177,9 > 76,9
Méthamphétamine	2,4	150,1 > 91,1	150,1 > 119,1
Methiopropamine	2,0	155,9 > 124,9	155,9 > 96,9
Methoxamine	2,5	211,7 > 161,8	211,7 > 178,9
Methoxyphenidine (MeO-Diphenidine)	6,0	295,9 > 210,9	295,9 > 85,9
Methylone	2,0	207,9 > 159,8	207,9 > 131,8
Methylphenidate	4,1	233,8 > 84,0	233,8 > 90,9
Methylphenidate d10	4,1	244,4 > 93,9	244,4 > 100,8
Metizolam	8,8	328,9 > 274,9	328,9 > 259,9
MT-45	en attente de réception		
N,N-diallyl-5-methoxytryptamine (5-MeO-DALT)	5,1	270,9 > 109,9	270,9 > 173,9
Naphyrone	3,5	281,9 > 210,9	281,9 > 140,9
Nifoxipam	6,5	313,8 > 285,9	313,8 > 41,8
Noréphédrine	1,5	151,7 > 116,9	151,7 > 90,9
Norfentanyl	3,2	233,0 > 83,9	233,0 > 177,0
Norkétamine	3,0	225,9 > 126,7	225,9 > 88,8
Ocfentanyl	5,1	371,1 > 105,0	371,1 > 188,1
Para-fluorobutyrylfentanyl (PFBF)	en attente de réception		
Pentedrone	3,6	191,7 > 143,9	191,7 > 90,7
Pentylone	3,8	235,9 > 85,9	235,9 > 187,9
Phencyclidine (PCP)	6,0	243,8 > 85,7	243,8 > 90,7
Pseudoéphedrine	1,8	166,1 > 117,3	166,1 > 132,9
Pyrazolam	6,0	355,9 > 205,8	355,9 > 166,9
Scopolamine	2,3	304,8 > 138,0	304,8 > 156,1
Sufentanil	7,6	387,1 > 110,9	387,1 > 140,1
U-47700	6,1	329,1 > 284,0	329,1 > 172,9

Tableau 3: Liste des molécules recherchées (nom, temps de rétention et deux transitions spécifiques), suite.

Partie 2 : Applications cliniques

Dans le cadre de ses activités, le laboratoire de toxicologie reçoit quotidiennement des prélèvements hospitaliers. Il s'agit principalement de demandes de dosage des médicaments (suivi thérapeutique) et des stupéfiants usuels. Les cliniciens peuvent également demander un criblage toxicologique, en particulier à la suite d'une consommation de produit inconnu (notamment des NPS).

Ces nouvelles substances sont vendues comme étant légales, et sont, à tort, jugées plus sûres. Pour la plupart, elles sont disponibles directement dans la rue, lors de regroupements festifs (concerts, rave party, soirées privées), mais également sur internet. Il est extrêmement facile de s'en procurer, à bas prix, mais il n'est pas aisé de savoir ce qu'on a réellement acheté. Il n'est pas rare que le principe actif ou la concentration du produit ne soit pas mentionné sur l'emballage. Même, lorsque le principe actif est indiqué, son dosage peut être inexact et donc trompeur. Ce manque d'information entraine un grand nombre d'intoxications par erreur de dosage ou par potentialisation de plusieurs substances consommées simultanément. Les signes et symptômes en cas d'intoxication par NPS sont très superposables à ceux des médicaments et de stupéfiants classiques. Pour beaucoup de substances, aucune information n'existe dans la littérature. Pour d'autre, des études ont été faites chez les animaux, mais pas chez l'Homme. Généralement, dans l'urgence de la situation, ces intoxications sont soignées de façon symptomatique, sans analyse toxicologique. Cependant, dans certains cas qui représentent un intérêt scientifique ou médico-légal (consommation de substance classée, trafic, infraction à la législation sur les stupéfiants (ILS)), il est indispensable de réaliser une analyse toxicologique, même après l'hospitalisation, afin de documenter le cas et d'enrichir la littérature.

Durant ma thèse, plusieurs cas d'intoxication aux NPS ont été observés dans le cadre hospitalier. Ces cas sont décrits ci-dessous et ont donné lieu à la rédaction de 4 publications.

- Deux hommes homosexuels ont été trouvés nus à leur domicile, par un ami. Ce dernier a contacté les urgences du fait de leur comportement inhabituel (hallucinations visuelles, incohérence du langage, difficulté à articuler et agressivité importante). Ils ont rapidement indiqué être consommateurs réguliers de NPS. Des emballages de poudres, annotés « 25I-NBOMe » ont été retrouvés à leur domicile. Une méthode dédiée à cette substance a été développée et validée. Des concentrations à 37 et 72 ng/mL ont été mesurées dans les prélèvements de sang des deux hommes, en adéquation avec le tableau clinique et la présence de la poudre. Il a été notifié, dans la feuille de suivi médical, que les symptômes s'étaient rapidement atténués *[Article 1]*.

- Un jeune homme de 17 ans, en soirée chez des amis, pense consommer un buvard de LSD, acheté auprès d'un trafiquant, dans la rue. Après plusieurs phases d'hallucinations, il est retrouvé inconscient. A l'arrivée des urgences, le jeune homme fait un arrêt cardio-respiratoire, présente une mydriase bilatérale réactive, est en tachycardie et convulse. Après 8 jours d'hospitalisation, il est encore très agité, agressif et présente des troubles du langage et de la motricité. Les analyses de sang et d'urine ont révélé l'absence d'alcool, de médicament et de LSD. La méthode d'identification des NPS a mis en évidence la présence de 25I-NBOMe à 6,1 ng/mL dans le sang. Six mois et demi après les faits, le jeune homme est toujours hospitalisé dans un service de rééducation pour déficit neurologique. Dans le cadre d'une expertise judiciaire, deux mèches de cheveux ont été prélevées afin d'établir son profil de consommation. Il a été montré que seul le segment de cheveux qui correspondait à la période des faits était positif au 25I-NBOMe. Il s'agit donc d'une exposition unique à cette substance et également du premier cas d'identification dans des cheveux humains. Toute la difficulté de cette investigation réside dans l'interprétation de la concentration capillaire (4,9 pg/mg) puisqu'il n'existe aucun référentiel *[Article 2]*.

- Deux jeunes migrants ont été hospitalisés en urgence à la suite de vomissements et de malaises. Ils auraient fumé un mélange d'herbe et de poudre, donné par un inconnu quelques minutes auparavant. Grâce à la description d'un témoin, cet homme, trafiquant connu, a été arrêté et a avoué avoir donné aux deux victimes, une cigarette mélangée à du SGT-151, un cannabinoïde de synthèse. Des prélèvements de sang et d'urines ont été réalisés. Une étude *in vitro* sur microsomes hépatiques humains a été entreprise afin d'obtenir des informations sur le métabolisme du SGT-151. Le SGT-151 et ses métabolites ont pu être identifiés dans les prélèvements des deux migrants et du trafiquant. Ces résultats indiquent que les trois sujets étaient sous l'influence du SGT-151 au moment des prélèvements. L'interprétation des concentrations est difficile à établir puisqu'il y a peu d'information dans la littérature concernant cette substance. Les concentrations mesurées (autour de 1 ng/mL) sont faibles alors même que les effets cliniques étaient importants, ce qui confirme que le SGT-151 est beaucoup plus actif que le Δ9-tétrahydrocannabinol *[Article 3]*.

- Les benzodiazépines sont une classe de médicaments largement utilisées et font l'objet d'abus et de mésusage dans le monde entier. La recherche étendue autour de leur structure chimique a engendré la synthèse d'une grande variété de molécules actives qui n'ont jamais eu d'autorisation de mise sur le marché. Aujourd'hui, ces molécules sont reprises par des e-boutiques qui les vendent sous le terme «designer benzodiazepines». Récemment, il a été constaté une augmentation des cas d'intoxications liés à l'abus de ces substances. Afin de faire un état des lieux

des « designer benzodiazepines », et plus précisément de la pharmacologie et la toxicologie chez l'homme, une revue complète de la littérature a été préparée. Il en ressort que nos connaissances, en ce qui concerne les « designer benzodiazepines » sont très variables selon les molécules. L'offre est toujours en évolution et on observe l'émergence de molécules dont la dose active est de plus en plus faible. Cela fait des « designer benzodiazepines » un défi pour les laboratoires d'analyse et un risque pour la santé publique *[Article 4]*.

Dans l'ensemble des cas décrits, la confrontation entre les résultats toxicologiques et le tableau clinique, tel que rapporté par entretien avec le médecin (urgentiste, addictologue, psychiatre), a permis d'établir que l'intoxication était bien corrélée à l'utilisation de la molécule, à l'origine de la prise en charge hospitalière.

Article 1: Identification of 25I-NBOMe in two intoxications cases with severe hallucinations

Alice Ameline, Pascal Kintz, Caroline Blettner, Éric Bayle, Jean-Sébastien Raul

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CASE REPORT

Identification of 25I-NBOMe in two intoxications cases with severe hallucinations

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KEYWORDS
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25I-NBOMe;
Plasma;
UPLC-MS/MS;
Severe hallucinations

Summary The abuse of new psychoactive substances (NPS) has been dramatically increasing all around the world since the late 2000s. The availability of hundreds of NPS in the past decade is challenging for both public health and global drug policies. We present the intoxication of two subjects who had sniffed a white creamy powder, characterized as 25I-NBOMe [2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine]. Plasma samples were analyzed by ultra-high performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS). Concentrations of 25I-NBOMe were 37 and 72 ng/mL, respectively. Major impairment of both subjects, including visual hallucinations and alteration of behaviour were attributed to these high concentrations. The data were compared to the few reports available in the medical literature.
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Introduction

For several years, new synthetic drugs appeared on the Internet. These are the new psychoactive substances (NPS), which designate a wide range of molecules having the same pharmacological effects as various illegal substances such as ecstasy, amphetamines, benzodiazepines, opiates, or cannabis. Their chemical structures can be close to the original

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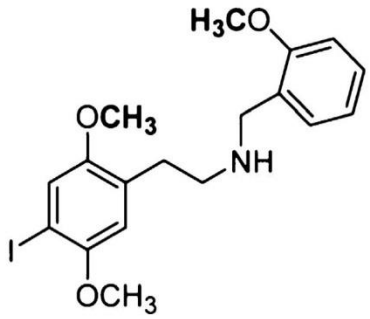

Figure 1. Chemical structure of 25I-NBOMe.

compound, but can be also very far from it. These substances can skirt the narcotic laws because they are not listed as controlled drugs. Generally sold on the internet, NPS are differentiated by their chemical names or trade names such as ''bath salts'', ''plant for nutrients'', ''incense''... According to a survey of the French Observatory Drugs and Drug Addiction [1], more than 382 substances circulated freely in the European Union between 2008 and 2015, including 176 in France. NPS are becoming more prevalent in the world of teenagers and parties, given the ease of purchase and use.

Among NPS, a group named phenethylamine NBOMe has gained importance in the recent years. N-Methyl-Benzyl-Oxy is the extension of the psychedelic family phenethylamine 2C, discovered by Dr. Alexander Shulgin and described in his book published in 1991 PIHKAL [2].

Among NBOMe, 25I-NBOMe (Fig. 1), or 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl) methyl]ethanamine, was discovered in 2003 by Ralf Heim [3] at the University of Berlin. It is a potent agonist of 5-HT2A receptors [4] in the brain. It can be consumed most often in the form of drops [5,6] or blotters and consumed by the transmucosal, sublingual or intranasal routes.

The NBOMe compounds have numerous psychedelic effects [5]: visual hallucinations, altered time and space, change of consciousness... They also have stimulating effects like euphoria or empathy. These effects can last from 5 to 10 hours [5]. They produce many side effects, including convulsions, paranoia, psychosis, coma up to death.

Analytically, the characterization of a consumption of 25I-NBOMe is not obvious. The literature is very poor in reporting cases where 25I-NBOMe was identified. Epidemiologically, only very few clinical cases of consumers were reported, indicating a low prevalence of this compound. Due to its possible misidentification, very few samples have been specifically tested for 25I-NBOMe. Indeed, consumers are generally accustomed to consume NPS and know how to manage their doses and the associated side effects [7]. Furthermore, for analysis of any NPS, it is necessary to have the pure standard substance to be able to build a reference spectrum and to identify the molecule in further biological specimens. Unfortunately, these standards are only available several months after the release of the substance on the Internet, and the large number of molecule is an economic problem to maintain an up to date reference library. The identification of 25I-NBOMe in blood is difficult, given that ingested doses are generally between 100 and 600 micrograms [8]. As a consequence, the use of tandem mass spectrometry appears as a pre-requisite to detect the very low circulating amount of the drug, in the sub ng/mL range and to entrance the specificity.

The objective of this publication is to present the analytical procedure established for assaying 25I-NBOMe in plasma of two subjects admitted at the Emergency room of the Hospital of Strasbourg.

Clinical case

Two gay men were discovered naked in their home. A friend of the couple called the medical rescue department because he found they acted strangely. Both reported visual hallucinations, incoherent speech, dysarthria and a significant aggressive behaviour. The emergency team noted a highly disinhibited behaviour and gestures of sexual nature (masturbation).

At their arrival at the hospital at 10:00 pm, both subjects reported having sniffed at 6:30 pm several drugs, ordered via the Internet. These drugs were methiopropamine, methylenedioxypyrovalerone (MDPV), amphetamines and opiates. Sildenafil was also abused by one of the addict, while the second reported alcohol consumption (two beers). A near death feeling was reported. Both individuals claimed to be regular consumers of NPS since 2014.

The clinical examination of the first patient highlighted a Glasgow score at 15, a coherent but highly productive speech, an absence of hallucinations, a reactive bilateral mydriasis, and a normal cardiopulmonary examination. The same clinical picture was observed for the second patient. The main symptoms were neurologic and psychiatric. Clinical observations went back to normal with appropriate behaviour around 3:00 am. After 5 hours of supervision, the two individuals were discharged from the hospital. A blood sample from each subject was collected and sent to the biochemistry laboratory for standard parameters (all within the normal range). Only 300 μL of plasma were available for the screening of 25I-NBOMe and MDPV at our laboratory.

During the first steps of rescue at home, the emergency team found three small plastic bags, each containing a white creamy powder. Two bags were labelled ''25I-NBOMe'' and the third was labelled ''MDPV''. A powder bag labelled 25I-NBOMe is presented on Fig. 2.

Materials and methods

The three bags of powder and a plasma sample from each patient were sent to the laboratory for 25I-NBOMe and MDPV analysis. There was no attempt to screen for other drugs, given the very low volume of specimens that were available (300 μL). A specific method to 25I-NBOMe was developed on an ultra-high performance liquid chromatography (Acquity class I) coupled to a Xevo TQD tandem mass spectrometer (UPLC-MS/MS) from Waters (Milford, MA, USA).

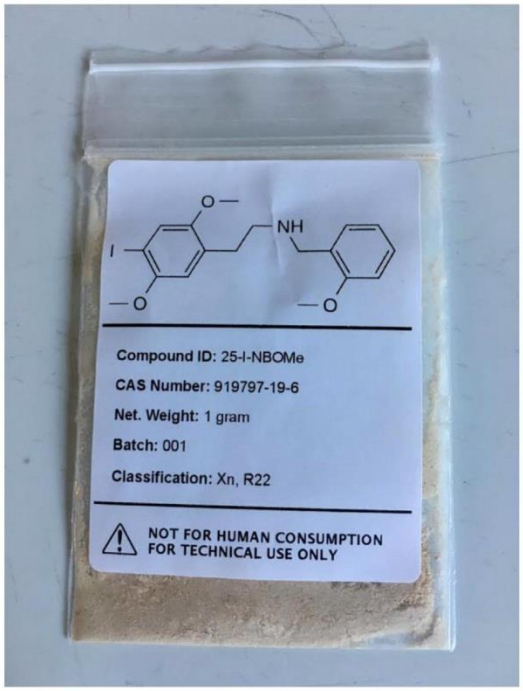

Figure 2. Picture of power bag annotated 25I-NBOMe.

Chemicals and reagents

Ammonium fomate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99-100%, AnalaR NORMAPUR and 36% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Ethyl acetate for liquid chromatography and acetonitrile for LC-MS were purchased from Merck (Molsheim, France). MDMA-d_5 was purchased from LipoMed (Arlesheim, Switzerland) and diluted to appropriate concentration using acetonitrile.

25I-NBOMe analysis

Both powders labelled 25I-NBOMe were first tested by LC-MS over a mass range from *m/z* 50 to *m/z* 600 to check for purity. Only one peak was observed and the powder was then estimated to be pure. However, this statement was not verified by alternative methods, such as NMR. As a consequence, the concentration values should be considered as semi-quantitative, at best. An aliquot was prepared at 1 g/L in acetonitrile.

25I-NBOMe was extracted from 300 μL plasma in presence of 1 ng of MDMA-d_5 used as internal standard, with 300 μL borate buffer pH 9.5 and 2 mL of ethyl acetate. After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 30 μL of ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained at 50 °C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid

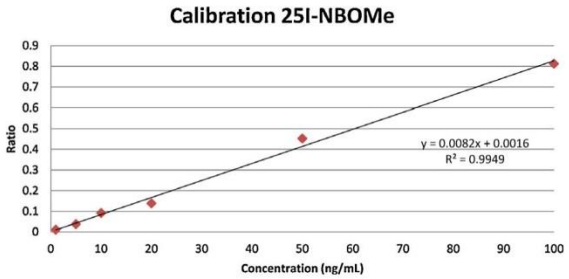

Figure 3. Calibration curve of 25I-NBOMe between 1 and 100 ng/mL.

in acetonitrile LC-MS (mobile phase B) as mobile phases at flow rate of 0.4 mL/min. The initial gradient was 80% mobile phase A and the final gradient, at 7 min, was 30% mobile phase A. An injection volume of 10 μL was used in all cases. A Xevo triple quadrupole mass spectrometer was used for the detection of the drugs. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of 25I-NBOMe and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149 °C; desolvation gas nitrogen heated at 600 °C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation of both species was performed. Collision energy was adjusted to optimize the signal for the 2 most abundant product ions of 25I-NBOMe: *m/z* 428.1 > 121.2 (CV: 22V) and 428.1 > 90.6 (CV: 36V), and the most abundant product ion of internal standard *m/z* 199.1 > 165.1 (CV: 12V). Transition *m/z* 428.1 to 121.2 was used for quantification of 25I-NBOMe. These transitions have already been described in the literature. MassLynx 4.1 software was used for quantification.

Results and discussion

Three hundred microliters plasma was spiked with the powder found in the pocket of the patients. Linearity was observed for 25I-NBOMe concentrations ranging from 1 to 100 ng/mL, with a correlation coefficient of 0.9949. The calibration curve is presented on Fig. 3. The limit of detection was estimated to be 0.1 ng/mL, with a S/N ratio of 3. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in plasma (matrix effect < 20%, after analysis of 10 various blank plasma).

25I-NBOMe tested positive in the two plasma samples at 37 and 72 ng/mL. Chromatograms obtained after extraction of the plasma of both subjects are presented on Figs. 4 and 5. These concentrations are high in comparison with previous reports and may explain the initial effects of the drug observed in two individuals. MDPV was detected in the plasma of subject 1 at 59 ng/mL. The plasma of subject 2 was free of MDPV. No other drug was identified.

As indicated earlier, only few papers have reported the detection of 25I-NBOMe in human biological specimens. In 2014, Poklis et al. [8] reported a fatal case involving

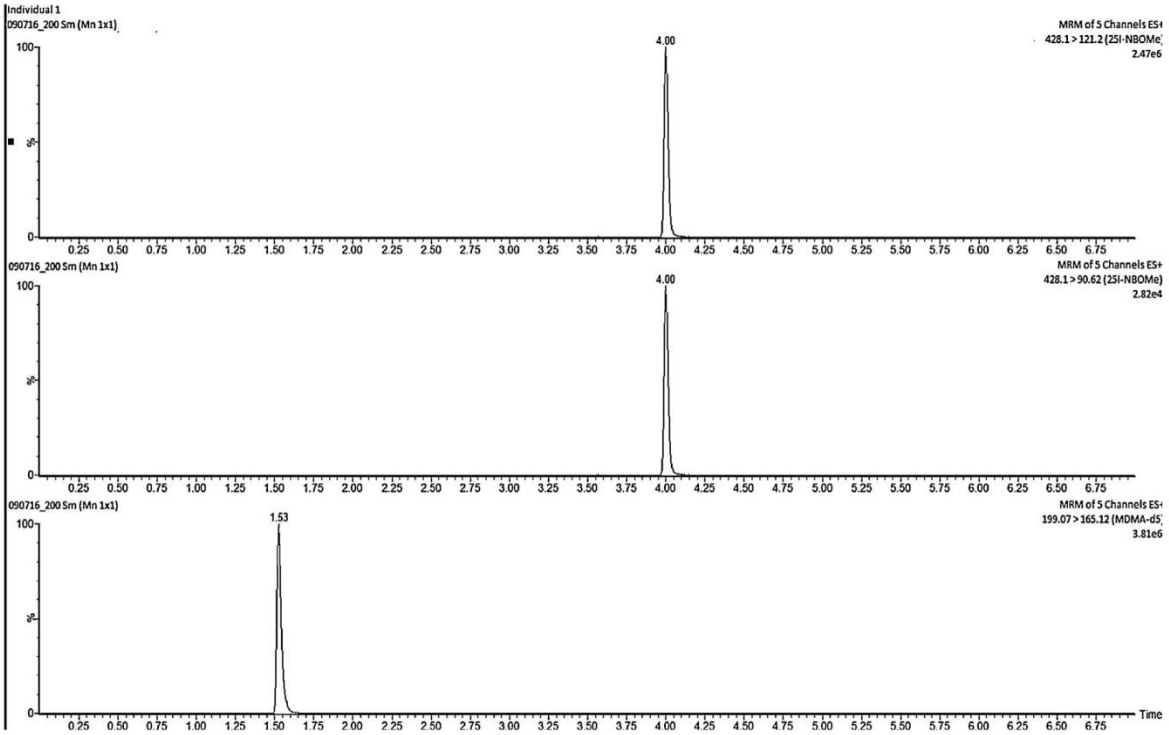

Figure 4. Chromatogram obtained after extraction of the plasma of subject 1. 25I-NBOMe concentration was 72 ng/mL. From top to bottom: 2 transitions for 25I-NBOMe (*m/z*: 428.1 > 121.2 and 428.1 > 90.62) and 1 transition for MDMA-d_5 (*m/z*: 199.07 > 165.12).

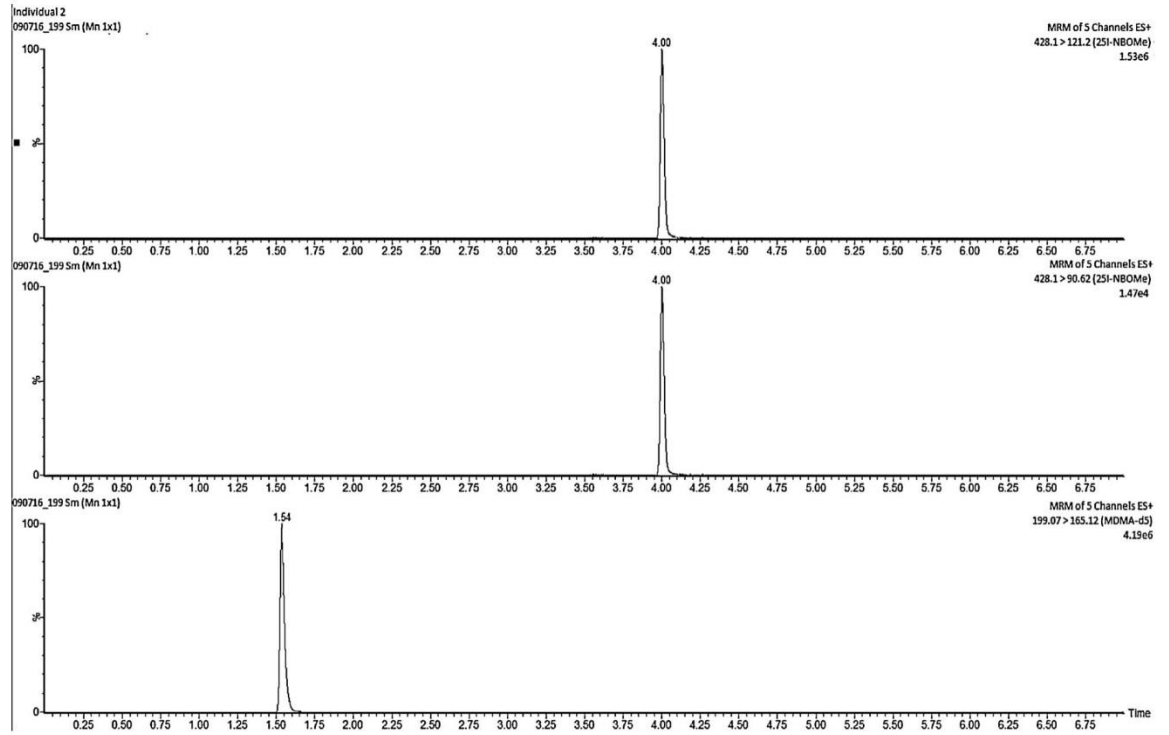

Figure 5. Chromatogram obtained after extraction of the plasma of subject 2. 25I-NBOMe concentration was 37 ng/mL. From top to bottom: 2 transitions for 25I-NBOMe (*m/z*: 428.1 > 121.2 and 428.1 > 90.62) and 1 transition for MDMA-d_5 (*m/z*: 199.07 > 165.12).

25I-NBOMe. A healthy 19-years-old mal ingested blotter paper infused with ''acid'' the day before his death by falling from his apartment balcony. Toxicological analysis of the decedent revealed 25I-NBOMe at 0.41 ng/mL in heart blood, 0.40 ng/mL in peripheral blood, 2.86 ng/mL in urine, 0.099 ng/mL in vitreous humor, 2.78 ng/mL in the brain, 5.64 ng/mL in the liver and 12.1 ng/mL in bile. It was established that hallucinations due to 25I-NBOMe have contributed to his fall.

In 2014, Boucher et al. [9] reported an intoxication case of a mixture of three NBOMe: 25I-NBOMe, 25C-NBOMe and 25H-NBOMe. A 29-year-old man, with schizophrenic and drug addiction histories, sniffed a drop of the mixture. After one hour, the subject developed signs of hallucinations, changes of behaviour, mydriasis, hypothermia and excessive sweating. Neurological failure caused coma and required invasive ventilation for 24 hours. Magnetic resonance imaging documented bilateral and symmetrical lesions of the basal ganglia. After one month, the patient had still memory impairment and significant abnormalities in executive functions. No blood was available for analytical investigations.

In 2015, Heiger et al. [10] described a series of self-reported exposures to 25I-NBOMe. Ten patients with an average age of 17 years (14—20 years), 8 males and 2 females, were admitted to a local emergency department after ingestion and/or insufflation of a drug referred to as ''25I''. Of 10 patients, six reported taking 25I alone; other substances were ethanol, 2,5-dimethoxy-4-ethylphenethylamine, marijuana and ketamine. Most common effects included tachycardia (90%), hypertension (70%), agitation (60%) and hallucinations (50%). Three patients required intubation, and all admitted patients were given intravenous benzodiazepines for sedation. The duration of toxicity was usually less than 24 hours in uncomplicated cases. Blood was analyzed in one single case, obtained from a patient presenting agitation and tachycardia. The serum 25I-NBOMe concentration was 0.76 ng/mL. All patients were discharge in good conditions once the symptoms were reversed.

Oosthuizen et al. [11] reported during the 2016 TIAFT meeting the case of the death of a 23-year-old female who was nasally insufflating an unknown white powder, which was believed to be synthetic LSD. Using LC-QToF, the following drugs were identified in her aortic blood: 28 ng/mL of 25I-NBOMe, 1 ng/mL of 25H-NBOMe and 0.7 ng/mL of 25C-NBOMe.

Shanks et al. [12] reported the deaths of two male teenage who consumed 25I-NBOMe and 25B-NBOMe in Indiana during 2014. 25I-NBOMe was detected in postmortem heart blood at 19.8 ng/mL for the second teenage.

Finally, Walterscheid et al. [13] have identified 25I-NBOMe in two fatalities without reporting the concentrations. In addition, in the same paper, they found in the serum of a 18-year-old mal presenting severe agitation and hallucinations a concentration of 0.76 ng/mL.

In all these cases involving a 25I-NBOMe intoxication [14], the same pharmacological effects were observed: alteration of behaviour, tachycardia, hypertension, agitation, aggression, visual and auditory hallucinations, mydriasis and excessive sweating. These symptoms have numerous forensic implications, as they can cause memory impairment, neurological and functional disorders.

25I-NBOMe is considered in France as a drug of abuse since a notice (*arrêté*) of 24th September 2015. More recently (27 September 2016), in the United States of America the Administrator of the Drug Enforcement Administration has placed 25I-NBOMe, including its optical, positional, and geometric isomers, salts and salts of isomers, whenever the existence of such salts, isomers, and salts of isomers is possible, into schedule I of the Controlled Substances Act.

Conclusion

We have presented 2 cases involving 25I-NBOMe poising with severe hallucinations and impairment. Symptoms spontaneously reversed after a few hours. Measured concentrations were high, in the light of the existing literature. Accordingly, the risk of missing 25I-NBOMe use or abuse during conventional toxicological screening is important. Since 25I-NBOMe appears not to be tested in many laboratories, one major concern about this drug regarding public health remains the lack of data on its potential acute toxicity and long-term side effects in humans. This is particularly true, as it has been reported in the recent literature [15,16] intoxication at a concentration lower than 1 ng/mL.

Disclosure of interest

The authors declare that they have no competing interest.

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Article 2: Retrospective demonstration of 25I-NBOMe acute poisoning using hair analysis

Alice Ameline, Audrey Farrugia, Jean-Sébastien Raul, Pascal Kintz

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CASE REPORT

Retrospective Demonstration of 25I-NBOMe Acute Poisoning Using Hair Analysis

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Abstract: ***Background***: The abuse of new psychoactive substances or NPS has been dramatically increasing all around the world since the last half of the year 2000 and has become a serious public health problem. NPS are a challenge for the worldwide forensic community due to the difficulties to accurately document the cases.

The N-benzylmethoxy (NBOMe) group is a new class of hallucinogenic designer drugs and has gained importance in recent years. 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)-methyl]ethanamine) is an analog of the 2C series of psychedelic phenethylamine drugs that contain an N-methoxybenzyl substituent, which significantly affects their pharmacological activities. It is a potent agonist of 5-HTA receptors and a severe hallucinogenic drug, with numerous irreversible psychedelic effects which can last from 5 to 10 hours. It is consumed most often in the form of drops or blotters by the transmucosal, sublingual or intranasal routes. The active dosage is very low, supposed to be less than 100 µg. The literature is poor in reporting cases where 25I-NBOMe was identified. Only very few clinical cases of overdosages were published, suggesting a low prevalence of this compound.

Methods: We present a retrospective demonstration of 25I-NBOMe acute poisoning with dramatic outcome, using hair analysis. Two hair strands, measuring 9.5 cm, were collected 6.5 months after drug consumption during a forensic clinical evaluation of brain dysfunctions after cardiorespiratory arrest and were analyzed by ultra-high performance liquid chromatography system coupled to a tandem mass spectrometry (UPLC-MS/MS) and using two specific transitions: *m/z* 428.1 > 121.2 (quantification) and 428.1 > 90.6 (confirmation). Hair strands were segmented to determine the historic pattern of drug use and differentiate a single exposure from a chronic exposure. The hair test result for 25I-NBOMe was the following: not detected (0-2 cm), not detected (2-4 cm), 1.0 pg/mg (4-6 cm), 4.9 pg/mg (6-8 cm) and not detected (8-9.5 cm).

Result: The result of the segment 6-8 cm coincides with the date of consumption (calculated with a hair growth rate at 1 cm/month) and the low concentration detected in the segment 4-6 cm probably corresponds to the contribution of dormant hair. The toxicological significance of the measured concentrations is difficult to determine because this is the first case dealing with hair analysis for 25I-NBOMe.

Conclusion: The use of hair analysis for NPS is still at the initial stages. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between dosage frequency of use, and hair concentrations. Under these circumstances, NPS hair analysis should be cautiously interpreted by experienced forensic toxicologist.

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1. INTRODUCTION

The abuse of new psychoactive substances (NPS) has been dramatically increasing all around the world since the last half of the year 2000 and has become a serious public health problem [1].

Among NPS, a group named phenethylamine NBOMe has gained importance in the recent years. The NBOMEs were originally synthesized for research purposes as potent agonists of the 5-hydroxy-tryptamine (HT)-2_A receptor and are derivatives of the 2C series of psychedelic phenethylamine drugs [2-4]. The name comes from an added N-benzylmethoxy substituent. Thus, added NBOMe group has been shown to significantly increase the compound's affinity for the 5-$HT2_A$ receptor, mediating the hallucinogenic effects that are similar to those of LSD. However, the NBOMe have

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greater stimulatory properties due to an α-adrenergic receptor interaction [5]. Discovered by Ralf Heim and described in this PhD thesis in 2003 [6], 25I-NBOMe [2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine] (Fig. **1**) is generally sold on the internet and consumed most often in the form of drops or blotters, and can be consumed by the transmucosal, sublingual or intranasal route [7, 8]. The active dosage is very low, supposed to be less than 100µg. This compound has numerous psychedelic effects: visual hallucinations, altered time and space, change of consciousness. It also has stimulating effects like euphoria or empathy. It produces many side effects, including convulsions, paranoia, psychosis and even coma up to death [9]. These effects can last from 5 to 10 hours [10-12].

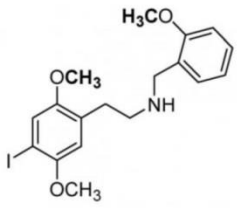

Fig. (1). Chemical structure of 25I-NBOMe.

Blood and urine remain the matrices of choice for toxicology analysis in case of a recent exposure to a drug, whereas hair analysis can enable to show repetitive and past consumption. Hair sample, an additional matrix, offers several advantages highlighting its large window of detection, non-invasive collection and storage at room temperature. It is also possible to perform segmental analysis, which allows the determination of the historic pattern of drug use. In such situations, the sample has to be cut as close as possible to scalp on vertex posterior region. This should allow to differentiate a single exposure from repetitive exposures, particularly when the response after a single administration is known [13].

Analytically, the characterization of a consumption of 25I-NBOMe is not obvious. The literature is very poor in reporting cases where 25I-NBOMe was identified. Only very few clinical cases of overdosages were published, suggesting a low prevalence of this compound [14]. The objective of this publication is to present a retrospective demonstration of acute poisoning with 25I-NBOMe using hair specimens collected 6.5 months after drug consumption, during a forensic clinical evaluation of brain dysfunctions. This case appears to be the first demonstration of NBOMe detection in human hair.

2. CLINICAL CASE

A 17-year-old man had probably consumed some LSD during a party with friends. After some hallucinations, he was found inanimate a few hours later. Upon the arrival of the medical emergency, the patient suffered from a cardiorespiratory arrest, with resumption of cardiac activity after 3 minutes of massage. He presented a reactive bilateral mydriasis, a tachycardia at 126 bpm and some convulsions. He was intubated at the scene and transported to an intensive care unit. The initial investigation at the emergency department revealed the following symptoms: hyperthermia with a core temperature of 40,6°C, pulse of 134 bpm with repolarization troubles, metabolic acidosis (pH 7.29, bicarbonates 20.5 mmol/L, lactate 2.7 mmol/L), rhabdomyolysis (CPK 12 765 Ul/L), acute renal failure (creatine 144µmol/L) and hepatocellular insufficiency (TP 13%, factor V 10%, TGO/TGP 3425/1943 UI/L). The patient was rehydrated and alkalinized. After 8 days, he was extubated. He was restless, aggressive, aphasic, apraxic and agnosic. Magnetic resonance imaging scan described some brain lesions [15].

Blood, urine and a yellow jelly, found near the young man by the medical emergency, were sent to the toxicology laboratory for drug screening, including ethanol. Testing for pharmaceuticals, LSD and ethanol was negative in both blood and urine samples. Testing for illegal drugs revealed 25I-NBOMe in the blood sample at 6,1 ng/mL. The yellow jelly was identified to be omeprazole, used in the treatment of gastroesophageal reflux. This compound was not detected in blood and urine.

Six and half months after the acute event, the patient was still hospitalized in a function re-education and rehabilitation service. He suffered from cognitive disorders with an infantile behavior, disorientation in time and space, attention deficits, memory problems and numerous difficulties to associate thoughts. Two hair strands, measuring 9.5 cm, were collected during a forensic clinical evaluation of brain dysfunctions to establish the pattern of poisoning by 25I-NBOMe.

3. MATERIALS AND METHODS

The hair strands were received in an envelope at the laboratory. The request was 25I-NBOMe analysis. Specimens are presented Fig. (**2**). A new specific method to 25I-NBOMe in hair was developed on an Acquity class I ultra-high performance liquid chromatography coupled to a Xevo TQD tandem mass spectrometer (UPLC-MS/MS) from Waters (Milford, MA, USA).

4. CHEMICALS AND REAGENTS

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99-100%, AnalaR NORMAPUR and 36% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Ethyl acetate for liquid chromatography grade and acetonitrile for LC-MS grade were purchased from Merck (Molsheim, France). 25I-NBOMe and MDMA-d5 were purchased from LipoMed (Arlersheim, Switzerland). Drugs were diluted to appropriate concentrations using acetonitrile.

5. 25I-NBOME ANALYSIS

The analysis of 25I-NBOMe was conducted on a hair strand of 9.5 cm, which was segmented into 5 segments (0-2 cm; 2-4 cm; 4-6cm; 6-8 cm; 8-9.5 cm). Based on a standard growth rate of 1 cm/month, it is anticipated that the segment 6-8 cm represents the period of acute poisoning. 25I-NBOMe was extracted from 50 mg decontaminated cut hair in the presence of 2 ng of MDMA-d5 used as internal standard after overnight incubation in 1mL borate buffer pH 9.5. Drugs were extracted by 5mL of ethyl acetate. After 15 min-



Fig. (2). Picture of the 2 hair specimens collected.

utes of agitation at room temperature, centrifugation and evaporation, to dryness, the residue was reconstituted in 250 µL of ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters Acquity HSS C18 column (150 x 2.1 mm x 1.8µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 87% phase A and the final gradient, at 15min, was 5% phase A. An injection volume of 10 µL was used in all cases. A Xevo TQD triple quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of 25I-NBOMe and the internal standard: capillary voltage at 1.5kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000L/h. In order to establish appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociated of both species was performed. Collision energy was adjusted to optimize the signal for the 2 most abundant product ions of 25I-NBOMe: *m/z* 428.1 > 121.2 (22 eV) and 428.1 > 90.6 (36 eV), and the most abundant product ion of internal standard *m/z* 199.1 > 165.1 (12 eV). Transition *m/z* 428.1 to 121.2 was used for quantification of 25I-NBOMe. These transitions have already been described in the literature [3, 16]. MassLynx 4.1 software was used for quantification.

6. RESULT AND DISCUSSION

6.1. Validation

Linearity was observed for 25I-NBOMe concentrations ranging from 1 to 100 pg/mg, with a correlation coefficient of 0.996. The calibration curve is presented on Fig. (**3**). QC samples (10 pg/mg and 50 pg/mg), analyzed in duplicate in six independent experimental assay, were used for determination a coefficient of variation for precision and accuracy. These CVs were lower than 20 %. The limit of detection and the lower limit of quantification are 0.5 pg/mg and 1 pg/mg respectively. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in hair. The matrix effect (< 20%) was investigated with spiked 25I-NBOMe at 10 pg/mg in twenty blank hair samples.

6.2. Toxicological Findings

By providing information on exposure to drugs over time, hair analysis may be useful in verifying self-reported histories of drug use in any situation in which a history of past rather than recent drug use is desired. During controlled studies, a drug user is not able to hide drug abuse since the window of detection is often long, i.e. months. Urine and blood tests are sometimes not useful to detect drug use when drugs are consumed every few days even when the tests are repeated. Hair analysis can also provide a retrospective calendar of an individual's drug use. For this, multi sectional analysis is required and involves taking a length of hair and cutting it into sections to measure drug use during shorter periods of time. The hair must be cut as close as possible to the scalp and particular care is also required to ensure that the individual hairs in the cut-off tuft retain their originally orientation. The most extensive study on sectional analysis for drugs of abuse has involved patients in rehabilitation centers. Segmental hair analysis is used to verify both their previous drug history and their recent enforced abstinence [17]. Hair analysis is suitable for accurately monitoring relative changes in drug intake in the same individual. In several studies, it has been shown that doubling of drug dose in the same individual results in an approximate doubling of the drug content of the hair, which is not always the case for blood and urine drug level due to transport saturation or extensive metabolism [13].

Currently, the young man is still hospitalized in a re-education and rehabilitation service. Temporal and spatial disorientation disorders persist. Reading is not possible and his graphologies are those of a child of 5 years old. The consumption of 25I-NBOMe seems to be at the origin of the cardiorespiratory arrest in view of molecules effects and knowing he had no cardiac history. The neurological sequelae may be consecutive to cerebral hypoxia, caused by the arrest.

The hair test result for 25I-NBOMe was the following: not detected (0-2 cm), not detected (2-4 cm), 1.0 pg/mg (4-6 cm), 4.9 pg/mg (6-8 cm) and not detected (8-9.5 cm). The results are presented on Fig. (**4**). Chromatogram obtained after extraction of hair is presented on Fig. (**5**). The result of the segment 6-8 cm coincides with the date of consumption (calculated with a hair growth rate at 1 cm/month) and the low concentration detected in the segment 4-6 cm probably corresponds to the contribution of dormant hair. The detection of a single drug exposure in hair, as described by Kintz [18], is based on the presence of the compound on interest in the segment corresponding to the period of the alleged event.

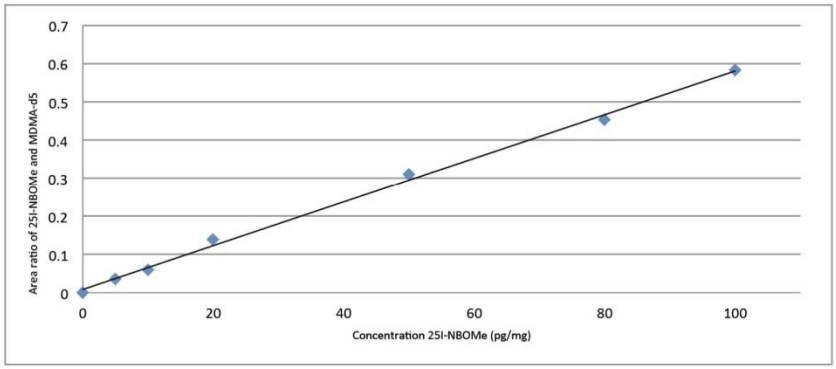

Fig. (3). Calibration curve of 25I-NBOMe between 1 and 100 pg/mg.

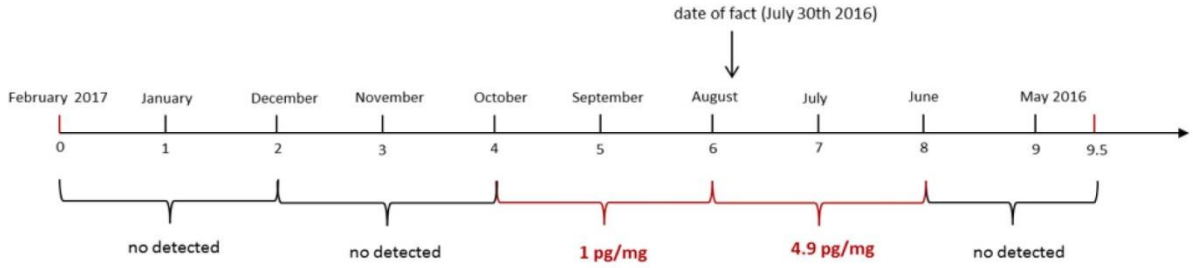

Fig. (4). Results of segmental hair analysis, demonstrating the positive response at the time of the acute exposure.

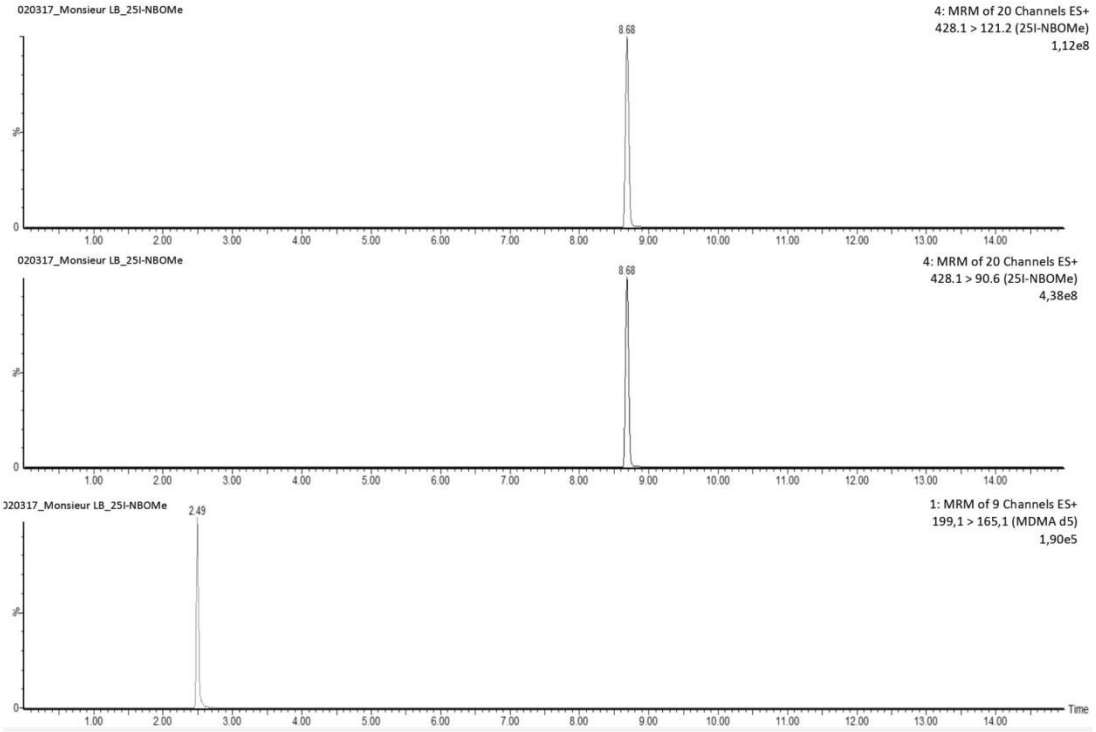

Fig. (5). Chromatogram obtained after extraction of the segment 6-8 cm. 25I-NBOMe concentration was 4,9 pg/mg. From top to bottom: 2 transitions for 25I-NBOMe (*m/z*: 428.1 > 121.2 and 428.1 > 90.6) and 1 transition for MDMA-d5 (*m/z*: 199.1 > 165.1).

However, in some cases, the drug can be detected in consecutive segments. To qualify for a single exposure in hair, Kintz [18] proposes to consider that the highest drug concentrations must be detected in the segment corresponding to the period of the alleged event and that the measured concentration be at least 3 times higher than those measured in the other segment(s).

The toxicological significance of the measured concentrations is difficult to determine because this is the first case describing human hair analysis for 25I-NBOMe and there is no hair data for other NBOMe analogues.

Furthermore, in a recent article, Salomone and al [19] describe difficulties to interpret results of hair analysis. The use of hair analysis for forensic issues concerning drugs collectively known as NPS is still controversial. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between dosage frequency of use and hair concentrations. The authors also considered different scenarios such as passive exposure vs. active consumption, mindful vs. unaware intake, and sporadic vs. chronic use. Under these circumstances, Salomone and al [19] conclude that NPS hair analysis should be cautiously interpreted by experienced forensic toxicologist.

CONCLUSION

We have presented a case of retrospective demonstration of acute poisoning with 25I-NBOMe, using hair analysis, collected 6.5 months after drug consumption. Hair analysis is essential to document a retrospective calendar of an individual's drug use and to discriminate a single exposure from repetitive exposures. The very low concentrations measured require the use of a tandem mass spectrometry to increase the specificity and sensibility of the test.

Several recent studies have presented hundreds of hair test results [20, 21] but 25I-NBOMe was never detected in hair, allowing the authors to present the first human case.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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

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Declared none.

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Article 3: Testing for SGT-151 (CUMYL-PEGACLONE) and its metabolites in blood and urine after surreptitious administration

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Special Issue

Testing for SGT-151 (CUMYL-PEGACLONE) and its Metabolites in Blood and Urine after Surreptitious Administration

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Abstract

The authors aim to report a case of surreptitious administration of a synthetic cannabinoid (SC) and the subsequent toxicological investigations to be able to document accurately the case for submission at a hearing. A dealer gave surreptitiously a substance to two juvenile migrants who experienced shakings and faintness. The laboratory received a blood sample from each of the two victims, who, according to the investigators, were probably exposed to SGT-151, a SC, also known as CUMYL-PEGACLONE. Blood and urine specimens from the dealer, who claimed being a user of SGT-151 were received at the same time. To characterize the metabolites of SGT-151, the drug was incubated with a pool of human hepatic microsomes and the cofactors required to ensure the functioning of the main Phase I and Phase II enzymes. The incubation media were analyzed by liquid chromatography coupled to high-resolution mass spectrometry. The metabolites identified following transformation by hepatic microsomes were mostly *N*-dealkylated SGT-151, mono-hydroxylated SGT-151 and di-hydroxylated SGT-151. The presence of SGT-151 (5.4 ng/mL) and its metabolite, *N*-dealkyl SGT-151, was confirmed in the dealer's blood. Two metabolites of SGT-151 (OH-SGT-151, diOH-SGT-151) were detected in the dealer's urine. SGT-151 (~1 ng/mL) and its metabolite *N*-dealkyl SGT-151 were detected in the blood samples of the two victims.

Introduction

In the last few years, considerable information about drug-facilitated crimes (rape, sexual assault, robbery, sedation of young or elderly persons, etc.) has accumulated. In these situations, the victims are subjected to non-consensual acts while they are incapacitated through the effects of a drug. Manipulation of tablets or cigarette spiked with an active drug has been observed in such situations. Victims usually report loss of memory during and after the event. For the perpetrator, the ideal substance is one that is readily available, is easy to administer, rapidly impairs the victim and causes anterograde amnesia (1, 2).

Cannabis is by far the most widely used drug of abuse in the world. Thus, it is not surprising to find THC or its metabolites in biological samples taken from victims of drug-facilitated crimes. Some of the psychoactive effects of THC such as euphoria, drowsiness, visual disorders and short memory troubles are expected by the perpetrator to facilitate the crime (3). Cannabis can be administered surreptitiously in pastries but those cases are rare. Like alcohol, most of the time, cannabis is used by the offender and shared

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with a more or less agreement of the victim, at least at the initial stage of the relationship. Cannabis can be shared by the victim and the offender, and later, the offender can take illegitimate advantage of the impaired victim. This can be considered more as an opportunistic crime rather than a proactive drug-facilitated crime (4).

Given the increasing prevalence of use and/or abuse of new psychoactive substances, it is not surprising to observe the deviation of synthetic cannabinoids (SC) in all fields of forensic toxicology, and therefore, it can be anticipated to detect them in situations where the drug is used as a chemical weapon to impair a victim. However, a search in databases (MedLine, Web of Science) was unable to produce a citation with the key words "synthetic cannabinoids" and "drug-facilitated crime" as consulted on 5 September 2018.

Therefore, we report here, what is assumed to be the first case in the literature, with the surreptitious administration by a drug dealer of SGT-151 to two victims.

Case history

In October 2017, the police was called at 10 pm by the supervisor of a youth hostel hosting minor children. Two 16-year old migrants presented seizures, followed by a collapse after having smoked a herbal mixture received some minutes before from an unknown man. They were rapidly rescued by a mobile emergency unit and a blood specimen (Vacutainer tube with a green top, using Li heparin as preservative) was collected. No urine samples of the victims were collected. Based on the description obtained from an employee of the hotel, the drug provider was arrested ~30 min after by the police. He confessed having given to the victims a cigarette laced with a white powder and indicated that it was SGT-151, a SC. Blood (Vacutainer tube with a green top, using Li heparin as preservative) and urine (dry tube with no preservative) were immediately collected. The powder of SGT-151 that was given to the migrants was not available for analysis.

A general screening for unknowns, including ethanol, medicinals, particularly psychoactive substances and standard drugs of abuse was achieved. All results returned negative.

Material and methods

Chemical

SGT-151 was obtained from GR8 Research Chemicals (the Netherlands). Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99–100%, AnalaR NORMAPUR and 36% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). β-glucuronidase (Helix pomatia), alamethicin (Trichoderma viride), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P) were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at −80°C until use. HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany). THC-d_3 was purchased from Lipomed (Arlersheim, Switzerland) and was diluted to appropriate concentrations using MeOH, according to the manufacturer's recommendations.

SGT-151 and metabolites analysis by UPLC–MS-MS®

Biological specimens were submitted to UPLC–MS-MS® analysis using a specific method. The drug was extracted from 1 mL of fluid (blood, hydrolyzed urine using 20000 UI of ß-glucuronidase) in presence of THC-d_3 used as internal standard, with borate buffer pH 9.5 and a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5, v/v). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters HSS C18 column maintained at 50°C. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in ACN LC–MS (mobile phase B) as mobile phases at flow rate of 0.4 mL/min. Elution profile: initial concentration of mobile phase B (13%) held until 0.5 min, increased to 50% at 10 min, then to 95% at 13.25 min, and finally held until 15 min. A Xevo triple quadrupole mass spectrometer was used. Ionization was achieved using electrospray in the positive ionization mode (ESI+). Collision energy was adjusted to optimize the signal for the two most abundant product ions of SGT-151 and its metabolites (Table I).

Linearity was verified from 1 to 20 ng/mL and in each batch, two controls of SGT-151 at 2 and 10 ng/mL were added to check accuracy and precision. Signal to noise criteria of ≥3 for LOD was used. The LOQ was considered as the first point of the linearity. Recovery was tested at 2 ng/mL and matrix effect was verified at 10 ng/mL ($n = 10$).

In vitro metabolism

The formation of SGT-151 metabolites was investigated by *in vitro* incubation with HLMs. Once thawed, HLMs (2 mg protein/mL for a final volume of 100 µL) were pre-activated by alamethicin (at 0.1-mmol/L) on ice in an intermediate volume of 50 µL in 0.1 M Tris-HCl–$MgCl_2$ (10 mM $MgCl_2$ and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50-µL dried residue of 100 mg/L of a SGT-151 methanolic solution. About 50 µL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37°C for 120 min and stopped by the addition of 200 µL of MeOH, subsequently centrifuged at 4°C for 10 min at 14000 rpm. About 10 µL of supernatant was immediately injected and analyzed by LC–HRMS.

LC–HRMS analysis

The chromatographic separation of samples obtained from incubations with HLMs or urine extracts (10 µL injected) was performed using the same ACQUITY HSS C18 column (150 × 2 mm, 1.8 µm, Waters) placed in a column oven at 50°C. The mobile phases consisted of ammonium formate buffer 5 mM, pH 3 (A) and ACN in 0.1% (v/v) formic acid (B); the flow rate was 0.4 mL/min. Elution

Table I. Retention time and MRM transitions for SGT-151 and its metabolites

Compound	Retention time	Transition 1 (m/z)	Transition 2 (m/z)
SGT-151	12.02 min	373.2 > 255.1	373.2 > 167.1
OH-SGT-151	11.70 min	389.2 > 271.1	389.2 > 201.1
diOH-SGT-151	9.19 min	405.2 > 287.1	405.2 > 213.1
N-dealkyl SGT-151	10.02 min	303.1 > 185.1	303.1 > 167.1

profile: initial concentration of mobile phase B (13%) held until 0.5 min, increased to 50% at 10 min, then to 95% at 13.25 min, and finally held until 15 min. Mass spectrometry data were acquired using a XEVO G2-XS QTOF (Waters, Manchester, UK) instrument controlled with MassLynx 4.1 software. Mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), ion spray voltage of 20 V, nitrogen as desolvation gas and argon as collision gas, source temperature at 140°C and desolvation temperature at 500°C with a desolvation gas (nitrogen) flow rate of 900 L/h. Conditions for the time of flight mass spectrometer scan mode were as follows: scan range 100–1000 m/z at 6 eV (low energy) and 50–1000 m/z with a collision energy ramp from 10 to 40 eV (high energy). The low energy scan mode is used to generate data similar to the full scan mode, while the high energy mode is closer to collision induced fragmentation.

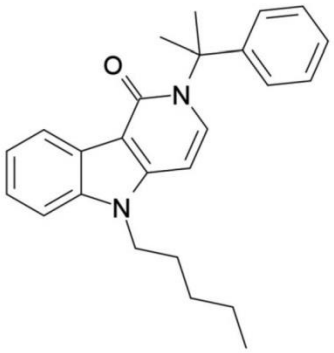

Figure 1. Chemical structure of SGT-151.

Metabolite identification

Data processing was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated software packages (Waters, Manchester, UK). A software algorithm (MetaboLynx™) was used for *in silico* prediction of SGT-151 bio-transformations. Taking into account available data on the metabolism of related substances, sets of theoretically possible bio-transformations of SGT-151 were created based on the structure of the drug. The automated metabolite profiling process was completed through the use of the fragmentation interpretation software tool MassFragment™ 4.1 (Waters), to enable software driven assignment of metabolite structures from fragmentation patterns. The LC–HRMS acquired data of HLM incubates are subsequently cross checked to *in silico* predicted bio-transformations: criteria used for identification of the targeted compounds included 0.003 ppm as exact mass error of the precursor ion as well as product ions.

Nuclear magnetic resonance analysis

To confirm the purity and the absence of any other organic substance, the SGT-151 powder was submitted to nuclear magnetic resonance (NMR) analysis. The NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 MHz equipped with a 5-mm quadruple nucleus probe at 295 K. The 1 H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 s, a relaxation delay of 2 s and a flip angle of 30°. The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. After manual grinding of the powder using a mortar for 5 min to obtain a homogenous sample, 7.5 mg was dissolved in 600 µL of methanol-D_4 and the ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations of SGT-151. ERETIC consisted of a digitally generated Gaussian peak (on single point

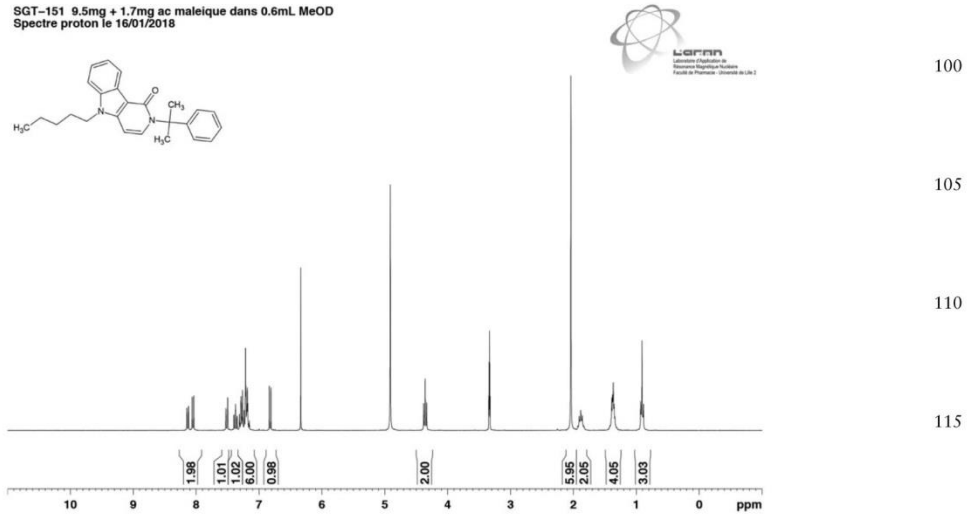

Figure 2. 1H-NMR (MeOD) analysis of the SGT-151 powder including signals related to SGT-151 [8.13 (1 H, ddd, ArH, J 7.8, 1.2, 0.7 Hz), 8.05 (1 H, d, ArH, J 7.8 Hz), 7.51 (1 H, dt, ArH, J 8.2, 0.8 Hz), 7.38 (1 H, ddd, ArH, J 8.3, 7.1, 1.2 Hz), 7.23 (6 H, m, ArH), 6.82 (1 H, d, ArH, J 7.8 Hz), 4.36 (2 H, t, N-CH2, J 7.2 Hz), 2.04 (6 H, s, CH3), 1.88 (2 H, q, CH2, J 7.2 Hz), 1.37 (4 H, m, CH2) and 0.9 (3 H, t, CH3, J 7.1 Hz)].

calibration calibrated using 15 mg maleic acid as a reference sample in 600 µL $CDCl_3$ and a control point using 10 mg of the same substance in 600 µL $CDCl_3$) inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the value peak area integrals in the spectrum give directly information about the concentration of protons sample purity (5).

Results and discussion

SGT-151 or 5-pentyl-2-(2-phenylpropan-2-yl)-2,5-dihydro-1*H*-pyrido[4,3-*b*]indol-1-one, also identified as CUMYL-PEGACLONE (Figure 1) has been described as a strongly binding cannabinoid receptor ligand with a K_i value in the low nanomolar range (6). The substance features a novel γ-carbolinone core structure, which is most likely synthesized to bypass generic legislative approaches to control SCs by prohibiting distinct core structures.

For identification and characterization of the purchased SGT-151 powder, NMR spectroscopy was applied (Figure 2). No organic impurity was detected in the powder using NMR. In addition, using the ERETIC method, NMR analysis revealed a purity index of 86%. The NMR profile matches with the data provided by Angerer *et al.* (6).

Based on pooled liver microsome assays and authentic human urine specimens, Mogler *et al.* (7) identified more than 20 metabolites, and proposed that a metabolite mono-hydroxylated at the γ-carbolinone core system and a metabolite with an additional carbonyl group at the pentyl side chain would be highly specific and sensitive markers to demonstrate SGT-151 exposure. Because these metabolites are not available from reference standard providers, it was necessary to perform the same study using HLMs and liquid chromatography coupled to high-resolution mass spectrometry to gain the retention times and spectral behaviors of the metabolites. This allowed transfer of the method to UPLC–MS-MS®.

Although not all metabolites that were identified in the study of Mogler *et al.* (7) were detected, it was decided to focus the attention on the most abundant responses (based on peak areas), i.e., the hydroxylated metabolite (most probably hydroxylated at the γ-carbolinone core), the di-hydroxylated metabolite and the *N*-dealkyl metabolite. Retention time and two MRM transitions are presented for each target compound within the UPLC–MS-MS® analysis in Table I. No Phase II metabolism study was achieved and the urine specimen was tested after enzymatic conjugate cleavage.

A mini-validation was achieved for SGT-151 in blood. The method was linear from 1 to 20 ng/mL, with a limit of quantification at 1 ng/mL, which is the first point of the linearity. The limit of detection was 0.4 ng/mL. Deviation of precision was <20%, which was considered acceptable, given the low concentrations to be measured. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood and the matrix effect was <15%. Recovery, tested at 2 ng/mL, was about 82%. Although SGT-151 and THC-d_3 are chemically different, the rationale for choosing THC-d_3 is its use during the screening procedure of cannabis and synthetic cannabinoids. In addition, deuterated SGT-151 seems not to be available from the reference standard suppliers.

SGT-151 was identified in the blood collected from the two victims and the dealer. Concentrations ranged from <1 ng/mL to 5.4 ng/mL (Table II). Due to a low amount of the sample collected

Table II. Concentrations of SGT-151 and presence/absence of the metabolites

Compound	Blood Vict 1	Blood Vict 2	Blood dealer	Urine dealer
SGT-151	<1 ng/mL	1 ng/mL	5.4 ng/mL	Not detected
OH-SGT-151	Not detected	Not detected	Not detected	Presence
diOH-SGT-151	Not detected	Not detected	Not detected	Presence
N-dealkyl SGT-151	Presence	Presence	Presence	Not detected

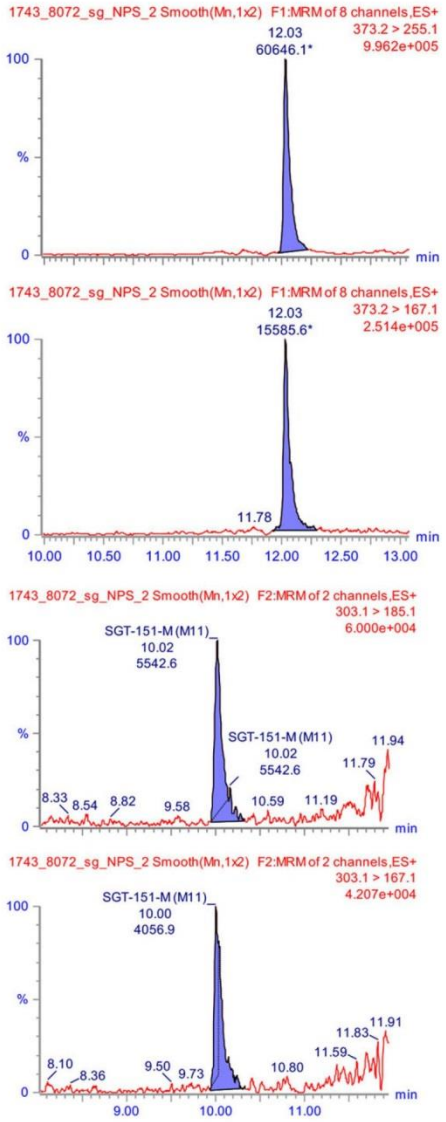

Figure 3. Chromatogram obtained after the extraction of the blood of victim 1 (top, two transitions for SGT-151, bottom, two transitions for *N*-dealkyl-SGT-151).

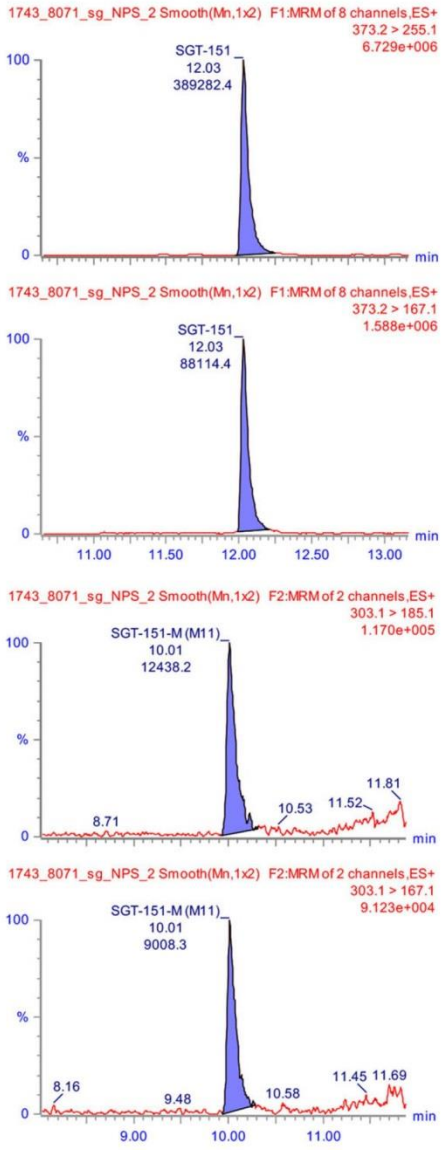

Figure 4. Chromatogram obtained after the extraction of the blood of victim 2 (top, two transitions for SGT-151, bottom, two transitions for *N*-dealkyl-SGT-151).

from the drug dealer, only 0.1 mL of blood was available for analysis, adding a level of uncertainty in the test as the raw measure was only slightly above the LOD. It is remarkable that the dealer had five times blood concentration compared with the victim. This can be due to the use of a higher dose or deeper smoking of the cigarette. In blood, it was consistently possible to identify the *N*-dealkyl metabolite. In the urine collected from the dealer, the OH-SGT-151

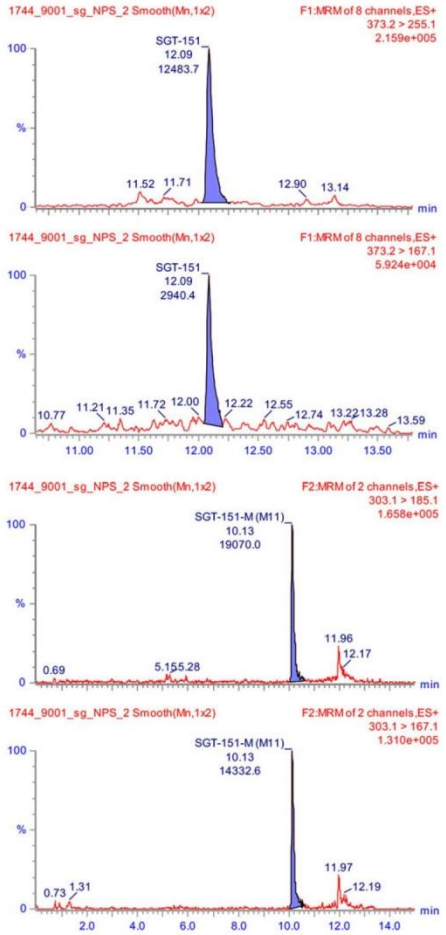

Figure 5. Chromatogram obtained after the extraction of the blood (only 0.1 mL was available) of the dealer (top, two transitions for SGT-151, bottom, two transitions for *N*-dealkyl-SGT-151).

and diOH-SGT-151 metabolites were identified, confirming the exposure to the drug. Typical chromatograms after blood and urine extraction are presented in Figures 3–6. No parent SGT-151 was detected in the urine specimen, indicating extensive metabolism. Due to the lack of reference material, there was no possibility to measure the concentrations of the detected metabolites. Possible pharmacological activity of the metabolite(s) is also unknown.

Although the pharmacodynamic data of SGT-151 have not yet been established, one can anticipate that all the three subjects were under the influence of the drug at the time of blood collection, as blood tested positive for the parent compound.

At this time, this paper represents the first report identifying the drug in France. It has been published that SGT-151 was first identified in Germany, in late 2016 (8) and since that date numerous specimens tested positive in that country.

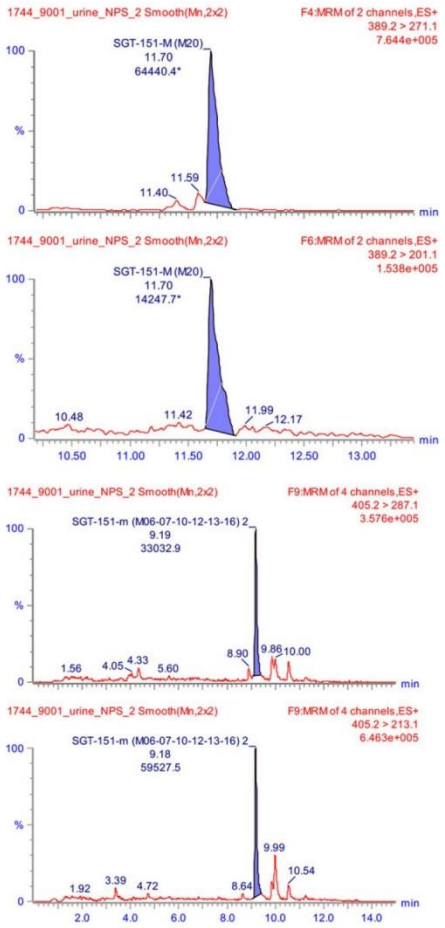

Figure 6. Chromatogram obtained after the extraction of the urine of the dealer (top, two transitions for OH-SGT-151, bottom, two transitions for diOH-SGT-151).

Conclusion

The presence of cannabis ingredients or Δ9-THC in biological samples of victims of DFC is a common finding. At this time, it seems that synthetic cannabinoids use has not yet been reported. The difficulty lies in the fact that this positive finding might or might not be related to the offense. In this case, based on the limited data available for SGT-151, the pathologist attributed to the drug the clinical signs. Police listed the case as a drug-facilitated crime (it was anticipated that the dealer wanted to impair the victims to prostitute them), but one can consider it more as an opportunistic crime rather than a proactive drug-facilitated crime. Knowledge of the victim's history of drug use is crucial to interpret the results. Due to a lack of reference material, particularly for synthetic cannabinoid metabolites, the biological proof can be difficult to establish. It can require tedious investigations, involving the most sophisticated tools (NMR, LC–HRMS and HLM preparation) that are now available to the forensic toxicologist.

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Article 4: Les “designer benzodiazepines”: qu’en sait-on aujourd’hui ?

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

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REVUE GÉNÉRALE

Les « designer benzodiazepines » : qu'en sait-on aujourd'hui ?

Designer benzodiazepines: What do we know about them?

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MOTS CLÉS
Benzodiazépine ;
Détection ;
Quantification ;
Effets ;
Analyse

Résumé Les benzodiazépines sont une classe de médicaments largement utilisées et faisant l'objet d'abus et de mésusage dans le monde entier. La recherche étendue autour de leur structure chimique a apporté une grande variété des molécules actives qui n'ont pas d'autorisation de mise sur le marché et qui sont reprises par les e-boutiques vendant les nouveaux produits de synthèse, sous le nom générique de « designer benzodiazepines ». Dans cette revue de la littérature, établie en septembre 2017, les auteurs se sont intéressés aux effets des « designer benzodiazepines », à leur métabolisme chez l'homme et aux méthodes analytiques applicables dans la détection et la quantification dans les milieux biologiques. Il en ressort que nos connaissances en ce qui concerne les « designer benzodiazepines » sont très variables selon la molécule d'intérêt. L'offre des « designer benzodiazepines » est toujours en évolution, et actuellement on observe l'émergence de molécules de plus en plus actives et de moins en moins étudiées par les scientifiques. Cela fait des « designer benzodiazepines » un défi pour les laboratoires d'analyse et pour la santé publique en général.
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KEYWORDS
Benzodiazepine;
Detection;
Quantification;
Effects;
Analysis

Summary Benzodiazepines are a class of drugs largely used and abused all over the world. The extensive research around this chemical structure resulted in a great variety of active compounds that never got a marketing authorization and which are now picked up by online e-merchants of new psychoactive substances. They are sold under the name ''designer benzodiazepines''. In this review of the literature available on September 2017, the authors will take interest in the effects and toxicity, in the human metabolism and in the analytical methods applicable to the detection and quantification in biological specimens of these drugs. It appears that our knowledge regarding the designer benzodiazepines varies, depending on particular molecule of interest. The supply of designer benzodiazepines is constantly changing, and recently the emergence of very active compounds with little scientific data has been observed. All this makes the designer benzodiazepines a challenge for testing laboratories and for public health in general.
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Introduction

Les benzodiazépines sont une classe de composés psycholeptiques avec des propriétés hypnotiques, anxiolytiques, myorelaxantes et anticonvulsivantes. Les premières molécules de cette classe, le chlordiazépoxide et le diazépam ont connu un grand succès, notamment en remplaçant les barbituriques comme une alternative plus sûre. Suite à cette percée sur le marché pharmaceutique, de nombreux laboratoires ont investi dans la recherche de nouvelles benzodiazépines, ce qui a conduit à la synthèse d'un très grand nombre de composés pharmacologiquement actifs mais qui se sont confrontés à un marché restreint, voire qui n'ont jamais été commercialisés en tant que médicament. Ces composés portent alors le nom de « designer benzodiazepines ». De nos jours, ces molécules échappent à la législation applicable aux médicaments et aux stupéfiants et ne sont plus protégées par leur brevet. Avec l'émergence des nouveaux produits de synthèse, ces molécules issues de la recherche pharmaceutique font progressivement leur apparition sur les sites Internet de vente sous l'appellation « composés chimiques de recherche » [1]. Une des stratégies de limitation de la diffusion de ces molécules par les autorités sanitaires et judiciaires consiste à inscrire ces produits sur la liste des stupéfiants. Certaines designer benzodiazepines sont utilisées comme médicaments dans un nombre restreint de pays comme le phénazépam en Russie. Contrairement aux autres classes de nouveaux produits de synthèse (NPS) les designer benzodiazepines sont utilisées à la fois dans le milieu festif mais aussi en « automédication » pour faire face au stress quotidien (insomnies, sevrage aux benzodiazépines de prescription, effets indésirables des drogues stimulantes) [1,2]. Elles sont donc considérées comme des molécules de substitution (« substance displacement ») [3].

Méthodes

Dans cette revue nous nous sommes intéressés aux nouveaux produits de synthèse de la classe des benzodiazépines et plus précisément à leurs effets et métabolisme chez l'homme ainsi qu'aux techniques analytiques décrites pour chaque molécule dans des milieux biologiques humains. Concernant le métabolisme des molécules, nous avons tenu compte des études in vitro réalisées sur des microsomes hépatiques humains et in vivo chez l'homme. Nous nous sommes servi des articles en libre accès trouvés sur PubMed, accessibles par la Bibliothèque nationale universitaire de Strasbourg ou exposés sur la plateforme ResearchGate, rédigés en anglais et en français. Nous avons commencé par les mots clés 'designer benzodiazepine' et 'NPS benzodiazepine' pour identifier les molécules sur lesquelles des publications ont été rédigées. Puis nous avons continué nos recherches pour chaque molécule en utilisant des mots clés tels que : 'metabolism', 'pharmacokinetics', 'effects', 'determination', 'detection' et 'characterisation'. Plusieurs documents spécifiques, comme des rapports d'OMS, ont été recherchés directement sur le moteur de recherche Google. Les molécules pour lesquelles nous avons trouvé assez d'information de sources réputées ont été retenues. Les structures moléculaires ont été tracées avec le logiciel PubChem Sketcher V2.4 disponible en ligne.

Pharmacologie

Les benzodiazépines agissent comme modulateurs allostériques des récepteurs de type A à l'acide gamma-aminobutirique (GABA-A). Ces récepteurs sont composés de cinq sous-unités qui forment deux sites de liaison pour le GABA et un site de liaison aux benzodiazépines. La fixation d'une benzodiazépine entraîne un changement de conformation du récepteur, ce qui augmente le flux de chlore lors de son activation par le GABA et induit une hyperpolarisation du neurone et une diminution des neurotransmissions. Cela mène à un effet inhibiteur dans tout le système nerveux central.

Les différents effets cliniques de chaque molécule sont dus à des différences de pharmacocinétique et d'affinité pour différents sous-types du récepteur GABA-A [4].

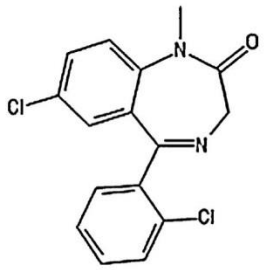

Figure 1. Structure chimique du diclazépam

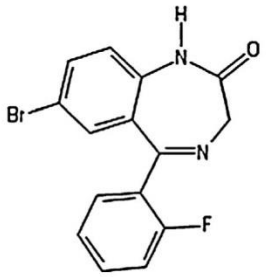

Figure 2. Structure chimique du flubromazépam

Toxicité

Les effets délétères des benzodiazépines dépendent de la dose du produit administrée. Les effets indésirables les plus fréquents à faible dose sont la somnolence, la fatigue et la léthargie. À plus fortes doses, des troubles de la coordination motrice, des vertiges, des changements abrupts de l'humeur et de l'euphorie peuvent se manifester. L'élimination lente de certaines benzodiazépines mène à leur accumulation dans les tissus riches en lipides et des signes de surdosage peuvent se manifester de façon retardée en cas de consommation répétée. Suite à une longue consommation, l'usager peut développer une tolérance et une dépendance aux benzodiazépines et l'arrêt brutal s'accompagne de symptômes de sevrage [4].

Des effets sévères s'observent quand les benzodiazépines sont utilisées en association avec les opioïdes. Les deux classes induisent une dépression respiratoire et leur effet est synergique [4]. Cette utilisation concomitante est répandue parmi les polyconsommateurs, car les benzodiazépines potentialisent l'euphorie due aux opioïdes [5]. On parle de ces effets comme des effets indésirables, mais ce sont parfois des sensations recherchées par les utilisateurs. Les benzodiazépines plus puissantes ont un fort effet amnésiant qui se manifeste par une amnésie antérograde. L'usager perd la mémoire pour quelques heures après l'administration [6]. C'est la mémoire épisodique, faisant partie de la mémoire à long terme, qui est affectée. Quant à la mémoire à court terme, elle ne semble pas être altérée [4].

Benzodiazépines

Diclazépam/chlorodiazépam

Le diclazépam (Fig. 1) ou chlorodiazépam est un analogue du diazépam, avec un atome de chlore en position 2′. Dans une étude d'auto-administration, le volontaire n'a pas déclaré d'effet perceptible dans les jours suivant l'administration d'un comprimé déclaré à 1 mg. Selon la même étude, le diclazépam est une benzodiazépine à longue durée d'action, avec un temps de demi-vie d'élimination terminale de 42 h [7].

Dans l'organisme, le diclazépam peut être N-desalkylé pour former le délorazépam, hydroxylé en position trois pour former du lormétazépam ou subir les deux biotransformations et donner du lorazépam. Ces trois métabolites subissent ultérieurement des réactions de phase II de glucuroconjugaison. Comme les métabolites du diclazépam sont des molécules actives mais également commercialisées par l'industrie pharmaceutique, cela peut être une source d'erreur dans l'interprétation des résultats des analyses toxicologiques [7].

Apres l'ingestion d'une dose déclarée à 1 mg de diclazépam, ce dernier peut être détecté jusqu'à 99 heures dans le sérum et son métabolite, le délorazépam, jusqu'à 10 jours. La concentration sérique maximale a été atteinte après 3 heures et correspondait à 3,4 ng/mL. Le volume de distribution est de 8 L/kg et la clairance de 165 mL/min. Pour les métabolites les taux sériques maximaux ont été atteints à 6 heures pour le lormétazépam (0,34 ng/mL), à 36 heures pour le délorazépam (2,0 ng/mL) et à 99 heures pour le lorazépam (0,4 ng/mL) [7].

Plusieurs cas légaux impliquant la consommation du diclazépam ont été rapportés dans une étude, avec des concentrations sanguines allant de 2,1 à 57 ng/mL et la médiane des concentrations de 13 ng/mL. Pour le cas à 57 ng/mL, le diclazépam a été détecté seul dans le sang et l'état du sujet de 18 ans a été décrit comme considérablement altéré [8].

Dans un cas de décès par mort toxique, imputé au déschloroétizolam (11 μg/L) l'analyse des urines a révélé la présence de lormétazépam et de lorazépam à 258 et 115 μg/mL, respectivement. La mise en évidence de ces deux molécules peut s'expliquer par la présence d'un sachet étiqueté « diclazépam » retrouvé sur le lieu de décès [9].

Au vu du faible dosage des comprimés commercialisés, la détectabilité du diclazépam par des méthodes immunochimiques est limitée [7]. Cependant, le kit Immunalysis® Benzodiazepine (Elisa) est capable de le détecter [10].

Plusieurs techniques de détection décrites pour le diclazépam sont présentées dans le Tableau 1.

Flubromazépam

Le flubromazépam (Fig. 2) est une benzodiazépine à longue durée d'action [2]. Dans une étude d'auto-administration, le volontaire a déclaré avoir eu envie de dormir pendant 3 jours après l'ingestion d'une capsule de 4 mg de flubromazépam [14]. Selon une analyse des forums d'usagers britanniques, les premiers effets apparaissent entre 30 minutes et 6 heures selon l'individu. Le flubromazépam a un profil semblable aux autres benzodiazépines avec des propriétés anxiolytique, myorelaxante et hypnotique à faibles doses et provoquant de la sédation et de l'amnésie à de plus fortes doses. En général, les effets sont décrits comme plaisantes et plus marqués qu'avec le diazépam [2].

Tableau 1 Méthodes de détection et de quantification du diclazépam.

Référence	Méthode	Milieu	Limite de détection (ng/mL)	Limite de quantification (ng/mL)	Applications
[11]	LC—MS/MS	Urine	2	2	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,15	0,50	Criblage des « designer benzodiazepines »
[13]	Microextraction, puis LC—MS/MS	Urine	1	3,5	Criblage des « designer benzodiazepines »

Dans l'étude d'auto-administration, après 4 mg per os, une concentration sérique maximale de 78 ng/mL a été observée 6 heures après l'ingestion. Le temps de demi-vie d'élimination est de 106 h, ce qui est en concordance avec la durée des effets ressentis par le sujet. Compte tenu d'un plateau de concentration stable entre 24 et 76 heures, le flubromazépam est susceptible de subir un cycle entérohépatique.

Dans l'organisme humain, le flubromazépam peut perdre l'atome de brome donnant le desbromo-flubromazépam. Ce métabolite débromé, ainsi que le flubromazépam peuvent être hydroxylés en position 3 (3-hydroxy-desbromo-flubromazépam et 3-hydroxy-flubromazépam), puis glucuronidés. Le flubromazépam peut aussi subir une hydroxylation sur une position aromatique non-déterminée. Le cycle diazépine peut subir l'hydrolyse chimique de la fonction imine. La détection du flubromazépam est possible jusqu'à 23 jours dans le sérum après administration et du 3-hydroxy-flubromazépam jusqu'à 19 jours. Le désbromo-flubromazépam peut prouver une administration plus récente car il peut être détecté seulement jusqu'à 7 jours après l'administration. Dans les urines, le flubromazépam a été dosé à des concentrations inférieures à 4 ng/mL. La cinétique des métabolites dans les urines est similaire à celle dans le sérum, avec la détection du OH-désbromo-flubromazépam jusqu'à 6 jours après l'ingestion et du OH-flubromazépam jusqu'à 28 jours [14].

En Norvège, 24 cas légaux impliquant le flubromazépam ont été étudiés. Les concentrations sanguines rapportées varient entre 4,7 et 1200 ng/mL avec une médiane de 55 ng/mL. Parmi ces cas, un sujet qui avait consommé du flubromazépam seul présentait un taux sanguin de 600 ng/mL et une altération légère de son état [8]. Dans une seconde étude portant sur la détection des designer benzodiazepines dans les urines, la gamme de concentration déterminée était de 2,7 à 30 ng/mL [11].

Dans certains cas, le long délai d'action du flubromazépam peut motiver les usagers à répéter leur consommation afin d'accélérer l'apparition des effets. Quant à la longue demi-vie plasmatique, elle peut mener à une accumulation lors de prises répétées et étalées dans le temps. Ce sont des circonstances potentielles de surdosage [2].

Le flubromazépam est détectable par le kit immunologiques Immunalysis® benzodiazepine (Elisa), dans du sang surchargé [10] et également par les tests immunologiques

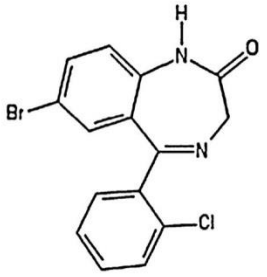

Figure 3. Structure chimique du phénazépam

CEDIA, HEIA, et KIMS II [15]. Plusieurs techniques de détection et de quantification ont été décrites pour le flubromazépam. Elles sont présentées dans le Tableau 2.

Phénazépam

Le phénazépam (Fig. 3) est une benzodiazépine 5 à 10 fois plus puissante que le diazépam. Elle a été développée en URSS où elle était largement prescrite pour le traitement des insomnies, de l'anxiété, de l'épilepsie et pour atténuer les symptômes de sevrage à l'alcool. En dehors de l'ex-URSS, le phénazépam n'est pas utilisé en médecine. Ses symptômes de toxicité peuvent persister plus de 5 jours (dépression du système nerveux central jusqu'à 3 semaines), ce qui rend le phénazépam particulier [17].

D'après un rapport de l'OMS, de nombreux cas de conduite sous l'influence de phénazépam ont été signalés aux États-Unis, en Angleterre et aux pays Scandinaves avec une très large variation des concentrations sanguines allant de 4 à 3600 ng/mL. Des cas de décès suite à des accidents de la route et des intoxications impliquant le phénazépam ont été décrits avec des concentrations allant de 5 à 1200 mg/L. La plupart de ces cas implique une polyconsommation, notamment en association avec des opioïdes [17]. Deux décès attribués au phénazépam ont été identifiés parmi 29 cas avec des concentrations sanguines de 970 et 1640 ng/mL et urinaires de 550 et 80 ng/mL. Pour les autres cas, les concentrations sanguines de 7 à 360 ng/mL et les concentrations urinaires allaient de 7 à 49 ng/mL [18]. Une intoxication se manifestant par de la confusion et désorientation suite à l'usage du phénazépam (taux de 490 ng/mL dans le sang) a été décrite en Écosse. Trois cas d'intoxication

Tableau 2 Méthodes de détection et de quantification du flubromazépam.

Référence	Méthode	Milieu	Limite de détection (ng/mL)	Limite de quantification	Applications
[16]	Électrophorèse capillaire—MS	Sérum	3,0	10,0 ng/mL	Criblage des « designer benzodiazepines »
[11]	LC—MS/MS	Urine	2,5	n/a	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,15	0,50 ng/mL	Criblage des « designer benzodiazepines »
[13]	Microextraction, puis LC—MS/MS	Urine	3	10 ng/mL	Criblage des « designer benzodiazepines »

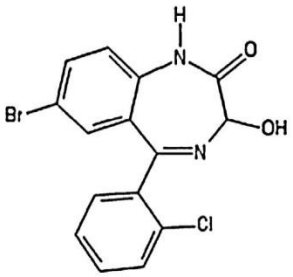

Figure 4. Structure chimique du 3-hydroxy-phénazépam

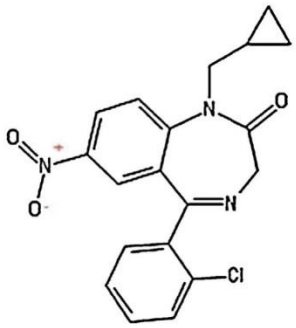

Figure 5. Structure chimique du cloniprazépam

au phénazépam par inhalation ont été rapportés en Suède. Les sujets présentaient de la somnolence, des troubles de l'élocution et de l'ataxie [17].

Après administration à des volontaires d'une dose de 5 mg de phénazépam per os, du 3-hydroxy-phénazépam a été détecté seulement dans les urines. Dans un décès impliquant le phénazépam, la 5-bromo-(2-chlorophenyl)-2-aminobenzophenone (ABPH), métabolite hydrolysé, a été détecté dans le foie. Un dernier métabolite, la 6-bromo-(2-chlorophenyl)-quinazoline-2-one (QNZ) est mentionné par plusieurs sources [17,19].

Le phénazépam peut être détecté par le kit Immunalysis® Benzodiazepine (Elisa), au moins dans du sang surchargé [10]. Plusieurs études se sont intéressées au dosage du phénazépam dans des milieux biologiques. Les méthodes de détection et de quantification du phénazépam sont présentées dans le Tableau 3.

3-hydroxy-phénazépam

Le 3-hydroxy-phénazépam (Fig. 4) est un métabolite actif du phénazépam [17] et du cinnazépam [27]. Dans l'organisme, il ne subit pas de biotransformation de phase I. C'est également le cas des autres 3-hydroxy-benzodiazépines comme le lorazépam et l'oxazépam [28].

Les taux sanguins fémoraux du 3-hydroxy-phénazépam, rapportés dans 27 décès impliquant l'administration du phénazépam varient entre 20 et 246 ng/mL et les taux urinaires — entre 17 et 274 ng/mL [18].

La détectabilité du 3-hydroxy-phénazépam par des techniques immunologiques est fortement variable. La réactivité croisée pour les tests CEDIA et HEIA est de 113 % (à 200 ng/mL) et de 464 % (à 100 ng/mL) respectivement mais de 48 % (à 500 ng/mL) et 65 % (à 200 ng/mL) pour les techniques EMIT II Plus et KIMS II [15].

Le dosage du 3-hydroxy-phénazépam est réalisable par LC—MS/MS après une extraction liquide—liquide. Les limites de détection et de quantification sont de 7 et de 16 ng/mL respectivement. Cette méthode a été appliquée dans plusieurs milieux biologiques et organes postmortem [18].

Nitrobenzodiazépines

Cloniprazépam

Chez l'homme, il n'existe aucune source fiable concernant les effets et la puissance du cloniprazépam (Fig. 5). À ce jour, les seules sources d'information sur ce sujet sont les forums des usagers.

Les connaissances sur le métabolisme du cloniprazépam sont limitées à des essais in vitro sur des microsomes hépatiques humains. Sept métabolites de phase I ont été détectés. Le cloniprazépam peut être d'abord réduit en 7-aminocloniprazépam, désalkylé en clonazépam ou hydroxylé en position 3 pour donner l'hydroxycloniprazépam. La réduction et la désalkylation

Tableau 3 Méthodes de détection et de quantification du phénazépam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification	Applications
[16]	Électrophorèse capillaire—MS	Sérum	1,5 ng/mL	5,0 ng/mL	Criblage des « designer benzodiazepines »
[11]	LC—MS/MS	Urine	5 ng/mL	5 ng/mL	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,15 ng/mL	0,50 ng/mL	Criblage des « designer benzodiazepines »
[13]	Microextraction, puis LC—MS/MS	Urine	3 ng/mL	10 ng/mL	Criblage des « designer benzodiazepines »
[20]	Dérivatisation avec TBDMS, puis GC-ECD	Sang	5 ng/mg	10 ng/mg	Criblage Benzodiazépines et leurs métabolites
[21]	Extraction SLE, puis GC	Sang, urine	5 ng/mg	20 ng/mg	Criblage des Benzodiazepines
[22]	Extraction en phase solide, puis LC—MS/MS	Sang	0,5 ng/mL	10 ng/mL	Méthode spécifique
[23]	Extraction liquide—liquide, puis LC—MS/MS	Salive	0,75 ng/mL	—	Criblage des médicaments psychotropes
[24]	Extraction par élution, puis UPLC—MS/MS	Sang	0,1 ng/mL	0,35 ng/mL	Criblage des médicaments psychotropes
[25]	Extraction SLE, puis UPLC—MS/MS	Sang	0,1 ng/mL	1,1 ng/mL	Criblage des benzodiazépines
[26]	Derivatisation avec TBDMS GC/MS	Sang	—	—	Criblage des benzodiazépines
[18]	LC—MS/MS	Fluides et tissus variées postmortem	0,3 ng/mL (7 pour oh)	0,7 ng/mL (16 pour oh)	Méthode spécifique

donnent du 7-aminoclonazépam. L'hydroxycloniprazépam peut être à son tour oxydé en cétocloniprazépam ou désalkylé en hydroxyclonazépam. Le clonazépam peut être hydroxylé en position 4′ pour donner l'hydroxyclonazépam. La formation du clonazépam et du 7-aminoclonazépam peut mener à une interprétation erronée des résultats d'analyse, en concluant une administration de clonazépam [28].

La détection du cloniprazépam et de ses métabolites est possible par LC—MS/MS [28], mais n'a pas encore été décrite dans les milieux biologiques.

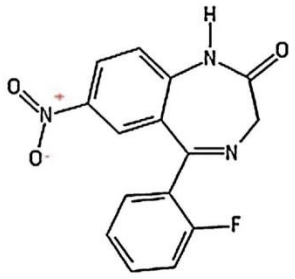

Figure 6. Structure chimique du fonazépam

Fonazépam

Le fonazépam (Fig. 6) est un métabolite actif du flunitrazépam, issu de la N-déméthylation de ce dernier. L'absence du groupement méthyle sur l'azote diminue son affinité pour le récepteur GABA-A et donc son action. Il est modérément sédatif et peut mener à un état léthargique. Il peut aussi causer un défaut de coordination, ce qui peut mener à des accidents. Lors d'un usage récréatif et à des doses élevées, des effets paradoxaux peuvent être attendus (agitation, convulsions) [29].

Des essais in vitro sur des microsomes de foie humain ont permis la détection des métabolites de phase I. Le 7-aminonorflunitrazépam est issu de la réduction du groupement nitro. Deux métabolites hydroxylés ont été détectés. L'hydroxylation peut se faire en position 3 donnant le nifoxipam et en position non identifiée sur le groupement fluorophényle [28].

Le fonazépam peut être détecté dans les urines par les tests immunologiques les plus courants. Etant un des

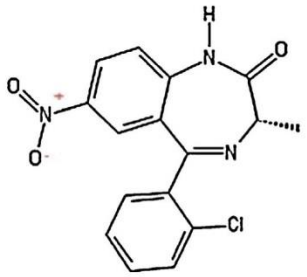

Figure 7. Structure chimique du méclonazépam

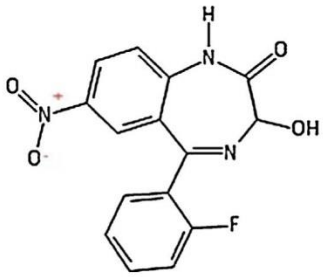

Figure 8. Structure chimique du nifoxipam

principaux métabolites du flunitrazépam, de nombreuses techniques de détection ont été rapportées dans des milieux biologiques variés [29].

Méclonazépam

Le méclonazépam (Fig. 7) est un analogue du clonazépam, méthylé en position 3, qui a des propriétés schistosomicides. Dans une étude, des volontaires sains se sont vus administrer des doses de 1, 2 et 4 mg de méclonazépam. Les sujets ont montré une première diminution des performances pour certains tests psychométriques à partir de 2 mg. Après l'administration de 4 mg de méclonazépam, les sujets ont montré une diminution des performances pour l'ensemble des tests psychométriques. Ils se sont aussi décrits comme somnolents, faibles, maladroits, mentalement lents, rêveurs et incompétents [30].

L'analyse des urines a mis en évidence deux métabolites, l'aminoméclonazépam et l'acétaminoméclonazépam. Ce dernier est considéré comme le métabolite le plus pertinent à rechercher dans les urines pour démontrer la prise du méclonazépam [31].

Une étude a rapporté une gamme de concentration urinaire allant de 1,6 à 190 ng/mL pour 45 échantillons analysés [11]. Une étude réalisée sur un volontaire sain a montré, après l'administration de trois doses (1 mg en intraveineuse, 1 mg per os et 4 mg per os) des pics plasmatiques entre 10 et 100 ng/mL [32].

Le méclonazépam et ses métabolites présentent une faible réactivité croisée pour les tests immunologiques CEDIA, EMIT II Plus, HEIA, et KIMS II [15]. Plusieurs techniques de détection du méclonazépam ont été décrites et sont présentées dans le Tableau 4.

Nifoxipam

Le nifoxipam (Fig. 8) est un métabolite actif du flunitrazépam, issu de sa 3-hydroxylation et déméthylation. Pour certains usagers, l'administration de 2 mg de ce composé provoque une faible sensation de relaxation et de sédation, alors qu'un dosage de 8 mg peut induire une sédation mais aussi un effet anxiolytique, une légère euphorie et des cauchemars. Pour d'autres usagers, l'administration de 1 mg accompagnée d'alcool entraîne un effet euphorique. Comme le flunitrazépam, le nifoxipam est extrêmement addictif à la fois physiquement et psychologiquement. Sa tolérance se développe en quelques jours d'utilisation continue et revient à l'état basal en 1 à 2 semaines après l'arrêt complet de la consommation [29]. Le délai d'action du nifoxipam varie entre 45 et 120 minutes avec des effets pouvant s'étaler sur 75 heures. D'autres sujets ont ressenti de l'euphorie après administration de 1 mg.

Dans l'organisme humain, le nifoxipam est réduit en amine (7-amino-nifoxipam), puis acétylé (7-acétamino-nifoxipam), ou directement glucuronidé sur l'hydroxyle en position 3 [31]. Dans les urines, le métabolite majoritaire, donc la meilleure cible pour les tests de dépistage, est le 7-acétamino-nifoxipam.

Le nifoxipam est détecté par les tests immunologiques les plus répandus [29]. Les techniques analytiques de détection et de quantification du nifoxipam dans les urines sont présentées dans le Tableau 5.

Nimétazépam

Le nimétazépam (Fig. 9) est une benzodiazépine, commercialisée au Japon comme hypnotique. Il est largement détourné de son usage en Asie [33] et des cas de

Tableau 4 Méthodes de détection et de quantification du méclonazépam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification	Applications
[11]	LC—MS/MS	Urine	1 ng/mL	1 ng/mL	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS a temps de vol	Sérum	0,10 ng/mL	0,33 ng/mL	Criblage des « designer benzodiazepines »
[31]	Nano-chromatographie liquide—MS	Urine	—	—	Méthode spécifique
[32]	GLC	Plasma	0,1 ng/mL	0,6 ng/mL	Méthode spécifique

Tableau 5 Méthodes de détection et de quantification du nifoxipam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification	Applications
[11]	LC—MS/MS	Urine	10 ng/mL	10 ng/mL	Criblage des « designer benzodiazepines »
[31]	Nano-chromatographie liquide—MS	Urine	—	—	Méthode spécifique

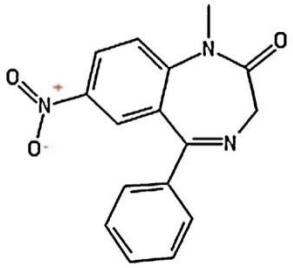

Figure 9. Structure chimique du nimétazépam

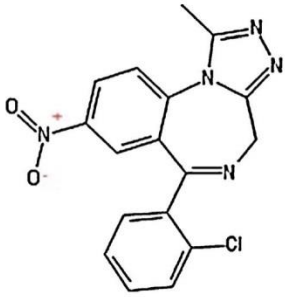

Figure 10. Structure chimique du clonazolam

complications psychiatriques ont été décrits à Singapour [34].

Par déméthylation le nimétazépam donne le nitrazépam. Son groupement nitro peut être réduit en amine pour donner le 7-aminonimétazépam, qui est son métabolite principal. Sa concentration dans les urines est 10 fois supérieure au nimétazépam inchangé, ce qui en fait une cible potentielle pour démontrer la consommation de nimétazépam. Sur la position 3, il peut être hydroxylé en 3-hydroxy-nimétazépam. La succession de ces deux réactions mène au 3-hydroxy-7-aminonimétazépam. Ce dernier peut être glucuronidé sur son groupement hydroxyle en position 3. Le nitrazépam issu de la N-déméthylation subit les mêmes biotransformations. Le nimétazépam est rapidement métabolisé, mais le 7-aminonimétazépam peut être détecté dans les urines plusieurs jours après l'ingestion [35].

L'analyse des urines de 7 sujets ayant subi des agressions sexuelles sous nimétazepam ont révélé des concentrations jusqu'à 5,2 ng/mL de nimétazépam et jusqu'à 3,9 ng/mL de nitrazépam. Les concentrations du 7-aminonimétazépam varient entre 25,3 et 152 ng/mL [35].

Le Tableau 6 présente les méthodes décrites pour la détection et la quantification du nimétazépam.

Triazolobenzodiazépines

Clonazolam

Le clonazolam (Fig. 10) est une triazolobenzodiazépine qui est active à des concentrations sanguines très faibles [36].

L'étude des métabolites éliminés chez l'homme par voie urinaire, permet de reconstituer le métabolisme du clonazolam. Sa fonction nitro peut être réduite en amine et ultérieurement acétylée. L'aminoclonazolam et l'acétaminoclonazolam peuvent être glucuronidés sur leur cycle triazole. D'après le métabolisme des autres triazolobenzodiazépines, on peut supposer que l'hydroxylation

Tableau 6 Méthodes de détection et de quantification du nimétazépam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification (ng/mL)	Applications
[16]	Électrophorèse capillaire—MS	Sérum	1,5 ng/mL	5,0	Criblage des « designer benzodiazepines »
[33]	LC—MS/MS	Urine	0,25 ng/mL	5,0	Méthode spécifique
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,10 ng/mL	0,33	Criblage des « designer benzodiazepines »
[35][a]	LC—MS	Urine	—	0,1	Méthode spécifique

[a] 7-aminonimétazépam.

Tableau 7 Méthodes de détection et de quantification du clonazolam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification	Applications
[11]	LC—MS/MS	Urine	5 ng/mL	5 ng/mL	Criblage des « designer benzodiazepines »
[31]	Nano-chromatographie liquide—MS	Urine	—	—	Méthode spécifique

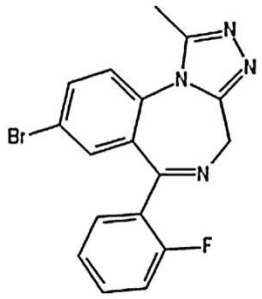

Figure 11. Structure chimique du flubromazolam

du clonazolam qui donne l'hydroxy-clonazolam, a lieu au niveau du cycle triazole. Deux métabolites hydroxylés et glucuronidés ont été détectés, dont l'un est probablement sur le cycle triazole et l'autre sur une position aromatique [31].

Les concentrations sanguines du clonazolam, rapportées dans 7 cas en Norvège, varient de 1,9 à 11 ng/mL avec une concentration médiane de 5,3 ng/mL [8]. Ces concentrations faibles pourraient rendre difficile sa détection avec les tests immunologiques [36]. Dans les urines, il présente une bonne réactivité croisée pour les tests CEDIA, HEIA, EMIT II Plus et KIMS II, mais à des concentrations plus élevées de 200, 100, 500 et 200 ng/mL respectivement. Néanmoins, un cas clinique a révélé des résultats positifs aux quatre précédents tests pour une concentration urinaire de 20 ng/mL, possiblement dû à une réactivité croisée des métabolites [15].

Les méthodes analytiques décrites pour le clonazolam sont présentées dans le Tableau 7.

Flubromazolam

Le flubromazolam (Fig. 11) est l'analogue triazolé du flubromazépam. D'après une analyse des forums suédois, il est principalement hypnotique et sédatif, et les usagers développent très vite une tolérance. Pour certains usagers, il est aussi myorelaxant et anxiolytique. D'autres déclarent une forte sensation de bien-être et d'euphorie. Les caractéristiques proéminentes du flubromazolam sont une perte de contrôle de la consommation et un sevrage long et parfois sévère. On retrouve dans la littérature des cas d'incidents où les usagers ont été hospitalisés en urgence pour des traitements psychiatriques ou arrêtés par la police [6].

Une étude d'auto-administration décrit les effets de 0,5 mg de fubromazolam sur un volontaire. Quatre-vingt-dix minutes après l'administration, l'effet myorelaxant s'est manifesté, ainsi qu'une légère fatigue. Après 3 heures, le volontaire est fortement sédaté et ce pendant plus de 10 heures. Pendant cette période, il s'est endormi à plusieurs occasions. Il présentait des troubles du langage et a développé une amnésie partielle d'une durée supérieure à 24 heures. L'après-midi suivant, le sujet a de nouveau ressenti des effets de sédation [37].

Sept métabolites ont été identifiés dans les urines. La monohydroxylation du flubromazolam est possible en position 4, en alpha (sur le groupement méthyle du cycle triazole) et sur une position aromatique non-déterminée. L'hydroxylation en position 4 et en alpha donne le métabolite dihydroxylé. La glucuronidation peut se faire directement sur le cycle triazole, ou après hydroxylation sur les positions 4 ou alpha. Comme les structures spécifiques du flubromazolam sont préservées dans tous ses métabolites, il n'y a pas de risque de confusion avec les métabolites des benzodiazépines médicales. Le flubromazolam et l'alpha-hydroxy-flubromazolam, son métabolite majoritaire, sont les cibles les plus pertinentes pour la détection dans les urines (jusqu'à 6,5 jours et jusqu'à 8 jours après l'administration respectivement) [38].

Après une dose orale de 0,5 mg, le profil cinétique du flubromazolam présente trois pics sériques : à 5 heures (7,4 ng/mL), à 8 heures (8,6 ng/mL) et à 30 heures (5,2 ng/mL). Il est suggéré que le flubromazolam subisse le cycle entérohépatique. La détection par la technique immunologique CEDIA a rendu des résultats négatifs pour tous les échantillons de sérum, mais positifs sur les urines durant 5 jours. L'analyse des cheveux après 2 semaines a révélé 0,60 pg/mg et après 4 semaines des concentrations de 0,44 pg/mg et 0,40 pg/mg dans les segments 0—1 cm et 1—2 cm respectivement [37].

Un cas d'intoxication aiguë au flubromazolam a été décrit. Un homme de 27 ans présentait un coma, une insuffisance respiratoire, une hypotension et une ischémie dans le système nerveux central après une prise de 3 mg. Le flubromazolam a été recherché dans le sérum (59 ng/mL) et dans les urines (105 ng/mL). Après un traitement antidotique et symptomatique, son état s'est amélioré [39].

Une étude de la réactivité croisée du flubromazolam a montré qu'il est détecté par les tests immunologiques CEDIA, EMIT II Plus, HEIA, et KIMS II dans les urines à des concentrations de 9 à 672 ng/mL [15]. La détection et la quantification du flubromazolam dans des milieux biologiques ont été décrites selon plusieurs méthodes présentées dans le Tableau 8.

Tableau 8 Méthodes de détection et de quantification du flubromazolam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification	Applications
[16]	Électrophorèse capillaire—MS	Sérum	1,5 ng/mL	5,0 ng/mL	Criblage des « designer benzodiazepines »
[11]	LC—MS/MS	Urine	2 ng/mL	—	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,10 ng/mL	0,33 ng/mL	Criblage des « designer benzodiazepines »
[38]	UHPLC—MS/MS	Urine	—	—	Méthode spécifique

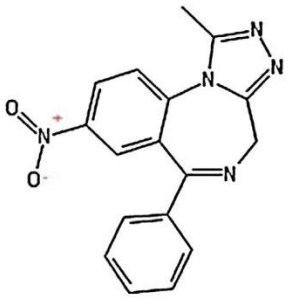

Figure 12. Structure chimique du nitrazolam

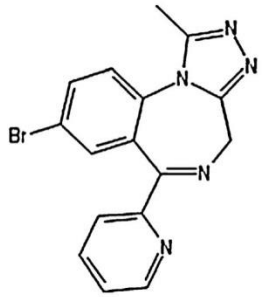

Figure 13. Structure chimique du pyrazolam

Nitrazolam

Il n'existe aucune source fiable concernant les effets et la puissance du nitrazolam (Fig. 12) chez l'homme. À ce jour, les seules sources d'information sont les forums des usagers.

Le nitrazolam, comme d'autres nitrobenzodiazépines, subit la réduction de son groupement nitro donnant le 8-amino-nitrazolam. Un métabolite hydroxylé a été détecté. Cet hydroxylation a probablement lieu en position 4 ou alpha de la molécule. La détection du nitrazolam et de ses métabolites a été réalisée par LC—MS/MS lors d'une étude in vitro [28]. Sa détection dans les milieux biologiques n'a pas été décrite.

Pyrazolam

Des expériences sur un volontaire et sur des microsomes de foie humain ont montré que le pyrazolam (Fig. 13) est peu métabolisé dans l'organisme et est éliminé inchangé par voie rénale. Après l'administration de 1 mg de pyrazolam, la concentration sérique maximale a été mesurée à 3 heures. Après une étude cinétique, le temps de demi-vie plasmatique a été estimé à 17 heures. Cinq heures 20 minutes après l'administration, la concentration urinaire était de 160 ng/mL [40]. Dans un autre cas en Norvège (conduite sous influence), la concentration sanguine du pyrazolam était de 74 ng/mL [8].

Les appareils AxSYM (Fluorescence Polarization Immunoassay) et Konelab (*homogeneous enzyme immunoassay*) ne détectent pas le pyrazolam à cause de ses faibles concentrations sériques après l'administration de 1 mg [40]. Le kit Immunalysis® Benzodiazepine (Elisa) semble être capable de le détecter à la fois dans du sang surchargé et dans des cas cliniques [10]. Les tests immunologiques CEDIA, HEIA, et KIMS II le détectent dans les urines à des concentrations supérieures à 44 ng/mL [15]. Plusieurs études ont décrit la détection et la quantification du pyrazolam. Elles sont présentées dans le Tableau 9.

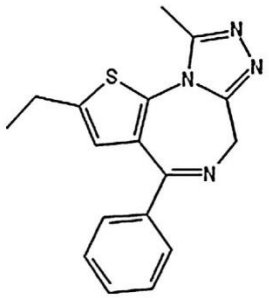

Figure 14. Structure chimique du déschloroétizolam

Thiénodiazépines

Déschloroétizolam/thiénalprazolam

Le deschloroétizolam (Fig. 14) est une thiénodiazépine moins puissante que l'étizolam [41]. Un cas de décès impliquant le déschloroétizolam est décrit dans la monographie du diclazépam [9]. Une étude décrit une concentration urinaire de 130 ng/mL [11].

Des études in vitro ont identifié quatre métabolites de phase I, dont trois hydroxylés et un dihydroxylé. D'après les similitudes de structure avec l'étizolam, les sites

Tableau 9 Méthodes de détection et de quantification du pyrazolam.

Référence	Méthode	Milieu	Limite de détection (ng/mL)	Limite de quantification (ng/mL)	Applications
[16]	Électrophorèse capillaire—MS	Sérum	15	50	Criblage des « designer benzodiazepines »
[11]	LC—MS/MS	Urine	4	10,0	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,10	0,33	Criblage des « designer benzodiazepines »

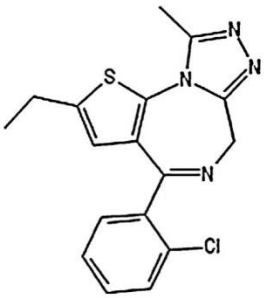

Figure 15. Structure chimique de l'étizolam

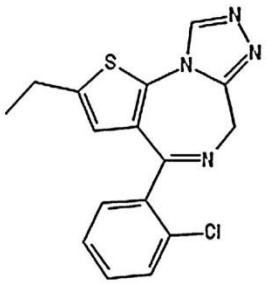

Figure 16. Structure chimique du métizolam

d'hydroxylation sont probablement 9-méthyle, 2-éthyle et la position 6. La combinaison des hydroxylations qui donne le métabolite dihydroxylé n'est pas connue. Les métabolites de phase I subissent probablement des biotransformations de phase II [36].

Dans des urines surchargées, le déschloroétizolam présente une réactivité croisée de 142, 88 et 103 % pour les tests immunologiques CEDIA (100 ng/mL), EMIT II Plus (200 ng/mL), et KIMS II (200 ng/mL) respectivement [15]. Plusieurs techniques de détection sont présentées dans le Tableau 10.

Étizolam

L'étizolam (Fig. 15) est un médicament disponible sur ordonnance en Italie, au Japon et en Inde. Il est utilisé en tant qu'anxiolytique. L'étizolam a un temps de demi-vie de 3 à 5 h, d'où une faible accumulation à long terme [42]. Des cas de dépendance à l'étizolam ont été décrits [43]. Dans un cas de prise en charge d'une patiente dépendante pour laquelle la dose a été réduite de 0,3 mg/semaine, aucun syndrome de sevrage n'a été observé [44]. Plusieurs cas de décès impliquant l'étizolam, le plus souvent en association avec d'autres produits psychoactifs, ont été décrits, les concentrations sanguines étaient de 7,2 à 300 ng/mL [45].

Après une utilisation prolongée d'étizolam, un phénomène de blépharospasme peut apparaître.

Plusieurs cas de prise d'étizolam ont été décrits dans une étude norvégienne avec des concentrations sanguines de 19 à 170 mg/L et une médiane de 50 mg/L. Deux cas impliquent l'étizolam seul : un homme de 34 ans sans altération de son état présentait une concentration sanguine de 31 ng/mL et un autre de 19 ans avec une concentration sanguine de 120 ng/mL, dont l'état n'a pas été déterminé [8].

Les métabolites majeurs de l'étizolam sont l'alpha-hydroxy-étizolam et le 8-hydroxy-étizolam. Les deux présentent une activité pharmacologique comparable à l'étizolam et ont des temps de demi-vie d'élimination plus longs [45]. Après l'incubation dans des microsomes de foie humain, cinq métabolites ont été détectés. Les expérimentateurs supposent que quatre métabolites sont monohydroxylés et que le cinquième est un aldéhyde [9].

L'étizolam peut être détecté par le kit Immunalysis® Benzodiazepine (Elisa), kit qui a été utilisé avec du succès dans au moins un cas légal [10]. Des méthodes analytiques applicables à l'étizolam sont présentées dans le Tableau 11.

Métizolam

Le métizolam (Fig. 16) est un analogue déméthylé de l'étizolam. Dans une étude d'auto-administration de 2 mg, le volontaire a déclaré une légère sédation sans déficience particulière [46].

Le métabolisme de phase I du métizolam a été étudié dans des microsomes de foie humain. Trois métabolites ont été détectés : deux sont monohydroxylés (l'un en position 6 et l'autre sur le groupement éthyle) et le troisième est dihydroxylé (sur les deux positions possibles) [28]. Selon une autre étude, le métizolam subit une hydroxylation sur le groupement éthyle et sur un azote du cycle triazole. Un autre métabolite monohydroxylé a été détecté dans des quantités insuffisantes pour la détermination de sa structure. Dans les urines, le métabolite hydroxylé sur le groupement éthyle ainsi que son glucuronide, et le métabolite N-hydroxylé ont été détectés [47].

Tableau 10 Méthodes de détection et de quantification du déschloroétizolam.

Référence	Méthode	Milieu	Limite de détection (ng/mL)	Limite de quantification (ng/mL)	Applications
[16]	Électrophorèse capillaire—MS	Sérum	1,5	5,0	Criblage des « designer benzodiazepines »
[11]	LC—MS/MS	Urine	2	5	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,15	0,50	Criblage des « designer benzodiazepines »

Tableau 11 Méthodes de détection et de quantification de l'étizolam.

Référence	Méthode	Milieu	Limite de détection	Limite de quantification	Applications
[16]	Électrophorèse capillaire—MS	Sérum	1,5 ng/mL	5 ng/mL	Criblage des « designer benzodiazepines »
[11]	LC—MS/MS	Urine	2 ng/mL	5 ng/mL	Criblage des « designer benzodiazepines »
[12]	LC à ultra haute résolution—MS à temps de vol	Sérum	0,15 ng/mL	0,50 ng/mL	Criblage des « designer benzodiazepines »
[13]	Microextraction, puis LC—MS/MS	Urine	3 ng/mL	10 ng/mL	Criblage des « designer benzodiazepines »
[48]	Dérivation avec TMS, puis GC—MS/MS	Sang	—	—	Méthode spécifique

Le métizolam peut être détecté dans les urines jusqu'à 46 heures après l'administration [47]. Son identification et sa quantification, après ingestion d'un comprimé de 2 mg, ont été décrites dans des milieux biologiques alternatifs : salive (Cmax de 0,77 ng/mL à 2 h), sueur (186 pg/patch après 72 heures), cheveux (0,27 pg/mg après 3 semaines), poils de barbe (0,73 et 0,28 pg/mg après 4 et 10 jours respectivement) et air expiré (Maximum 9 pg/filtre à 1 heure) [46]. Aucune technique d'analyse pour la détection ou la quantification du métizolam dans le sang n'a été proposée.

Limitations

Pour de nombreuses des molécules les données humaines sont insuffisantes. Les études sur les animaux ont été exclues car leurs résultats ne peuvent pas être directement extrapolées chez l'homme. Une autre source potentielle des données concernant les effets des designer benzodiazepines sont les blogues et les sites destinés à leur consommation tels que « drugs.tripsit.me », « bluelight.org », « psychoactif.org » ou « drugs-forum.com ». bien que la fiabilité des témoignages anonymes reste discutable, ces sources sont les seules qui permettent d'obtenir des renseignements sur les designer benzodiazepines les plus récentes. Ces sources n'ont pas été exploitées telles quelles mais par le biais d'articles les référençant.

L'offre des designer benzodiazepines étant en pleine expansion, la liste des molécules de notre article n'est pas exhaustive.

Conclusion

Les designer benzodiazepines sont une classe de nouveaux produits de synthèse en pleine expansion. Avec la mise en place de barrières légales pour les premières designer benzodiazepines (inscription sur la liste des substances contrôlées, voire interdites), les usagers et les e-commerçants se tournent vers des molécules moins connues. Ainsi, pour certaines designer benzodiazepines, de nombreuses informations concernant leurs effets, leur toxicité ainsi que des méthodes d'analyse sont disponibles dans la littérature, alors que pour d'autres, très peu d'informations ont été publiées. Pour les molécules les plus récentes sur le marché (fluclotizolam, flunitrazolam, phénazolam, bromazolam, flualprazolam et autres) il n'existe qu'une seule source concernant leurs propriétés physicochimiques : PubChem.

Les décès impliquant uniquement des designer benzodiazépines sont exceptionnels, mais elles sont souvent impliquées dans des intoxications suite à la polyconsommation de substances illicites. De plus, à cause de leur effet amnésiant, elles peuvent être utilisées dans des situations de soumission chimique.

L'interprétation des analyses peut être difficile liée aux similitudes de structure entre les designer benzodiazepines et les métabolites des médicaments délivrées sur ordonnance, et entre les métabolites des designer benzodiazepines et les médicaments. Il est donc nécessaire d'étudier les métabolites lors du développement de méthode. De

plus, certaines molécules classées comme des designer benzodiazepines dans une juridiction peuvent être reconnues comme des médicaments dans d'autres et donc obtenues de manière tout à fait légale par des patients.

Dans le futur, on peut s'attendre à l'expansion de cette classe avec différents sous-types de benzodiazépines, voire des modulateurs GABA-A non-benzodiazépines (imidazopyridines, cyrazolopyrimidines, cyclopyrrolones...), de structures similaires aux « Z-drugs ».

Déclaration de liens d'intérêts

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

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Partie 3 : Applications médico-judiciaires

L'analyse toxicologique médico-judiciaire, suggérée par le médecin légiste et décidée par un magistrat ou un OPJ sur autorisation d'un magistrat, se doit de répondre simplement aux critères suivants :

- ✓ établir, confirmer ou infirmer un diagnostic d'intoxication
- ✓ évaluer l'importance de l'intoxication pour interpréter les résultats
- ✓ apprécier l'altération du comportement au moment des faits (homicide, agression, …)
- ✓ rechercher des facteurs ayant pu contribuer au processus fatal dans des circonstances inhabituelles (produits dopants, exposition dans un cadre professionnel, alimentation contaminée, …)

L'expert en toxicologie, chargé de répondre à ces critères, est en lien direct avec la justice et a pour mission de l'éclairer dans le cadre d'accidents de la voie publique, d'infractions à la législation sur les stupéfiants, d'agressions sexuelles et soumissions chimiques, et de recherches des causes de la mort.

D'après l'arrêté du 29 septembre 2017, complété par la circulaire du 6 novembre 2017, la recherche des NPS est obligatoire dans le cadre de l'expertise toxicologique de référence.

Parmi les intoxications aux NPS, certaines peuvent être fatales. Ainsi, plusieurs cas de décès, impliquant une ou plusieurs nouvelles substances psychoactives, ont été rapportés en France ces dernières années. L'enquête DRAMES (Décès en Relation avec l'Abus de Médicament Et de Substances), mise en place en 2002 par l'Agence nationale de sécurité du médicament et des produits de santé (ANSM), a constaté, ces dernières années, une augmentation des décès. En 2012, les NPS étaient impliqués dans moins de 1% des décès toxiques, alors qu'en 2015 et 2016 ils étaient impliqués dans 3% des cas, augmentation récemment confirmée en 2017.

Au cours de ma thèse, le laboratoire de toxicologie a été confronté à plusieurs situations médico-judiciaires originales, impliquant des NPS. Ces situations sont décrites ci-dessous et ont donné lieu à la rédaction de 6 publications.

- Suite à un accident de la route, un homme de 35 ans a été conduit aux urgences dans un état de confusion générale (incohérence du langage, perte de l'équilibre et pupilles dilatées). D'après plusieurs témoins, il conduisait à contre sens. Un sachet annoté « AB-PINACA 10g, legal research chemical reagent only » a été retrouvé dans sa poche. L'analyse RMN de cette poudre a confirmé l'identité de la molécule et a révélé une pureté de 93,5 %. Les analyses toxicologiques ont mis en évidence la présence d'AB-PINACA, un cannabinoïde de synthèse développé par Pfizer en 2009, dans

le sang du sujet (5,7 ng/mL), dans ses urines (< 1 ng/mL), dans ses cheveux (195 pg/mg) et dans ses poils pubiens (5 pg/mg). Généralement, les concentrations mesurées dans les poils pubiens sont plus élevées que dans les cheveux, du fait de la contamination par les urines et une vitesse de pousse plus lente. D'après nos résultats, il semble que l'AB-PINACA se métabolise rapidement et complètement. La molécule mère, absente des urines, ne peut donc contaminer les poils pubiens *[Article 5]*.

- Un homme de 31 ans a été découvert décédé au domicile d'un ami. Des stupéfiants (4-MEC et GHB), du matériel pour sniffer, des bouteilles d'alcools et des sextoys ont été retrouvés à proximité du corps. L'autopsie était peu informative et rapporte l'absence de signe de traumatisme, mais une congestion massive des viscères pouvant résulter d'une phase agonique longue. Des prélèvements de sang périphérique, d'urine, de contenu gastrique, de bile et de cheveux, ont été collectés au décours de l'autopsie. Le dosage de l'alcool sanguin a révélé une alcoolémie à 0,58 g/L, confirmée par un résultat à 0,76 g/L dans les urines. Le GHB sanguin était à 172 mg/L, avec des urines à 706 mg/L. Il s'agit, d'après la littérature scientifique, de concentrations dites toxiques. La présence de 4-MEC a été confirmée par une méthode spécifique d'identification. La concentration sanguine était à 507 ng/mL, potentiellement létale. L'analyse capillaire n'a pas mis en évidence la présence de 4-MEC. Ce résultat est en faveur d'une primo-exposition à la 4-MEC. Compte tenu des résultats toxicologiques, le médecin légiste a conclu que le décès était d'origine toxique, confirmé par ses propres observations, secondaire à une poly-intoxication associant alcool éthylique, 4-MEC et GHB, vraisemblablement dans un contexte de Chemsex. Ce terme désigne le fait de consommer des produits psychotropes pendant des activités sexuelles afin de les rendre plus intenses, plus longues et de faciliter la désinhibition des partenaires. On observe depuis quelques années, une augmentation importante de cette pratique *[Article 6]*.

- La 3-méthylmethcathinone (3-MMC), isomère positionnel de la méphédrone (4-MMC), est une cathinone de synthèse souvent utilisée, en association avec d'autres substances, dans un contexte de Chemsex (voir publication précédente). Quatre sujets ayant consommés de la 3-MMC (deux intoxications et deux décès) sont étudiés dans cette publication. L'intérêt est d'obtenir des informations sur la détectabilité de la 3-MMC dans diverses matrices biologiques, sur les concentrations mesurées lors d'intoxication et de décès, et sur les substances consommées en association *[Article 7]*.

- Comme décrit dans la publication précédente, la 3-MMC est une substance stimulante et hallucinogène, fréquemment utilisée en contexte Chemsex. Il existe peu d'information la concernant

dans la littérature. Il est ainsi impossible de déterminer de façon définitive une concentration létale, puisque cette substance est chimiquement instable et qu'aucune étude, impliquant son administration, n'a été réalisée. Ce travail permet donc de discuter des difficultés d'interprétation et confirme qu'il n'est pas possible de fixer un seuil décisionnel de toxicité *[Article 8]*.

- Un homme de 41 ans a été retrouvé sans vie à son domicile. Il était médicalement suivi pour des troubles psychiatriques et pour toxicomanie. Lors de l'examen externe du corps, du sang périphérique, du sang cardiaque, des urines et des cheveux ont été collectés. Sept sachets de poudres et de cristaux, supposés être des NPS, ont été retrouvés près du corps. L'utilisation de méthode standard, telle que la LC-MS/MS, n'a pas permis d'identifier la nature chimique des 7 substances. Une stratégie analytique et un développement de méthode en GC-MS et RMN ont été nécessaires et ont permis d'identifier ces poudres et cristaux avec certitude et d'évaluer leur pureté. Les analyses toxicologiques ont montré que le sujet est décédé à la suite d'une consommation de 3-MeO-PCP, avec une concentration dans le sang périphérique potentiellement létale (489 ng/mL) et qu'il était consommateur régulier de ce produit, tel que objectivé par l'analyse des cheveux (15,6 ng/mg) *[Article 9]*.

- Une femme de 39 ans a été découverte décédée à son domicile, après avoir été agressée par son conjoint. Tous deux étaient consommateurs réguliers de produits psychotropes. L'agresseur a avoué avoir étranglé sa compagne à l'aide d'une corde, alors qu'ils étaient sous l'influence de plusieurs substances. Les analyses toxicologiques ont mis en évidence la présence de 3-MeO-PCP dans le sang de la victime, ainsi que diverses substances (alcool, diazépam, nordiazépam, cocaïne, benzoylecgonine et cocaéthylène). L'analyse capillaire a révélé une consommation répétée de 3-MeO-PCP sur les 6 derniers mois. Dans le sang de l'agresseur, prélevé 1h après les faits, plusieurs substances ont été identifiées (alcool, diazépam, nordiazépam, oxazépam, méthadone, EDDP, THC et ses métabolites), mais aucune trace de 3-MeO-PCP.
Ces investigations ont mis en évidence que la jeune femme était sous l'influence de 3-MeO-PCP au moment de son décès, pouvant avoir altéré sa capacité à se défendre. Il s'agit de la première identification de 3-MeO-PCP dans les cheveux, avec des concentrations entre 731 et 893 pg/mg *[Article 10]*.

Dans chacun de ces cas, la confrontation entre les résultats toxicologiques et les observations faites lors de l'autopsie par le médecin légiste, ont permis d'établir que le décès était bien corrélé à la consommation de la molécule identifiée.

Article 5: Testing for AB-PINACA in human hair: distribution in head hair versus pubic hair

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SHORT COMMUNICATION

WILEY

Testing for AB-PINACA in human hair: Distribution in head hair versus pubic hair

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Abstract

Synthetic cannabinoid receptor agonists were first identified in herbal products in 2008 advertised as a legal replacement for cannabis. These herbal incense are usually called "spice" and among these, one product in particular has gained popularity: AB-PINACA (N-[(2S)-1-Amino-3-methyl-1-oxobutan-2-yl]-1-pentyl-1H-indazole-3-carboxamide). This drug has been discovered to have a stronger binding to human cannabinoid CB_1 and CB_2 receptors than Δ^9-THC.While some articles have been published regarding the presence of AB-PINACA in biological fluids such as blood and urine, none reports the presence of AB-PINACA in hair. We have developed and validated a method for detection of AB-PINACA in hair using a liquid chromatography –tandem mass spectrometry system and applied it to head and pubic hair obtained in a case of intoxication. The validation procedure demonstrated a limit of detection and a limit of quantification of 0.5 and 1 pg/mg, respectively and acceptable linearity, repeatability, and reproducibility. AB-PINACA tested positive in the blood (5.7 ng/mL) and less than 1 ng/mL was found in urine. The analysis of the hair specimens resulted in an unusual distribution of the drug between head and pubic hair. AB-PINACA was identified at a higher concentration in head hair (195 pg/mg) versus in pubic hair (5 pg/mg). The very low concentration of AB-PINACA in the urine after consumption, due to rapid metabolism, could explain this infrequent distribution, as pubic hair can be contaminated by urine. In any case, it cannot be excluded that the high concentration in head hair may be due to environmental contamination.

KEYWORDS

AB-PINACA, hair, LC–MS/MS, synthetic cannabinoid

1 | INTRODUCTION

In the last 10 years, forensic and clinical laboratories worldwide found themselves faced with the problem of new psychoactive substances (NPS).

NPS are a range of drugs that have been designed to mimic established illicit drugs, such as cannabis, cocaine, ecstasy, LSD, and pharmaceuticals, such as benzodiazepines and opiates. Although the NPS terminology seems to imply the creation of new drugs, the majority of these substances are produced by altering the molecular structure of well-known psychoactive drugs and they are sold under such names as house scents, bath salts, incense, herbal highs, or plant food, finding a wide and efficient distribution through e-commerce. These products are claimed to contain only natural non-illegal compounds and consequently have no limitations in their commercial distribution, in spite of having important psychoactive effects.[1]

The chemical structure (phenethylamines, piperazines, cathinones, tryptamines, synthetic cannabinoids) of NPS and their pharmacological and clinical effects (hallucinogenic, anesthetic, dissociative, depressant) help to classify them into different categories.

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In the recent past, 50% of newly identified NPS have been classified as synthetic cannabinoids (SCs) followed by new phenethylamines found at 17%.[2]

SCs refer to a number of synthetic molecules developed as potential drugs acting on human cannabinoid receptors type I and II (CB_1 and CB_2 receptors), originally designed with a therapeutically intent, often for the treatment of pain. However, it was difficult to isolate the therapeutic properties from unwanted psychoactive effects.

Toward the end of 2008, several cannabinoids were detected in smoking herbal mixtures named Spice Gold, Spice Silver, and Yucatan Fire. They did not contain tobacco or cannabis, but when smoked, produced effects similar to those of cannabis.[3]

These compounds are produced in a variety of similar chemical structures, with small changes in their synthesis procedure, in order to escape controls and inclusion on the list of prohibited substances. Although they are defined as SCs, many of these substances are not structurally related to so-called conventional cannabinoids, such as THC.

Most SCs are pharmacologically characterized as agonists to CB_1 receptors. However, structural differences between SC receptor agonists (SCRA) and Δ9-THC lead these synthetic compounds to possess increased biological activity.[4] They can be significantly more potent than the active ingredient in cannabis, with a higher risk of adverse effects, including cardiovascular effects, as well as psychosis, seizures, and coma.[5]

Inter alia, there have been a number of reported cases of intoxication in relation to the synthetic cannabinoid AB-PINACA.

AB-PINACA (N-[(2S)-1-Amino-3-methyl-1-oxobutan-2-yl]-1-pentyl-1H-indazole-3-carboxamide), originally developed by Pfizer Inc. (patent WO/2009/106980- indazole derivatives) as a synthetic cannabinoid receptor agonist, has been found outside medical settings as a synthetic constituent and found in herbal smoking mixtures that are sold under a variety of brand names. AB-PINACA is clandestinely manufactured and is also available in powdered form as a "research chemical." In its pure form, but mostly as a synthetic constituent added to a plant matrix (eg, Damiana (*Turneradiffusa*) or marshmallow (*Althaea officinalis*), AB-PINACA is generally consumed by smoking. This synthetic cannabinoid binds and activates human CB_1 and CB_2 receptors at low nanomolar concentrations, and induces a number of biological responses also triggered by Δ9-THC. Moreover, in vivo studies have shown that AB-PINACA is 2- to 14-fold more potent than the natural constituent of cannabis. This highlights how it represents a risk to public health and society, especially since no therapeutic use is recognized for AB-PINACA.[6]

A few papers have reported the detection of AB-PINACA in human biological samples but none in hair.

In 2018, Armenian et al[7] reported six cases of suspected intoxication from AB-PINACA and ADB-PINACA. AB-PINACA and its pentanoic acid metabolite were detected using a liquid chromatography–time of flight–mass spectrometry (LC–TOF–MS) in two of their cases. All the cases manifested typical signs of synthetic cannabinoid toxicity, including agitation, confusion, lethargy, and hypertension. Tachycardia, hyperthermia, and psychosis are also common symptoms of synthetic cannabinoids toxicity from full CB_1 and CB_2 receptor agonist. No concentration was reported.

Brianna et al, in 2015,[8] reported all impaired driving cases that were submitted to their laboratory in 2014 and that were positive for AB-PINACA and AB-CHIMINACA. The range of blood concentrations was 0.6–41.3 ng/mL for AB-PINACA. The majority of the concentrations observed were lower than 10 ng/mL. However, the case histories that were provided indicated apparent and severe impairment of the individual even at low concentrations. Slurred speech, confusion, lack of coordination, and lethargy were commonly observed.

In 2015, Thornton et al[9] reported a case of an unintentional exposure to AB-PINACA in a 10-month-old child, resulting in central nervous system depression and the need for mechanical ventilation. Her initial serum specimen was sent for analysis by liquid chromatography–quadrupole time-of-flight–mass spectrometry (LC–QTOF–MS). Results revealed the presence of AB-PINACA at 42 ng/mL and its metabolite AB-PINACA N-pentanoic acid at 345 ng/mL.

If few articles have been published regarding the presence of AB-PINACA in biological samples such as blood and urine, none reports the presence of AB-PINACA in keratin matrices. However, some articles have reported the presence of synthetic cannabinoids in the hair.

In 2017, Franz et al[10] validated a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the detection of synthetic cannabinoids in human hair. They tested 294 authentic samples, resulting in 163 positive specimens and showed a broad concentration range, from less than 1.0 up to 57 000 pg/mg, identifying 52 different synthetic cannabinoids in hair.

Sim et al, in 2017,[11] validated an analytical method for detection of AB-CHIMINACA and its metabolites in hair using an LC–MS/MS and they applied it to the hair samples from some suspects of synthetic cannabinoids use. Among 122 hair samples analyzed, 37 showed the presence of AB-CHIMINACA at concentrations ranged from 2.2 to 1512 pg/mg.

Salomone et al, in 2014,[12] developed an ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analytical method for the detection of 23 synthetic cannabinoids in hair samples in order to expand the number of screened compounds emerging in the European territory and evaluate the diffusion of cannabimimetics among different populations of drug consumers. They analyzed 344 hair samples of which 15 were found positive for at least one synthetic cannabinoid. The drug most frequently detected was JWH-073, generally at low concentration (range 1.6–50.5 pg/mg), followed by JWH-122 (range 7.4–2800 pg/mg). Other detected drugs included JWH-250, JWH-081, JWH-018, JWH-210, JWH-019, and AM-1220. For several positive samples, the synthetic cannabinoid concentration was lower than 50 pg/mg, underlining the need for established cut-off values for discrimination between chronic consumption and occasional use (or external contamination).

Hair analysis is useful to have some explanations about drug use but the possibility of contamination is always present. As with Δ^9-tetrahydrocannabinol (THC) numerous sources of external contamination have to be considered when interpreting the analytical results: side-stream smoke, contamination via handling of the herbal mixtures, incorporation via sebum and\ or sweat and much more. To address the problem of potential external contamination, metabolites should be analyzed. However certain metabolites can also formed

ex vivo or might be present in sebum or sweat. To evaluate the different aspects affecting hair analysis results for synthetic cannabinoids, Franz et al[13] tested the stability of two synthetic cannabinoids (AB-CHIMINACA and 5F-PB-22) in hair matrix and under thermal stress. The stability tests revealed that the metabolites are also formed in externally contaminated hair. In addition, hydrolysis products (5F-PB-22 3-carboxyindoleand AB-CHIMINACA valine) were detected as degradation products in smoke condensate and they could be deposited by condensation on the hair after thermal degradation during smoking. Therefore, interpretation of "metabolite" findings should be carried out with utmost care.

It is difficult to estimate the prevalence of AB-PINACA side effects, especially because patients at the emergency departments are rarely questioned about the use of these drugs and because in the case of fatal consumption it is not easy to recognize the signs of a death involving AB-PINACA during the autopsy. In addition, the drug is not always detectable in biological samples by conventional toxicological screening usually used in laboratories. For these reasons, consumption is rarely known, and clinical signs are rarely described, pointing the importance of anamnestic and circumstantial elements.

In the present study, we have developed a method for the detection of AB-PINACA in human hair specimens obtained from an impaired driving case.

2 | CASE REPORT

A 35-year-old male was injured during a traffic accident. Some witnesses indicated that the subject was driving in the wrong direction. He was rescued and transferred to the emergency department of the city hospital. Rescuers remarked that he was in a state of semi-vigilance, with slow reflexes. On arrival at the hospital, he appeared confused, with dilated pupils and slow speech; he was unable to maintain his balance. His vital signs were normal. The blood alcohol test was negative and a standard drug screen test performed also came back negative. A plastic bag of white powder with a label "AB-PINACA, 10 g, legal research chemical reagent use only" was found in the subject's pocket. Police investigations revealed that he was an "occasional" drug user. After calling the toxicologist, the collection of blood with NaF as a preservative, urine without preservative, head hair, and pubic hair was executed and the bag containing the white powder was sent for analysis to the forensic toxicology laboratory.

Blood and urine were kept at +4°C until analysis. Head hair (0.5 cm, dark brown) and pubic hair specimens were stored in an envelope at room temperature.

3 | MATERIAL AND METHODS

3.1 | Standard and reagents

Certified solution of MAM2201-d5 at a concentration of 1 mg/mL was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). AB-PINACA used to make the working solutions for the calibration curve was the powder contained in the packet found during the inspection, after correction with the purity index obtained during proton nuclear magnetic resonance (1H-NMR) analysis (correction by a factor 1.06 by weight).

Methanol for HPLC Isocratic Grade (MeOH), ethyl acetate (HPLC grade), acetonitrile for (HPLC grade), acid formic, dichloromethane, n-hexane, and diethyl ether were obtained from V.W.R. Chemicals ProLabo (Fontenay-sous-Bois, France). Sodium carbonate and sodium bicarbonate were purchased from Merck (Darmstadt, Germany). Isoamyl alcohol and disodium tetraboratedecahydrate were obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France). Ammonium formate was purchased from Alfa Aesar Thermo Fischer (Karlsruhe, Germany). Water was dispensed by a Miele E 318 G7749 Aqua Purificator (Miele-Einweg-Harze, Germany).

Sodium carbonate buffer, pH 10.2, was prepared by adding 1.87 g of sodium bicarbonate and 0.29 of sodium carbonate to 250 mL of distilled water and adjusting the pH to 10.2 with 0.1 M sodium hydroxide. Saturated borate buffer, pH 9.5, was prepared by adding disodium tetraboratedecahydrate using a magnetic agitator to facilitate dissolution until pH 9.5.

Mobile phase A (formate buffer adjusted to pH 3 in deionized water) was prepared by adding 0.157 g of ammonium formate to 500 mL of deionized water and adjusting the pH to 3.

Mobile phase B (0.1% formic acid in acetronitrile) was prepared by adding 1.00 mL of formic acid to 1 L of acetonitrile.

MAM2201-d5 was used as internal standard (IS) at a concentration of 100 ng/mL in methanol. For calibration standards, adequate dilutions were made from the solution of AB-PINACA at 1 g/L.

3.2 | AB-PINACA analysis

3.2.1 | Powder analysis

To confirm the purity and the amount of the AB-PINACA powder, a nuclear magnetic resonance (1H-NMR) analysis was performed. Briefly, the 1H-NMR spectra were recorded on AVANCE 300 (Bruker Biospin, Wissembourg, France) operating at 300 MHz equipped with a 5 mm quadruple nucleus probe (QNP) probe at 295 K. The 1H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 seconds, a relaxation delay of 2 seconds and a flip angle of 30°. The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. After thin manual grinding of the powder using a mortar for 5 minutes to obtain an homogenous item, 7.5 mg was dissolved in 600 µL of methanol-D4 and the ERETIC (Electronic Reference To access In vivo Concentrations) method was used for the determination of absolute concentrations of AB-PINACA. ERETIC consisted of a digitally generated Gaussian peak (on single point calibration calibrated using 15 mg of maleic acid as a reference sample in 600 µL CDCl3 and a control point using 10 mg of the same substance in 600 µL CDCl3) inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the value peak area integrals in the spectrum give directly information about the concentration of protons sample purity.[14] To test inorganic impurities ICP/MS analysis was performed.

About 2 mg of the powder was mineralized in polypropylene tubes with 500 µL of Suprapur 65% nitric acid at 70°C for 60 minutes. Before testing, the residue was 1/40 diluted with 0.1% nitric acid and 0.01% Triton X-100. Rhodium (Sigma, Saint Quentin Fallavier, France) at 1 ng/mL was added as an internal standard.

Heavy metals and other elements (32 elements in total) were semi-quantitated in the powder by inductively coupled plasma mass spectrometry (ICP–MS) on a Perkin Elmer NexION 300 apparatus (Waltham). The RF generator power was 1.4 kW. The plasma gas flow rate of argon was as follows: nebulization, 0.95 L/min, plasmagen 13 L/min and auxiliary 0.90 L/min.

The aim of the test was not to determine the concentrations of the elements but to verify any unusual enrichment of an element.

3.2.2 | Blood analysis

A 7-point calibration curve was prepared using the following concentrations: 0.5, 1, 2, 5, 10, 20, and 50 ng/mL of AB-PINACA.

A sample volume of 1.0 mL of human whole blood was subjected to an alkaline liquid–liquid extraction (LLE). The specimen was spiked with 10 µL of 100 ng/mL deuterated internal standard (MAM-2201 d5), 1 mL of sodium carbonate buffer, and 5 mL of a 90:10 (v/v) hexane: ethyl acetate mixture. Specimens were vigorously shaken by Multi-Flask Shaker for 15 minutes, then centrifuged at 3000 rpm for 15 minutes. Subsequently, the organic supernatant was transferred to a glass conical vial and evaporated to dryness under a gentle stream of nitrogen at 35°C. Following dryness, specimens were reconstituted with 50 µL of 5mM ammonium formate buffer adjusted at pH 3 and transferred to injection vials.

3.2.3 | Hair analysis

The hair specimens (head hair and pubic hair) strands were decontaminated twice with 5 mL of dichloromethane for 2 minutes and dried. The specimens were of dark brown color, with a length of 0.5 cm and not oriented. They were cut into snippets and extracted all at once and 20 mg were incubated in 1 mL buffer borate pH 9.5 for 15 hours at 40°C, in the presence of 0.2 ng of MAM2201-d5 used as IS. Then, 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5) was added. After agitation for 15 minutes and centrifugation (15 minutes, 3000 rpm), the organic phase was collected and evaporated to dryness under a nitrogen flow. The residue was reconstituted in 50 µL of 5mM ammonium formate buffer adjusted at pH 3.

A 6-point calibration curve was prepared using the following concentrations: 1, 5, 10, 50, 100, and 250 pg/mg.

The decontamination washes were combined and evaporated. After addition of the IS and 50 µL of 5mM ammonium formate buffer adjusted at pH 3, the mixture was tested for AB-PINACA. No drug was identified.

3.2.4 | Instrument conditions

LC was performed using a Waters Acquity HSS C18 column (150 x 2.1 x 1.8 µm). The column compartment was regulated at 50°C. A 10-µL injection with a 0.4 mL/min flow of formate buffer adjusted to pH 3 (A) and 0.1% formic acid in acetonitrile (B) was used. The gradient elution was as follows: The initial composition (30% B) was increased from 30 to 60% over 2.5 minutes, 60 to 90% over 6.5 minutes and returned to initial conditions over 3 minutes. AB-PINACA and MAM-2201 were eluted at 4.01 and 6.05 minutes, respectively. The total run time was 11 minutes.

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

The following conditions were found to be optimal for the analysis of AB-PINACA and the internal standard: capillary voltage at 1.5 kV; source block temperature at 150°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation was performed. Cone voltage and collision energy were adjusted to optimize the signal of the 4 most abundant products ions of AB-PINACA: m/z 331.5 > 145.3; 331.5 ≥ 215.5; 331.5 > 286.5; 331.5 > 314.5. For the internal standard, the following transitions were used: m/z 379.2 > 115; 379.2 > 141.1; 379.2 ≥ 169.1; 379.2 > 237.1 (Table 1). The underlined transitions were used for quantification.

MassLynx 4.1 software was used for quantification.

3.2.5 | Validation procedure

The hair test method was validated for linearity, repeatability, reproducibility and detection and quantitation limits according to the SFTA (Societé Française de Toxicologie Analytique) guidelines.[15] Three calibration curves that included six points (concentrations ranging from 1 to 250 pg/mg), obtained over a three-day period, were established for the study of linearity.

Quantification was achieved by plotting the peak area ratios of AB-PINACA to the IS versus concentration followed by linear regression analysis.

Repeatability and reproducibility were determined for two QC levels, 10 pg/mg and 250 pg/mg. For the repeatability, six replicates of each QC level were processed the same day. For the reproducibility, each QC level was processed three times, three different days over a period of two weeks.

The limit of detection (LOD) is the lowest concentration of the compound that can be detected with a signal-to-noise ratio greater than 3:1 for the specific transition. The limit of quantification (LOQ) was defined as the first point of the calibration curve.

TABLE 1 AB-PINACA and IS MAM2201-d5 specific transitions

AB-PINACA	Precursor	Product Ion	Cone Voltage (V)	Collision Energy (eV)
	331.5	145.3	22	35
	331.5	215.5	22	22
MAM2201-d5				
	379.2	141.1	55	45
	379.2	169.1	55	27

4 | RESULTS AND DISCUSSION

For the identification and the characterization of the AB-PINACA powder, 1H-NMR spectroscopy was applied. This confirmed the chemical structure which was in accordance with the literature.[16] No impurity was detected in the AB-PINACA powder and purity was estimated at 93.5% using ERETIC method.[14] Information about the NMR results is available in the Supporting Information.

For the method validation, the six-point calibration curve showed good linearity in the concentration range 1–250 pg/mg with a correlation coefficient ranging from 0.9771 to 0.9945 during the 3 tests. The LOD and the lower LOQ are 0.5 and 1 pg/mg respectively. Repeatability was 11.9 and 18.4% at 10 and 250 pg/mg, respectively, and reproducibility was13.6 and 19.1%, at 10 and 250 pg/mg, respectively. Validation results are reported in Table 2.

AB-PINACA was found in the blood, head hair, and pubic hair of the subject at the following concentrations: 5.7 ng/mL, 195 pg/mg and 5 pg/mg (Table 3). The chromatogram obtained after extraction of subject's head hair is available in the Supporting Information. In addition, although the method was not validated, urine was tested for AB-PINACA using the extraction method described for blood. The rough response of AB-PINACA was lower than the signal obtained with a calibrator at 1 ng/mL.

The main advantage of hair as a testing matrix is the ability to provide a historical detail of an individual's exposure to drugs following chronic use, but it is also possible to detect drugs in hair following a single exposure.[17] Unlike many traditional biological samples collected for toxicological investigations such as blood, urine, or oral fluid, drugs incorporated into the hair matrix remain relatively stable for many months or even years. The drug passes from the circulating fluids into the hair and remains tightly bound there.[4]

As indicated earlier, not many articles have reported the detection of AB-PINACA in human biological samples.

AB-PINACA blood concentration found in our case is in agreement with the few data reported in the literature.[8,9] Brianna et al[8] reported that the concentrations of AB-PINACA detected in their cases ranged from 0.6 to 41.3 ng/mL and that the majority of the concentrations observed were lower than 10 ng/mL. Despite the low concentrations, severe impairment of the individual was however observed. Similar to the cases observed by Armenian et al,[7] confusion, dilated pupils, slurred speech, and lack of coordination are common signs of AB-PINACA toxicity.

When testing head and pubic hair, a higher concentration in head hair compared to that found in pubic hair was found (Table 3). This distribution is unusual because, in most studies, a greater concentration of the molecule was found in pubic hair when compared to head hair.

In 2007, Kintz et al[18] performed a study in which they analyzed head hair and pubic hair from eight social drinkers to research ethyl glucuronide, a metabolite of ethanol that can be detected in hair. In all eight specimens, the ethyl glucuronide concentration was below the LOQ (10 pg/mg) in head hair but a high concentration was identified in pubic hair, in the range 12–1370 pg/mg.

In 1994, Cirimele et al[19] tested 43 human head and pubic hair samples to investigate cannabinoids (THC and its metabolite THC-COOH). Concentrations found in head hair ranged from 0.26 to 2.17 ng/mg for THC and 0.07 to 0.33 ng/mg for THC-COOH. In pubic hair, THC concentrations ranged from 0.34 to 3.91 ng/mg and THC-COOH concentrations from 0.07 to 0.83 ng/mg. In most cases, the highest cannabinoid concentration was found in pubic hair.

Offidani et al in 1993[20] investigated the distribution of some drugs in hair samples taken from different parts of the body (head, pubis, and axillae) of 15 subjects who died of a drug overdose. The concentration levels observed for these drugs differed in relation to the anatomic region. In particular, morphine head hair concentrations ranged from 0.23 ng/mg to 5.3 ng/mg and the highest levels were observed in pubic hair with concentrations between 0.18 and 31.7 ng/mg. Similar results were obtained for cocaine and benzodiazepines. Pubic hair concentrations were always the highest.

Similar results were obtained by Mangin et al in 1993.[21] The authors reported the concentrations of opiates in head, pubic, and axillary hair obtained from 20 fatal heroin cases. The highest morphine concentrations were found in pubic hair (0.80–41.34 ng/mg), followed by head hair (0.62–27.1 ng/mg), and axillary hair (0.40–24.20 ng/mg).

The incorporation of drugs into hair is due to the transportation by passive diffusion of these substances present in blood into the growing cells located at the base of the follicle of the hair where the drugs become trapped and bound in the keratin matrices during the keratogenesis. Therefore, drug incorporation into hair is dependent on the drug concentration in blood, which is dependent on the dose of drug ingested. However, there are many other factors that must be considered: contributions from sweat, sebaceous and apocrine gland secretions; and external contamination from drug handling, smoke, drug deposit by contaminated hands, or simply by environmental contamination. Incorporation of drugs into hair also depends on local factors, which are variable according to the anatomical regions involved, ie, excessive sweating for axillary hair or urine contamination of pubic hair. Another important element is the difference in the biology of these various types of hair. Scalp hair has a higher growth rate, as it ranges from 0.3 to 0.5 mm/day, while pubic hair has a lower growth rate (approximately 0.2 to 0.3 mm/day).

TABLE 2 Validation parameters of AB-PINACA in hair

Linearity	1–250 pg/mg
Correlation coefficient	0.977–0.994
LOD	0.5 pg/mg
LOQ	1 pg/mg
QC 10 pg/mg	
CV repeatability (%)	18.4
CV reproducibility (%)	19.1
QC 250 pg/mg	
CV repeatability (%)	11.9
CV reproducibility (%)	13.6

TABLE 3 Results of blood, head hair, and pubic hair analysis in the reported case

	Blood (ng/mL)	Head Hair (pg/mg)	Pubic Hair (pg/mg)
AB-PINACA	5.7	195	5

Therefore, pubic hair, with a low grow rate, may be able to concentrate drugs more than head hair. Finally, the high levels of drugs in urine may contaminate pubic hair, particularly in subjects with poor hygiene practices.[22] This property has been used as a discrimination test for alcohol abstinence, by testing pubic hair for ethyl glucuronide,[18] which have a greater dynamic to the presence of alcohol when compared to head hair.

The fact that a higher concentration of AB-PINACA was found in head hair rather than in pubic hair can be justified by a scarce presence of AB-PINACA in urine due to an extensive metabolism. Some papers reported the study of the metabolism of AB-PINACA.

Wohlfarth et al, in 2015,[23] reported the metabolite profiling of AB-PINACA. They performed a metabolism study of AB-PINACA in human hepatocytes and they also analyzed two urine specimens from suspected AB-PINACA cases using high-resolution mass spectrometry (HRMS). They identified 23 metabolites but the most intense were AB-PINACA carboxylic acid (hydrolysis product), carbonyl AB-PINACA (ketone formation), and hydroxypentyl AB-PINACA (oxidation). AB-PINACA was only detected at low concentration or even not detected in urine.

In 2017, Richter et al[24] investigated the phase I and the phase II metabolism of six NPS from different drug classes (including AB-PINACA) using ps9 (pooled human s9 fraction) or pHLM/pHLC (pooled human liver microsomes combined with cytosol). They found that AB-PINACA is extensively metabolized and that the predominate biotransformations were the same observed by Wohlfarth et al.[23] Hydrolysis, mono-, and d-hydroxylation metabolites were also demonstrated in a series of human liver microsome experiments.[25]

Similarly to THC, contamination is also possible also for SCs. From the literature, it can be very difficult to distinguish an environmental cannabis-contaminated subject from a subject consuming cannabis. Therefore, while a negative result can exclude long-term use or strong contact with drugs, a positive result has to be interpreted very cautiously and cannot definitively account as a confirmed sign of substance exposure. Some authors have even challenged the identification of a metabolite (THC-COOH for cannabis), as evidence of use.[13-26]

Irrespective of the findings, a complete interpretation of hair test results can be a critical step, which should be achieved with other intelligence available, for example medical history, witness statements, and the circumstances of the matter.

Therefore, one cannot exclude that the high difference between the head hair test and the pubic hair test can be part of contamination by the environment.

5 | CONCLUSION

NPS are not subject to clinical evaluation before release on the market. It is only when an accident, an overdose, or a fatality occurs that an NPS can be classified and legally controlled or its medical use defined. The ease with which NPS are bought on the Internet has prompted an increase in their consumption and represents a great risk for public health. In this study, the authors presented a case of intoxication involving AB-PINACA. It seems that AB-PINACA is rapidly totally metabolized and therefore difficult to detect in urine. It is generally accepted that higher concentrations in pubic hair versus head hair can be the consequence of contamination by urine. However, if the drug is not present in urine, this can result in an unusual pattern of concentrations between the two types of hair. The analysis of the distribution of this molecule between biological fluids and different hair has never been done, allowing the authors to present this initial study.

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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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Article 6: Aspect toxicologique d'un phénomène en plein essor: le Chemsex. Description d'un cas médico-légal aux conséquences fatales, impliquant la 4-MEC.

Alice Ameline, Adeline Blanchot, Nadia Arbouche, Jean-Sébastien Raul, Pascal Kintz

La revue de médecine légale, accepté pour publication.

La Revue de Médecine Légale

Aspect toxicologique d'un phénomène en plein essor : le Chemsex. Description d'un cas médico-légal aux conséquences fatales, impliquant la 4-MEC.
Toxicological aspect of a booming phenomenon: Chemsex. Description of a fatal medico-legal case involving 4-MEC.

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Résumé:	Le Chemsex, contraction des termes anglais « chemical » et « sex », provient de la culture gay anglo-saxonne et désigne le fait de consommer des produits psychotropes pendant des activités sexuelles pour les rendre plus intenses, plus longues et faciliter la désinhibition des partenaires. On observe depuis une dizaine d'années une augmentation de cette pratique dans la population des hommes ayant des relations sexuelles avec d'autres hommes (HSH). Les substances les plus fréquemment utilisées sont le GHB/GBL, les cathinones de synthèse (méphédrone, 4-MEC et 3-MMC) ainsi que la cocaïne et les médicaments de la performance sexuelle. Les auteurs rapportent un cas de décès d'origine toxique, secondaire à une poly-intoxication associant alcool éthylique, GHB et 4-méthyléthylcathinone (4-MEC), dans un contexte de Chemsex. L'analyse du sang périphérique a montré une concentration potentiellement létale de 4-MEC à 507 ng/mL, la présence, à concentration très toxique, de GHB à 172 mg/L, ainsi que de l'alcool éthylique à 0,58 g/L. Il s'agissait probablement d'un sujet débutant dans cette pratique, puisque l'analyse des cheveux s'est révélée négative. Plusieurs études décrites dans la littérature permettent de faire un état des lieux de la situation du Chemsex en France et ainsi d'alerter les instances de santé publique et d'appuyer les programmes de réduction des risques et de prise en charge. Chemsex, a contraction of the English terms "chemical" and "sex", comes from the gay culture and refers the use of psychotropic drugs during sexual activities to make them more intense, longer and to facilitate relaxation between the participants. Over the past ten years, this practice has increased in the population of men who have sex with men (MSM). The most frequently used substances are GHB / GBL, synthetic cathinones (mephedrone, 4-MEC and 3-MMC), in addition to narcotics and usual drugs. The authors report a case of toxic death, due to poly-intoxication involving ethyl alcohol, GHB and 4-MEC, in a context of Chemsex. Peripheral blood analysis showed a potentially lethal concentration of 4-MEC at 507 ng/mL, the presence of GHB at 172 mg/L, a highly toxic concentration, and ethyl alcohol at 0.58 g/L. Several studies published in the medical literature make it possible to make an inventory of the situation of Chemsex in France and thus to alert the public health authorities. This allows supporting the programs of reduction of the risks and assumption of responsibility.

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Aspect toxicologique d'un phénomène en plein essor : le Chemsex. Description d'un cas médico-légal aux conséquences fatales, impliquant la 4-MEC.

Toxicological aspect of a booming phenomenon: Chemsex. Description of a fatal medico-legal case involving 4-MEC.

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Résumé

Le Chemsex, contraction des termes anglais « chemical » et « sex », provient de la culture gay anglo-saxonne et désigne le fait de consommer des produits psychotropes pendant des activités sexuelles pour les rendre plus intenses, plus longues et faciliter la désinhibition des partenaires. On observe depuis une dizaine d'années une augmentation de cette pratique dans la population des hommes ayant des relations sexuelles avec d'autres hommes (HSH). Les substances les plus fréquemment utilisées sont le GHB/GBL, les cathinones de synthèse (méphédrone, 4-MEC et 3-MMC) ainsi que la cocaïne et les médicaments de la performance sexuelle. Les auteurs rapportent un cas de décès d'origine toxique, secondaire à une poly-intoxication associant alcool éthylique, GHB et 4-méthyléthylcathinone (4-MEC), dans un contexte de Chemsex. L'analyse du sang périphérique a montré une concentration potentiellement létale de 4-MEC à 507 ng/mL, la présence, à concentration très toxique, de GHB à 172 mg/L, ainsi que de l'alcool éthylique à 0,58 g/L. Il s'agissait probablement d'un sujet débutant dans cette pratique, puisque l'analyse des cheveux s'est révélée négative. Plusieurs études décrites dans la littérature permettent de faire un état des lieux de la situation du Chemsex en France et ainsi d'alerter les instances de santé publique et d'appuyer les programmes de réduction des risques et de prise en charge.

Mots clés Chemsex, polyconsommation, HSH, 4-MEC, GHB, alcool éthylique

Summary

Chemsex, a contraction of the English terms "chemical" and "sex", comes from the gay culture and refers the use of psychotropic drugs during sexual activities to make them more intense, longer and to facilitate relaxation between the participants. Over the past ten years, this practice has increased in the population of men who have sex with men (MSM). The most frequently used substances are GHB / GBL, synthetic cathinones (mephedrone, 4-MEC and 3-MMC), in addition to narcotics and usual drugs. The authors report a case of toxic death, due to poly-intoxication involving ethyl alcohol, GHB and 4-MEC, in a context of Chemsex. Peripheral blood analysis showed a potentially lethal concentration of 4-MEC at 507 ng/mL, the presence of GHB at 172 mg/L, a highly toxic concentration, and ethyl alcohol at 0.58 g/L. Several studies published in the medical literature make it possible to make an inventory of the situation of Chemsex in France and thus to alert the public health authorities. This allows supporting the programs of reduction of the risks and assumption of responsibility.

Key words Chemsex, poly-drug consumption, MSM, 4-MEC, GHB, ethyl alcohol

Introduction

Le Chemsex, contraction des termes anglais « chemical » et « sex », provient de la culture gay anglo-saxonne et désigne le fait de consommer des produits psychotropes pendant des activités sexuelles pour les rendre plus intenses, plus longues et faciliter la désinhibition des partenaires [1]. L'utilisation de substances psychotropes licites ou illicites à des fins sexuelles n'est pas un phénomène nouveau. Toutefois, on observe depuis une dizaine d'années l'augmentation de cette pratique dans la population des hommes ayant des relations sexuelles avec d'autres hommes (HSH). En France, comme dans d'autres pays d'Europe, cette tendance a entrainé l'émergence de nouvelles modalités de rencontres, telles que les sites internet et les applications mobiles géolocalisées, et a favorisé la consommation de nouveaux produits, notamment les nouveaux produits de synthèse (NPS) [2]. Les substances les plus couramment associés au Chemsex sont l'acide gamma-hydroxybutyrique et la gamma-butyrolactone (GHB et GBL), les cathinones (tels que la méphédrone (4-MMC), la 4-méthyléthcathinone (4-MEC), la 3-méthylméthcathinone (3-MMC), etc.), la cocaïne, les médicaments de la performance sexuelle (sildénafil-Viagra, tadalafil-Cialis etc.) et plus rarement la kétamine et la méthamphétamine.

Le GHB, qui était la substance la plus répandue jusqu'à aujourd'hui, est très souvent remplacé par la GBL, dont la forme circulante est le GHB, qui est moins chère et plus facilement disponible dans le domaine de l'industrie (vendue liquide sous l'intitulé « G ») [3]. Les cathinones sont fréquemment utilisées car elles sont très facilement accessibles sur Internet, peu onéreuses (entre 10 et 20 euros le gramme), et possèdent des caractéristiques proches de celles de l'amphétamine. Parfois, plusieurs substances sont consommées simultanément. Cette association de produits stimulants a pour effet de provoquer des sensations d'euphorie et d'augmenter l'excitation et l'endurance sexuelle. Sur le plan des effets secondaires, cette polyconsommation peut causer à court terme des nausées, des vomissements, des troubles du rythme cardiaque, des vertiges, voire même une perte de connaissance [4]. A long terme, un risque d'addiction peut s'observer. En cas de surdosage, l'intoxication aigue peut entrainer la mort [2].

Cet article présente un cas d'intoxication fatale au GHB, associé à la 4-MEC et à l'alcool, lors d'une pratique HSH sous l'influence de produits psychotropes.

Cas clinique

Un matin tôt, le corps sans vie d'un homme âgé de 31 ans, pesant 95 kg pour 1.81 m, a été découvert au domicile d'un de ses amis. Le corps a été retrouvé en décubitus ventral au sol du salon, la tête reposant sur le canapé, vêtu uniquement d'un string. Les manœuvres de réanimation, entreprises par son ami, seraient restées vaines. Des stupéfiants (4-MEC et GHB), du matériel pour sniffer, des

bouteilles d'alcools et des sextoys ont été découverts à proximité du corps. Les opérations de scanner, d'examen externe et d'autopsie médico-légale ont permis de mettre en évidence :

- l'absence de lésion traumatique suspecte évocatrice de l'intervention d'un tiers ou de nature à expliquer le décès au scanner et lors de l'autopsie ;
- un syndrome asphyxique et une congestion multiviscérale marqués, non spécifiques d'une cause de décès ;
- une congestion et un œdème pulmonaire massifs ;
- la présence de particules textiles noires, déposées de façon circonférentielle au niveau des poignets et des chevilles, ainsi que des lésions ecchymotiques d'allure récente à la face antérieure des deux poignets, pouvant avoir été occasionnés au cours de jeux sexuels.

A l'issue de ces opérations, la cause exacte du décès n'a pu être clairement identifiée. En vue d'investigations toxicologiques, des prélèvements de sang cardiaque, de sang périphérique, de contenu gastrique, d'urine, de bile et de cheveux ont été effectués par le médecin légiste. Les échantillons ont été stockés à +4 °C jusqu'aux analyses, à l'exclusion des cheveux, conservés à température ambiante. Aucune demande d'analyse d'anatomie pathologie n'a été ordonnée par le magistrat.

Analyses toxicologiques

Les analyses ont été réalisées dans le cadre de l'expertise toxicologique de référence, selon l'arrêté du 29 septembre 2017 (JORF n° 0223 du 5 octobre 2017, texte n° 12), en utilisant des méthodes validées du laboratoire [5-6]. Brièvement, le dosage de l'alcool éthylique a été réalisé sur l'échantillon de sang périphérique et d'urines par Headspace-chromatographie gazeuse couplée à un détecteur à ionisation de flamme, selon la méthode officielle. Les solvants et les substances volatiles ont été recherchés dans les urines par Headspace-chromatographie gazeuse couplée à un spectromètre de masse. La recherche immunologique des médicaments et des stupéfiants, à partir de sang et des urines, a été faite par la technique Elisa (Alere). La mesure de la carboxyhémoglobine a été réalisée sur du sang cardiaque par CO-oxymétrie (Radiometer ABL80 Flex). Le GHB a été dosé dans le sang périphérique après précipitation par l'acétonitrile et dérivation par silylation, par chromatographie gazeuse couplée à un spectromètre de masse (Agilent). Les investigations toxicologiques ont été complétées par un criblage exhaustif des médicaments et des stupéfiants par chromatographie liquide couplée à un détecteur à barrettes de diodes (Shimadzu) et par chromatographie liquide couplée à un spectromètre de masse (Waters XEVO TQD). Les Nouvelles Substances Psychoactives (NPS) ont été recherchées dans le sang périphérique par chromatographie liquide couplée à un spectromètre de masse en tandem (Waters XEVO TQD) en mode MRM (multiple

reactions monitoring) en utilisant 2 transitions spécifiques pour chaque composé. Une méthode dédiée à la 4-MEC a été développé par chromatographie liquide couplée à un spectromètre de masse en tandem après extraction liquide-liquide en milieu alcalin.

Résultats

Le dosage de l'éthanol dans le sang périphérique a révélé une alcoolémie à 0,58 g/L, confirmée par un résultat à 0,76 g/L dans les urines. Ces résultats indiquent une consommation d'alcool peu de temps avant le décès. L'analyse des solvants et des substances volatiles dans les urines n'a pas révélée de xénobiotique autre que l'éthanol. Le dosage de la carboxyhémoglobine a donné une valeur de 6,4% qui est comprise dans la limite des valeurs normales. Le GHB sanguin était à 172 mg/L, confirmé par une positivité des urines à 706 mg /L. Ces concentrations, très toxiques, montrent également une consommation de GHB peu de temps avant le décès et en grande quantité [7]. Les concentrations sanguines normales de GHB post-mortem, sans consommation, sont généralement inférieures à 50 mg/L, la plupart du temps inférieures à 10 mg/L [7-8], du fait d'une possible production endogène.

La recherche exhaustive des médicaments inscrits à la Pharmacopée française, des stupéfiants usuels (opiacés dont héroïne, cocaïne, dérivés de l'amphétamines et cannabis), des NPS et des produits de substitution (buprénorphine, méthadone) a permis de caractériser un usage de 4-MEC, un analogue de synthèse de la cathinone. La présence de 4-MEC a été confirmée par la méthode spécifique d'identification (chromatographie liquide couplée à la spectrométrie de masse en tandem) de ce composé. Dans le sang périphérique, la concentration était de 507 ng/mL. Cette concentration est potentiellement létale [9]., mais ne reflète pas la concentration réelle au moment du décès. En effet, comme cela a déjà été décrit pour la méphédrone (4-MMC), la 4-MEC est un composé instable qui se dégrade spontanément dans le temps, même dans des prélèvements conservés au réfrigérateur. La 4-MEC peut devenir indétectable dans le sang total après 14 jours, [10-11]. Ainsi, la concentration mesurée dans cette expertise représente une valeur minimale d'exposition à la 4-MEC, les analyses ayant été réalisées environ 4 semaines après l'autopsie pour des raisons administratives (signature de devis). Comme il est d'usage au laboratoire, le sang cardiaque n'a pas été analysé quantitativement, les phénomènes de redistribution post mortem pouvant gêner l'interprétation finale.

L'analyse des cheveux, de couleur noire et d'une longueur d'environ 1 cm, n'a pas permis de mettre en évidence la 4-MEC. L'absence de 4-MEC dans les cheveux est très en faveur d'une primo-exposition à la molécule dans les semaines précédant le décès, l'analyse des NPS ne posant pas de problème particulier sur le plan analytique.

Discussion

Compte tenu des résultats toxicologiques, des observations lors de l'autopsie (syndrome asphyxique marqué associé à un œdème et une congestion pulmonaire massifs), des produits retrouvés à proximité du corps et du contexte de Chemsex, le médecin légiste a conclu que « le décès de Monsieur X. est d'origine toxique, secondaire à une poly-intoxication associant alcool éthylique, GHB et 4-MEC, vraisemblablement dans un contexte de Chemsex ».

Pour les auteurs, ce cas est intéressant car il souligne une pratique dangereuse, alors même que le phénomène de Chemsex se banalise. Il ne s'agit plus d'une situation marginale, comme en atteste l'augmentation des cas de décès.

Les données de prévalence des pratiques Chemsex demeurent néanmoins faibles. Une série d'enquêtes quantitatives ainsi que des observations de nature plus qualitative issues d'investigations ethnographiques et de services de soins apportent toutefois des éclairages sur l'ampleur actuelle de ces conduites [2]. Le Centre d'addictovigilance de Paris a récemment analysé tous les cas de Chemsex extraits des bases de données NotS, DRAMES et soumission chimique, entre janvier 2008 et août 2018 [12]. Une comparaison des données avant et après l'année 2014 a été réalisée afin d'établir l'évolution de cette pratique. Selon cette étude, 235 cas de Chemsex dont 24 décès (10,21 %) ont été collectés et analysés. Il s'agit exclusivement d'hommes, âgés entre 28 et 50 ans. Une augmentation du nombre de cas a été observée au fil des années (1 cas en 2008, contre 50 au cours des 8 premiers mois de l'année 2017), avec une diffusion à l'ensemble des régions de France et une extension à toutes les tranches d'âge. Sur les 235 dossiers, 345 substances psychoactives ont été renseignées et concernent principalement des dérivés de la cathinone (4-MEC et 3-MMC). Une évolution des produits consommés a été observée au cours de la période d'étude. En 2014, la méphédrone, la naphyrone (NRG-3), la 4-MEC et la méthylènedioxypyrovalerone (MPDV) étaient les substances les plus retrouvées. En ce qui concerne la polyconsommation, cette addition de composés est retrouvée dans 64 % des cas, principalement en association avec le GHB (dans 19 % des cas). La forte augmentation de consommation coïncide avec une augmentation récente du nombre de décès.

En parallèle de cette étude, le Centre d'addictovigilance a également développé un programme d'analyse des produits consommés par les pratiquants de Chemsex. De janvier 2016 à décembre 2017, 66 échantillons ont été collectés, ainsi que 39 questionnaires remplis de façon anonyme. Les échantillons sont principalement des NPS psychostimulants de type cathinones de synthèse (alpha-pyrrolidinopentiophenone (alpha-PVP), 4-fluoro-alpha-pyrrolidinopentiophenone (4F-PVP), 3-MMC, 4-MEC, etc.) et amphétamines de synthèse (2-fluoro-méthamphétamine (2-FMA), méthamphétamine, 3,4-méthylènedioxy méthamphétamine (MDMA), 3-fluorophenmetrazine (3-FPM), 3,4-dichloromethylphenidate (3,4-CTMP) et bk-4-Bromo-2,5-dimethoxyphenethylamine (BK-2CB)). 88% des résultats sont en adéquation avec les substances annoncées dans les questionnaires.

Concernant leurs teneurs, une très grande variabilité a été observée, ce qui tend à augmenter les risques de surdosage. En prenant comme exemple les 18 échantillons de 4-MEC collectés, la pureté médiane est à 70%, avec une pureté minimale à 13 % et une maximale à 100 %. Ceci prouve que les NPS circulant n'ont pas tous la même teneur et donc des effets plus ou moins importants, ce qui rend la consommation très aléatoire.

Une autre étude, menée par le service de pharmacologie et toxicologie du CHU de Garches, a mesuré la proportion de consommation de substances contrôlées dans la population HSH par analyse capillaire, au décours de l'étude ANRS Ipergay [13]. Une mèche de cheveux a été prélevée tous les 4 mois chez 69 volontaires. Quinze NPS ont été identifiés chez 27 sujets, soit 39 %. La 4-MEC a été identifiée 11 fois et arrive en deuxième position par ordre de fréquence. Aucun cas de consommation de NPS seule n'a été observé. Les NPS sont associées à la cocaïne dans 25 cas (93 %), à la MDMA dans 20 cas (74 %) et à la kétamine dans 19 cas sur les 27 cas positifs (70 %).

Toutes ces données permettent d'avoir une vision nationale du phénomène de Chemsex, qui apparait être en plein essor depuis quelques années. Ces études servent d'outil de surveillance et doivent permettre d'alerter les instances de santé publique et d'appuyer les programmes de réduction des risques et de prise en charge [2].

Conclusion

Alors que les produits psychotropes sont utilisés de longue date en contexte sexuel, l'usage des nouvelles substances psychoactives (NPS) par des homosexuels masculins semble se développer ces dernières années. Une augmentation des cas de décès liés au phénomène Chemsex est décrite dans la littérature, essentiellement à l'étranger, mais aussi parfois sur le territoire national, en général sous forme d'études épidémiologiques. Une revue complète de la littérature a été récemment publiée sur ce sujet [14]. Comme le démontre le cas ici décrit, la polyconsommation peut avoir des conséquences dramatiques. Une première et unique consommation de 4-MEC peut être fatale, en association avec du GHB et de l'alcool éthylique. Dans ces cas, l'examen externe et l'autopsie n'apportent pas d'élément spécifique permettant de conclure à la cause exacte du décès. Une demande d'analyse toxicologique complète apparaît alors indispensable afin de l'établir. Les instances de santé publique et les professionnels en contact avec des HSH doivent rester vigilants et développer des programmes de réduction des risques et de prise en charge.

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Article 7: Abuse of 3-MMC and forensic aspects: about 4 cases and review of the literature

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ORIGINAL ARTICLE

Abuse of 3-MMC and forensic aspects: About 4 cases and review of the literature

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Summary In recent years, the recreational use of new psychoactive substances (NPS) has increased all around the world. They are designed to cause similar effects to controlled drugs and are considered as alternative to illegal drugs. Among NPS, derivatives of cathinone, one of the psychoactive ingredients of the plant khat, have emerged in the recreational drug market. 3-methylmethcathinone or 3-MMC is a structural isomer of mephedrone (4-MMC) and a psychostimulant and hallucinogenic drug. Furthermore, to get intense recreational effect, 3-MMC can often be combined with other drugs, by chemsex practitioners. Users report a soft feeling of energy and increased concentration, as well as a feeling of well-being, euphoria and unbridled libido. There is a risk of significant psychological dependence after repetitive 3-MMC exposures. Very limited data about clinical, psychological and forensic aspects of 3-MMC abuse are available in the medical literature. The authors report 4 cases of subjects abusing 3-MMC and other drugs, involving two intoxications and two deaths. Biological samples were analysed by ultra-high performance liquid chromatography system coupled to a tandem mass spectrometry (UPLC-MS/MS™). 3-MMC was detected in concentrations ranging from 3 to 613 ng/mL in blood, and from 14 pg/mg to 17.1 ng/mg in hair. In view of these values, and those from the very limited literature, it is difficult to establish an impairment concentration or a lethal threshold of 3-MMC.
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Introduction

In recent years, the recreational use of new psychoactive substances (NPS), or research chemicals (RC), has increased all around the world. They are designed to cause similar effects to controlled drugs and are considered as alternative to illegal drugs. NPS pose a challenge for forensic toxicologists and physicians (particularly in emergency rooms or in addiction centres) because of the lack of information on these substances. However, due to numerous alarms, both in the medical community but also in the general population, they are considered of great interest for public health authorities. Along with their extensive metabolism and the difficulties to purchase reference material, for toxicologists, the increasing number of NPS represents a major issue in routine casework. Although the main response to this threat is to make the selling and buying of these substances illegal, new compounds are available through the Internet at a regular frequency. Moreover, despite the classification of some substances as illegal, they are still circulating among people interested for new experiences. Since 2010, one new NPS was identified per month in drug seizures in France, while one new NPS was identified per week in Europe [1].

Among NPS, analogues of cathinone, one of the psychoactive ingredients of khat (a plant growing in Yemen and East Africa), have emerged in the recreational drug market [2]. Available for sale on many websites, mephedrone (4-MMC) is probably, among these derivatives, the one that has so far been the most successful and the first cathinone to have emerged. Classified as a controlled drug during spring of 2010 after several death cases, mostly in the UK and Scandinavian countries, this drug has had time, by the craze it has aroused, to create a real phenomenon in the gay party environment. After its classification, another molecule of the family of cathinone is more and more spread among users: 3-methylmethcathinone or 3-MMC is a structural isomer of mephedrone and a psychostimulant and hallucinogenic drug (Fig. 1). 3-MMC is listed as a NPS that made its appearance in 2011, but belongs to the generic cathinone family that has been forbidden since July 2012 (https://ansm.sante.fr/S-informer/Communiques-Communiques-Points-presse/Classement-comme-stupefiants-de-l-ensemble-des-drogues-de-synthese-de-la-famille-des-cathinones-Communique/(language)/fre-FR). 3-MMC was a perfect replacement drug for mephedrone, which was subject to legal control since 2010. This NPS has the appearance of white fine powder or small white crystals with characteristic smell-like liquorice that users may swallow, sniff, drop, smoke or inject [2]. Furthermore, for an intense recreational effect, 3-MMC can be combined with other drugs. In the gay population, this combination takes place at ''chemsex'' parties where cathinones could be associated with gamma-hydroxybutyric acid/gamma-butyrolacone (GHB/GBL), cocaine, erectile dysfunction drugs (sildenafil, tadalafil) and more rarely ketamine and methamphetamine, in order to enhance sexual experiences [3,4].

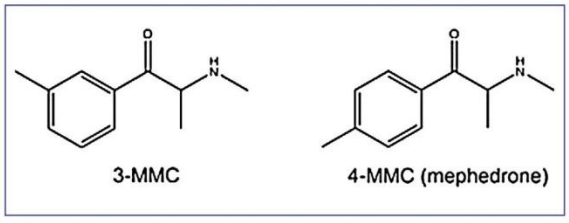

Figure 1. Chemical structure of 3-MMC and 4-MMC, two structural isomers.

According to abusers, the effects of 3-MMC are less potent than those of mephedrone (two to five times less powerful, depending on users). Its effects can be compared to those of MDMA and amphetamines, but with less intensity. This molecule is stimulating (on a physical and psychological level), euphoric and empathogenic. Unlike mephedrone and MDMA, 3-MMC does not mark the body too much: no dilated pupils and no tight jaws. But of course, if a greater amount is consumed, some marks can appear to the user. Several forums describe the different ways to consume 3-MMC, as well as the duration of the effects. There is no controlled study available in the medical literature. The oral route (by parachuting) is the most commonly used route of administration [5,6]. Doses vary between 100 and 150 mg, depending on the users. The first effects appear between 15 minutes and 1 hour and dissipate after 4 to 6 hours. This mode of consumption is preferred because it is not painful, compared to the nasal route. When the 3-MMC is snorted, the first effects are felt after about ten minutes (against two to four minutes for mephedrone). Users indicate that the first line is very painful (burning nose and flowing, very unpleasant sensation in the throat). The recommended dose is between 20 and 80 mg, depending on the tolerance and the first effects appear between 10 and 15 min, and then dissipate after 2 hours. When sniffed, the effects last less than after oral administration. The effects dissipate after two hours when one to two lines have been consumed. In parachuting [7], the effects appear between 15 min and 1 hour after the ''drop'' and can be felt until 4 to 6 hours after the consumption. Users report a soft feeling of energy and increased concentration, as well as a feeling of well-being, euphoria and unbridled libido. The effect would be constant and clean, far from the roller coaster sensations that cocaine, MDPV or ethylphenidate can produce [8].

About clinical effects, some reports are available in the literature. The most frequently reported problems were agitation, seizures, hallucination, hypertension, tachycardia, hyperthermia, bradycardia, depression and concentrations difficulties [9–11]. Most patients need hospital care for only one day but some for longer.

There is a risk of significant psychological dependence after repetitive 3-MMC exposures [12]. This stimulant, acting over a short period of time, adapts particularly well to all social situations: first in the evening with friends and then in after party, to finish at work or in class, giving the illusion of being able to manage all usual activities. The doses are quickly increased, and users try to reduce hiding the exhaustion or a difficult descent. In the short term, the risks are the same as ecstasy or cocaine. Strong side-effects are increasing with duration and dosage: loss of appetite, lack of sleep, increased heart rate and blood pressure, hyperthermia, bruxism (clenching of the jaws), palpitations, and brain zaps (sensations of electric shock at the level of the head). In the long term, 3-MMC is strongly suspected of being toxic to the nervous, cardiac (risk of heart attack) and

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vascular systems. It is recommended for people with heart problems or hypertension to avoid consuming cathinones [13,14]. Psychological problems and paranoid delusions can occur especially in case of repeated consumption. Chemsex practitioners use 3-MMC associated with GHB/GBL for its intoxicating effects, such as euphoria, reduced inhibitions, sedations and muscle relaxation [15—17]. In the present paper, the authors describe 4 cases of 3-MMC consumption resulting in two behavioural alterations and two deaths.

Cases histories

Case 1: A 33 year-old man was found wandering the street after killing his wife and his 2 years old daughter in an outburst of violence, 24 hours earlier. A blood specimen was immediately collected and chest hair was collected one month later.

Case 2: A 38 year-old man was found deceased in his home. There was no trace of violence or traumatic lesions on the body. An autopsy was performed and femoral blood, urine and hair specimens were collected.

Case 3: A 31 year-old man was found unconscious. When the emergences arrived at the scene, he presented a partial paralysis of his body and a coronary syndrome (obstruction of one or more coronary arteries). The man was known for chemsex practices. A bag of powder, annotated 3-MMC, was found in his jeans pockets. A blood specimen was collected during the medical examination.

Case 4: A 50 year-old man was found hanged in a swapping nightclub. He was known for chemsex practices. A medicolegal autopsy was requested. Cardiac blood and urine were collected during body examination.

Blood and urine were kept at 4 °C until analysis. Hair specimens were stored at room temperature.

Materials and methods

Chemical

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99—100%, AnalaR NORMAPUR and 36% hydrochloric acid were purchased from VWR Prolabo (*Fontenay-sous-Bois, France*). HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany).

3-MMC was purchased from LGC Standards (Molsheim, France), at concentration at 1 mg/mL. MDMA-d5 was purchased from LipoMed (Arlersheim, Switzerland). Drugs were diluted to appropriate concentrations using acetonitrile and methanol, according to the manufacturer's recommendations.

Toxicological screening tests

A drug of abuse screen by enzyme-linked immunosorbent assay (Elisa — Alere) was performed on blood. Ethanol was determined in blood and urine samples using headspace gas chromatography with flame ionization detection (HS-GC-FID). A volatile screening was realized with headspace gas chromatography-mass spectrometry (HS-GC-MS). Drugs screening was performed on blood with high performance liquid chromatography-diode array detection (HPLC-DAD) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS™).

3-MMC analysis

A specific method to detect 3-MMC was developed on an Acquity class I ultra-high performance liquid chromatography coupled to a Xevo TQD tandem mass spectrometer (UPLC-MS/MS™) from Waters (Milford, MA, USA). 3-MMC was extracted from 1 mL of blood in presence of 2 ng of MDMA-d5 used as internal standard, with 1 mL borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5). After 15 minutes of agitation at room temperature, centrifugation (3000 rpm for 15 min) and evaporation to dryness at 45 °C, the residue was reconstituted in 50 μL of 5 mm ammonium formate buffer adjusted at pH 3.

Hair stand was decontaminated twice with dichloromethane and cut into small pieces. Fifty milligrams were incubated overnight at 50 °C in 1 mL borate buffer pH 9.5, in presence of MDMA-d5. 3-MMC was extracted by 5 mL of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5). The rest of the extraction is the same as for the blood.

Chromatography was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained at 50 °C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 87% phase A and the final gradient, at 15 min, was 5% phase A. An injection volume of 10 μL was used in all cases. A Xevo TQD triple quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis of 3-MMC and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149 °C; desolvation gas nitrogen heated at 600 °C and delivered at a flow rate of 1000L/h. In order to establish appropriate multiple reactions monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociated of both species was performed. Cone voltage and collision energy were adjusted to optimize the signal for the 2 most abundant product ions of 3-MMC: m/z 178.1 > 143.9 (32 V and 28 eV, quantification) and 178.1 > 91.1 (32 V and 20 eV, qualification), and the most abundant product ion of internal standard m/z: 198.9 > 164.9 (22 V and 14 eV). MassLynx 4.1 software was used for quantification.

Results and discussion

Linearity in blood was observed for 3-MMC concentrations ranging from 10 to 1000 ng/mL, with a correlation coefficient of 0.996. QC samples (10 ng/mL and 500 ng/mL), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of

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Table 1 Results of 3-MMC in blood samples, and other molecules detected.

Case	3-MMC (ng/mL)	Other molecules (ng/mL)
1	3	THC = 0.5; THC-COOH = 14
2	613	Amphetamine = 938; GHB = 154 mg/L
3	392	4-MEC = 4.5; GHB = 234 mg/L; Nordiazepam = 199; Oxazepam = 26; Bromazepam = 149
4	462	Methoxamine = 70; Nordiazepam < 10; Bromazepam < 10

Table 2 Results of 3-MMC in hair samples, and other molecules detected.

Case	3-MMC (pg/mg)	Other molecules (pg/mg)
1	14	Methoxamine = 260; Ethylphenidate = 41; THC= 2229; Cocaine = 193; Pholcodine = 239; Zopiclone = 416; Hydroxyzine = 239
2	17100	Amphetamine = 14800

variation for precision and accuracy. These CVs were lower than 20%. The limit of detection and the lower limit of quantification are 0.1 and 10 ng/mL respectively. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood. The matrix effect (< 20%) was investigated with spiked 3-MMC at 100 ng/mg in twenty blank blood samples.

In hair, linearity was observed for concentrations ranging from 5 to 5000 pg/mg, with a correlation coefficient of 0.989. QC samples (20 pg/mg and 1000 pg/mg), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision and accuracy. These CVs were lower than 20%. The limit of detection and the lower limit of quantification are 1 and 5 pg/mg respectively. When needed, the extracts were diluted to meet the criteria of linearity.

Discrimination between 3-MMC and 4-MMC (mephedrone) is not possible using liquid chromatography, as both drugs are eluted at the same retention time and produce the same ion transitions. The use of gas chromatography is necessary to separate the 2 isomers, as after derivatization they have different retention times [18,19]. This was verified in the tested biological specimens.

The screening of blood during routine lab testing revealed a negative result for drugs of abuse with immunosorbent assay Elisa, except for case 1 where cannabis was detected and for case 2 where amphetamine was detected. This demonstrates that 3-MMC is not detectable using standard amphetamines/methamphetamines immunoassay kits. No ethanol was detected. The screening for volatile substances was negative. All 4 cases were positive for 3-MMC by UPLC-MS/MS™. The results are presented in Table 1 (blood) and Table 2 (hair).

3-MMC was detected in concentrations ranging from 3 to 613 ng/mL in blood. In cases 2 and 4, concentrations of 3-MMC in the urine were also determined, at 19.7 and above 10 mg/L, respectively. According to the literature, 3-MMC is not stable in time. One stability study indicated that over a period of two weeks of storage more than 50, 70 and even 95% losses were observed at −20 °C, 4 °C and room temperature, respectively [19]. Therefore, one can anticipate that the measured concentrations in blood obtained from the 4 cases where much lower than those corresponding at the time of collection or death. Careful interpretation is needed when interpreting 3-MMC blood concentrations as this can result in under-estimating the authentic initial concentration. Few cases of 3-MMC overdoses have been described in the literature. These cases, as well as the measured concentrations, are presented in Table 3. The metabolism of NPS is still under investigation and up to now very little is known about drug disposition in humans. Regarding 3-MMC, only one study [22] describes the detection of 3-MMC and its two main metabolites: 3-methylepehdrine and 3-methylnorephedrine (Fig. 2). However, these metabolites were not investigated in this study. Cases reporting concentrations in the hair are presented in Table 4.

In view of these concentrations, it is difficult to define a concentration, which means impairment or a fatality threshold. There is no correlation between the measured concentration and the type of intoxication (fatal or not). This is probably due to the chemical instability of the drug, as well as the simultaneous abuse of other substances and their concentrations. The authors recommend achieving the analytical tests as soon as possible after blood collection or autopsy.

3-MMC is rarely consumed alone, and often combined with other psychoactive substances. For ten years, in France as in other countries, new practices of drug consumption and potentially harmful for men having sex with men, have been noted. Chemsex (or slam, in the case of injection of stimulants in sexual context) mark a renewal in the quest for carnal pleasures. At the same time, they expose their partners to sexual or drug-related risks. This is a real public health concern because abuse may involve subjects without knowledge or experience of drug consumption. NPS are products with poorly known effects. Clinical reports are often scares and not documented with toxicological analyses.

It is common to find a simultaneous consumption of several substances associated to 3-MMC. This was verified by the results of the hair tests. This poly-consumption is intended to obtain a synergistic, complementary effect or even a drug enhancement. In the study conducted by

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Table 3 Table of 3-MMC cases from the literature, blood concentrations.

References	Cases	Alive or deceased	Concentrations
[10]	Retrospective measured of 50 suspected use of NPS presenting to hospitals in Sweden, between 2012 and 2014	Alive	2—1490 ng/mL
[18]	A 26 year-old female who was stopped by the police because of her conspicuous way of driving	Alive	39.9 ng/mL
[20]	Retrospective analyses of 95 cases between 2013 and 2015	Alive and Deceased	DUID cases: 1—171 ng/mL Traffic accidents: < 1—29 ng/mL Drug possession: 2—408 ng/mL Intoxication: < 1—1600 ng/mL Other: < 1—61 ng/mL
[11]	Man who consumed 500 mg of 3-MMC and other substances (5-APB and ethanol). He did not recover and died 4 h after the use of drugs	Deceased	1600 ng/mL
[19]	69 year-old man, practicing chemsex	Deceased	330 ng/mL (femoral blood)
[21]	32 year-old man, found dead at home after sniffing 1 or 2 lines of a white powder	Deceased	249 ng/mL (femoral blood)

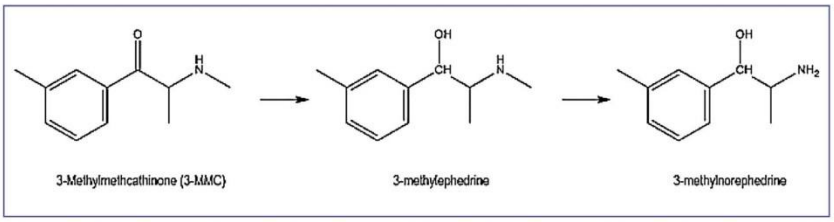

Figure 2. Chemical structures of 3-MMC, 3-methylephedrine and 3-methylnorephedrine.

Table 4 Table of 3-MMC cases from the literature, hair or pubic hair concentrations.

References	Cases	Alive or deceased	Concentrations
[22]	Man, charged with dealing NPS-containing materials	Alive	25.8 ng/mg in pubic hair
[19]	A 69 year-old man, fell into cardiopulmonary arrest, during a party	Deceased	206.7 ng/mg in hair

Daskalopoulou et al. [23] involving chemsex, 32% of the subjects abused a single substance (poppers: 38%, cannabis: 36%, and cocaine: 12%), 21% used 2 substances and among those who used 3 or more substances (47%), there was a relatively higher consumption of mephedrone, 3-MMC, GHB/GBL, MDMA, methamphetamine and ketamine. The results of Sewell et al. [24] confirm this aspect with a higher proportion of significant consumption of mephedrone,

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3-MMC, Crystal meth and GHB in combination. Consumption associated with other substances (and more particularly benzodiazepines, hypnotics or cannabis) is also observed to get sedation and sleeping time or to accompany the descent phase which includes agitation and anxiety disorders. This poly-consumption is consistent with the 4 forensic case results since 3-MMC was never consumed alone.

Conclusion

The 4 cases presented in this paper illustrate the consumption of 3-MMC, in addition to other illicit substances. A toxicological analysis is the only way to highlight the consumption of substances. This is an interesting supplement for the interpretation of physicians. The screening of NPS becomes essential in a toxicology laboratory because it allows to determine the causes of intoxication or death in an increasing number of cases. The use of liquid chromatography is the most suitable analytical tool but does not discriminate isomers (like 3-MMC and 4-MMC), which requires the use of an alternative instrument, i.e. gas chromatography. From a legal point of view, the consumption of new psychoactive substances makes it possible to circumvent drugs laws and conventional screening tests. This is the reason why 3-MMC (not prohibited until 2012) quickly took the place of 4-MMC (prohibited since 2010) on the market of NPS.

More and more often described in the literature, ''chemsex'' is a recent phenomenon, concerning men who have sex with men, associated with poly drug consumption, in order to enhance sexual experiences. This trend is very dangerous since it may involve people without knowledge or experience of drug consumption and may have dramatic consequences. At this time, nothing is known about psychological disorders after long-term 3-MMC abuses.

Disclosure of interest

The authors declare that they have no competing interest.

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Article 8: Determination of a threshold fatal 3-MMC concentration in human: mission impossible

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COMMENTARY

Determination of a threshold fatal 3-MMC concentration in human: mission impossible

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In recent years, the recreational use of new psychoactive substances (NPS) has increased all around the world. They are designed to cause similar effects to controlled drugs and many were developed as "legal" alternatives. NPS pose a challenge for forensic toxicologists and physicians, particularly in emergency rooms or in addiction centers. Although the main response to this threat is to make the selling and buying of these substances illegal, new compounds are available illicitly at a regular frequency. Since 2010, one new NPS was identified per month in drug seizures in France, while one new NPS was identified per week in Europe (Lahaie et al. 2013).

Among NPS, derivatives of cathinone, one of the psychoactive ingredients of khat (*Catha edulis*) have emerged in the recreational drug market (Daveluy et al. 2017; Zaami et al. 2018). Available for sale on many websites, mephedrone (4-MMC) is probably, among these derivatives, the one that has so far been the most popular among illicit users. Another molecule of the family of NPS is more and more widespread among users: 3-methylmethcathinone or 3-MMC is a structural isomer of mephedrone and a psychostimulant and hallucinogenic drug. 3-MMC made its appearance in 2011 but belongs to the generic cathinone family that has been forbidden since July 2012. It was a perfect replacement drug for mephedrone, which was subject to legal control since 2010. This NPS has the appearance of white fine powder or small white crystals with characteristic smell-like liquorice that users may sniff, drop, smoke, or inject (Daveluy et al. 2017). Furthermore, for an intense recreational effect, 3-MMC can be combined with other drugs. In the gay population, this combination takes place at "chemsex" (recent phenomen concerning men who have sex with men) parties where cathinones could be associated with methamphetamine, sildenafil, gamma-butyrolactone/gamma-hydroxybutyric acid or cocaine, in order to enhance sexual experiences (Monteil-Ganiere et al. 2018). The effects of 3-MMC are less potent than those of mephedrone (two to five times less powerful, depending on users). Its effects can be compared to those of MDMA and amphetamines, but with less intensity. This molecule is stimulating on a physical and psychological level, euphoric and empathogenic. The oral route is the most commonly used route of administration. Doses vary between 100 and 150 mg, depending on the users. The first effects appear between 15 min and 1 h, and dissipate after 4 to 6 h. This mode of consumption is preferred because it is not painful, compared to the nasal route. Users report a soft feeling of energy and increased concentration, as well as a feeling of well-being, euphoria and unbridled libido. The effect would be constant and clean, far from the roller coaster sensations that cocaine, MDPV or ethylphenidate can provide according to user reports (PsychoActif website 2014).

Few reports of clinical effects of overdose are available in the literature. The most frequently reported problems were agitation, seizures, hallucination, hypertension, tachycardia, hyperthermia, bradycardia, depression, and concentration difficulties (Sande 2016; Bäckberg et al. 2015; Adamowicz et al. 2014; Pichini et al. 2014). There is a risk of significant psychological dependence after repetitive 3-MMC exposures. The doses are quickly increased and users try to reduce hiding the exhaustion or a difficult down. In the short term, the risks are the same as methylenedioxymethamphetamine or cocaine. Strong side-effects are increasing with duration and dosage: loss of appetite, lack of sleep, increased heart rate and blood pressure, hyperthermia, bruxism (clenching of the jaws), palpitations, and brain zaps (sensations of electric shock at the level of the head). In the long term, 3-MMC is strongly suspected of being toxic to the nervous, cardiac (risk of heart attack) and vascular systems. Psychological problems and

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paranoid delusions can occur especially in case of repeated consumption. Ravers and club attendees use 3-MMC associated with GHB for its intoxicating effects, such as euphoria, reduced inhibitions, sedations, and muscle relaxation (Karila and Reynaud 2011; Kelly 2011). In the present paper, the authors describe 2 fatal cases of 3-MMC consumption observed between 2016 and 2017.

In this period, about 1000 post-mortem cases were investigated, where about 25% were toxic deaths. In case 1, a 38-year-old man was found deceased in his home with no trace of violence or traumatic lesions on the body (Strasbourg, France). An autopsy was performed and femoral blood was collected. Concerning case 2, a 50-year-old man, known for chemsex practices, was found hanged in a swinger's nightclub (Bordeaux, France). A medico-legal autopsy was requested. Cardiac blood was collected during body examination. All blood samples were kept at 4 °C until analysis. In both cases, autopsy was performed within 48 h after death notice.

A drug of abuse screen by enzyme-linked immunosorbent assay (ELISA) was performed on blood. Ethanol was determined using headspace gas chromatography with flame ionization detection (HS-GC-FID). A volatile screen was realized with headspace gas chromatography-mass spectrometry (HS-GC-MS). Drug screening was performed with high-performance liquid chromatography-diode array detection (HPLC-DAD) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). Chromatography was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained at 50 °C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B), with a flow rate at 0.4 mL/min. A Xevo TQD triple quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ES+). Cone voltage and collision energy were adjusted to optimize the signal for the 2 most abundant product ions of 3-MMC: m/z 178.1 > 143.9 (32 V and 28 eV) and 178.1 > 91.1 (32 V and 20 eV), and the most abundant product ion of internal standard m/z: 198.9 > 164.9 (22 V and 14 eV). MassLynx 4.1 software was used for quantification. Linearity was observed for 3-MMC concentrations ranging from 10 to 1000 ng/mL, with a correlation of coefficient of 0.996. QC samples (10 ng/mL and 500 ng/mL), analyzed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision and accuracy. These CVs were lower than 20%. The limit of detection and the lower limit of quantification are 0.1 and 10 ng/mL respectively. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood. The matrix effect (< 20%) was investigated with spiked 3-MMC at 100 ng/mg in 20 blank blood samples.

Discrimination between 3-MMC and 4-MMC (mephedrone) is not possible using liquid chromatography, as both drugs are eluted at the same retention time and produce the same ion transitions. The use of gas chromatography (GC) is necessary to separate the 2 isomers, as after derivatization they have different retention times (Maas et al. 2017; Jamey et al. 2016). In both cases, 3-MMC was confirmed by GC.

The screening of blood during routine lab testing revealed a negative result for drugs of abuse with immunosorbent assay ELISA. This demonstrates that 3-MMC is not detectable using standard amphetamines / methamphetamines immunoassay kits. No ethanol was detected and the screening for volatile substances was negative. The 2 cases tested positive for 3-MMC by UPLC-MS/MS at 613 and 462 ng/mL, respectively. Few cases of 3-MMC overdoses have been described in the literature with concentrations ranging between 249 and 1600 ng/mL (Adamowicz et al. 2014, 2016; Jamey et al. 2016; Bottinelli et al. 2017). In view of these concentrations, it is difficult to define a concentration which means impairment or a fatality threshold. There is no correlation between the measured concentration and the type of intoxication (fatal or not). This is probably due to the chemical instability of the drug. Jamey et al. (2016) published a stability study that indicated that over a period of 2 weeks of storage more than 50, 70, and even 95% loss of the drug was observed at − 20 °C, 4 °C, and room temperature, respectively. Therefore, one can anticipate that the measured concentrations in blood obtained from the 2 cases where much lower than those corresponding at the time of collection and of course time of death. Careful interpretation is therefore needed when interpreting 3-MMC blood concentrations as this can result in underestimating the authentic initial concentration.

According to the literature, it appears that 3-MMC is rarely consumed alone that are often combined with other psychoactive substances. For 10 years, in France as in other countries, new practices of drug consumption and potentially harmful for men having sex with men have been noted. Designated under the term "chemsex" or "slam" if it is the injection of stimulants in sexual context, these behaviors mark a renewal in the quest for carnal pleasures. At the same time, they expose their followers to sexual or drug-related risks. This is a real public health concern because abuse may involve subjects without knowledge or experience of drug consumption.

The 2 cases presented in this paper illustrate the consumption of 3-MMC causing death. In view of determined concentrations, it is difficult to define a concentration which means impairment or a fatality threshold. This can be explained by the chemical instability of 3-MMC, the significant lack of literature, and of course the lack of controlled study involving its administration to humans.

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Article 9: Identification and analytical characterization of seven NPS, by combination of ^{1}H-NMR spectroscopy, GC-MS and UPLC-MS/MS®, to resolve a complex toxicological fatal case.

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Identification and analytical characterization of seven NPS, by combination of ^{1}H NMR spectroscopy, GC–MS and UPLC–MS/MS®, to resolve a complex toxicological fatal case

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ABSTRACT

The authors report the case of a 41-year-old man known as a polydrug addict and found dead at home. The initial toxicological screening was inconclusive for a toxic death. However, 7 small bags of unknown powders and crystals, supposed to be NPS, were found near the body and sent to the laboratory 3 weeks later. This article focuses on the interest of nuclear magnetic resonance (NMR) spectroscopy to solve this fatal case. The 7 items were identified by ^{1}H NMR and their purities were measured by the quantitative NMR (qNMR) method using maleic acid as internal standard. The compounds were 4-chloro-α-pyrrolidinopentiophenone, dibutylone, 4-chloroethcathinone, 4-α-pyrrolidinohexuiphenone, 3,4-methylenedioxy-*N*-benzylcathinone, 3-methoxy-phencyclidine and α-pyrrolidinopropiophenone, with purities in the range 51–89%. In the biological specimens of the victim, only 3-methoxy-phencyclidine (3-MeO-PCP) was identified in cardiac blood, peripheral blood and urine at 743, 489 and 16,700 ng/mL. Hair (6 cm) concentration of the drug was 15,600 pg/mg. The other NPS were not detected. The peripheral blood concentration was higher than the values reported in previous intoxicated and fatal cases. The toxicological significance of the measured concentration in hair was difficult to establish because this is only the second case describing a test result for 3-MeO-PCP.
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1. Introduction

For several years, new synthetic drugs appeared on the Internet and constitute a wide group named New Psychoactive Substances (NPS), which designate a large range of molecules having the same pharmacological effects as various legal or illegal substances such as ecstasy, amphetamines, benzodiazepines, opiates, or cannabis. Their chemical structures can be close to the original compound, but can be also very far from it. Some of these substances can circumvent the narcotic laws because they are not listed as controlled drugs. Generally sold on the internet, NPS are differentiated by their chemical names, abbreviations or more general names, such as "bath salts", "plant for nutrients", "incense" ... According to a survey of the French Observatory Drugs and Drug Addiction [1], more than 382 substances circulated freely in the European Union between 2008 and 2015, including 176 in France.

According to the United Nations, since 2010, there has been a marked increase in the new types of NPS that have been detected [2]. For example, 166 substances were reported in 2009, while more than 670 are available in 2018 [2]. In fact, their widespread availability through the internet made the issue of NPS a global phenomenon with evidence that the majority of countries that reported the emergence of NPS were from Europe, followed by the Americas, and Oceania [3,4].

The National Agency for the Safety of Medicines and Health Products (ANSM) in France has carried out a study which collects, from forensic experts, cases of deaths related to an overdose involving psychoactive substances [5]. The objective of this study was to collect a compendium of deaths between 2012 and 2016. It appears that NPS were responsible for 36 deaths in France, with an increasing annual prevalence. From the 36 cases, 15 involved

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Fig. 1. Pictures of the 7 small bags of powders and crystals. They are all annotated, as "A-PVP", "ethyl", "4-MCC", "4-MPH", "MPEP", "MeO-PCP" and "X".

only one NPS (mostly synthetic opioids, methoxethamine, 5-APB and 5-MAPB), 13 involved a combination of several NPS, and in 12 cases NPS were associated with other psychoactive substances, such as cocaine, heroin or benzodiazepines. These NPS included cathinones (3-methylmethcathinone, 4-methylethcathinone, butylone, MDPV, mephedrone, methylone, mexedrone, pentedrone and PVP), benzofurans (5-APB, 5-APBD and 5-MAPB), arylcyclohexylamines (methoxetamine and methoxphenidine), designer benzodiazepines (diclazepam and deschloroetizolam), NBOMe (25C-NBOMe), synthetic opioids (ocfentanil), piperazines (ethylphenidate) and others (3F-phenmetrazine and methiopropamine). The number of cases involving at least one NPS has been increasing sharply since 2015, with the appearance of new products every year, including a fentanyl analogue in 2016 (i.e. furanylfentanyl). Data from the last 2 years in France indicate a prevalence of NPS-related toxic deaths of about 3% [5].

The French Society of Analytical Toxicology (SFTA) has published recommendations for carrying out toxicological analyses in a case of death involving NPS [6]. The purpose of these recommendations was to propose a reasonable approach in the implementation of an analytic strategy for determining the role of NPS. Several points were established, such as the minimum list of NPS to test for, the biological specimens to analyse, the possible metabolites to test for and some data about the stability of several analytes.

The city of Strasbourg, in which this case occurred, is particular because it has a close border with Germany and is not far from Luxembourg, Belgium and Switzerland. The NPS consumed in these countries are probably not the same as those consumed in France and many French abusers provide themselves with NPS on the other side of the borders. It is therefore very difficult to keep a data library of MS/MS spectra up to date. For example, flubromazolam has been observed in Germany, but to the best of our knowledge not in France [7]. In addition, it is difficult to obtain and purchase the reference standards of all NPS. Some are excessively expensive, and some do not even exist in the catalogue of referenced suppliers. Toxicology laboratories are therefore limited and must adapt to the material they can purchase and try to achieve legal exchanges between colleagues.

In addition to the difficulties associated with molecule characterization (identification, quantification, purity), analytical methods used routinely are not always sufficient to firmly identify NPS. For example, liquid chromatography, which is the most widely separative method used, may not discriminate positional isomers using standard LC protocols, such as mephedrone (4-MMC) and 3-methylmethcathinone (3-MMC), which are NPS very commonly abused. In LC–MS, their retention times may be identical as well as their fragmentation pattern. It is therefore necessary to use another method, such as gas chromatography (GC) [8,9] or nuclear magnetic resonance (NMR) spectroscopy to discriminate the species. NMR is a physical phenomenon in which spin bearing nuclei (most commonly ^{1}H, ^{13}C, ^{19}F, ^{31}P), placed in a strong magnetic field, may absorb and re-emit electromagnetic radiation. This method not only makes it possible to definitively identify a compound, but also to confirm the absence of other organic compounds and to determine the purity of a product [10,11].

This article focuses on the simultaneous use of ^{1}H NMR spectroscopy, GC–MS and UPLC–MS/MS® to solve a complex toxicological fatal case involving unknown small bags of powders and crystals, supposed to be NPS (Fig. 1).

2. Case history

A 41 year-old white man (1.72 m, 78 kg) was found dead at his home. His body was lying on the floor with several traces of needle marks and a large amount of dried blood around his mouth. There was no trace of violence or traumatic lesions on the body that could explain his death. Several small bags of powders and crystals were found near the body, as well as boxes of pharmaceuticals, containing oxazepam, methadone and tramadol. The man was known for psychiatric troubles and polydrug addiction. Only an external body examination was performed where cardiac blood, peripheral (femoral) blood, urine and hair (brown, 6 cm, in bulk) specimens were collected and stored at +4 °C until analysis (performed 6 weeks later), except hair, kept at room temperature. This delay was due to administrative reason with the Judge in charge of the case. A certain level of uncertainty may be associated with concentration determination, given the unknown stability of the various drugs.

3. Materials and methods

3.1. Chemical

Ammonium formate (99%) was provided by Alfa Aesar (Schiltigheim, France). Formic acid (99–100%), AnalaR NORMAPUR and hydrochloric acid (36%) were purchased from VWR Prolabo (Fontenay-sous-Bois, France). HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany).

MDMA-d_5 was purchased from Lipomed (Arlersheim, Switzerland) and was diluted to appropriate concentrations using methanol.

Maleic acid (99.98%) was provide by Merck (Darmstadt) as TraceCert® quality for quantitative NMR. Deuterated acetonitrile (ACN-d_3), deuterated methanol (MeOH-d_4), deuterated water (D_2O) and deuterated dimethylsulfoxide (DMSO-d_6) were purchased from Eurisotop (Saarbrücken).

3.2. Nuclear magnetic resonance analysis

5–10 mg of each powder was completely dissolved in deuterated acetonitrile and deuterated methanol (because of possible low solubility of compounds in acetonitrile). Several spectra were recorded: ^{1}H, $^{13}C\{^{1}H\}$, ^{13}C DEPT135, ^{1}H-^{1}H *g*COSY, edited ^{1}H-^{13}C *g*HSQC and ^{1}H-^{13}C *g*HMBC. In case of fluorinated compound, ^{19}F and $^{19}F\{^{1}H\}$ NMR spectra were also recorded. Based

on the combined analysis of these 1D- and 2D-data and the molecular mass and fragmentation patterns found by LC–MS full scan, a structure was established by the operator. This approach has already been proposed in the literature for other NPS [12,13].

In order to confirm the absence of other organic compounds such as synthetic impurities and to evaluate the purity of each sample, a stock solution of defined concentration (5.0 mg/mL) of maleic acid (Certified Reference Material) in either D_2O or DMSO-d_6 was prepared and used to prepare a sample of known analyte concentration and a proton spectrum was recorded (~10 mg/mL). The sample purity was defined by the following equation:

$$P_{sample} = \frac{I_{analyte}}{I_{CRM}} \times \frac{N_{CRM}}{N_{analyte}} \times \frac{M_{analyte}}{M_{CRM}} \times \frac{m_{CRM}}{m_{sample}} \times P_{CRM}$$

with:

P_{sample}: purity of the sample as mass fraction (%)
P_{CRM}: purity of the CRM as mass fraction (%)
$I_{analyte}$: integral of the analyte signal
I_{CRM}: integral of the CRM signal
$N_{analyte}$: number of analyte protons
N_{CRM}: number of CRM protons
$M_{analyte}$: molecular mass of the analyte (g/mol)
M_{CRM}: molecular mass of the CRM (g/mol)
m_{sample}: mass of sample (mg)
m_{CRM}: mass of CRM (mg)

The ^{1}H NMRspectra were recorded on AVANCE 400 (Bruker Avance III, France) operating at a proton Larmor frequency of 400 MHz equipped with a 5 mm double resonance broad band probe head (BBO + F) probe at 295 K. For quantitative NMR, the ^{1}H spectra were recorded with 16 scans, a 64-K time-domain data points with 8012 Hz spectral width, an acquisition time of 3409 s, a relaxation delay of 45 s and a flip angle of 90°. The Free Induction Decays (FIDs) were Fourier transformed (0.3 Hz broadening) and the phase and the baseline were corrected. In these measurement conditions, the value of the peak area integrals in the spectrum are directly proportional to the number of corresponding spins (i.e. nuclei) for each compound. This gives direct information about the respective concentration of protons for maleic acid and the compound of interest's sample purity and, using the above equation, direct access to sample purity, independently of the eventual presence of non-organic components [14]. The analyses of the spectra were performed using TopSpin 3.5 pl7.

3.3. Toxicological screening tests

Ethanol was tested by head space GC/FID on a Perkin Elmer system (TurboMatrix 40 & Clarus 580) using a standard validated procedure. Volatiles were determined by head space GC/MS on a Thermo system (Focus GC & DSQII). ELISA tests were achieved using Neogen® Microplate kits using the recommendations of the manufacturer. Pharmaceuticals and drugs of abuse (including NPS) screenings [15] were performed with ultra-performance liquid chromatography–diode array detection (Waters UPLC–PDA®) and ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS®, Waters). Methadone and EDDP were tested with a specific method by UPLC-MS/MS® system, according to previous applications [16,17].

To our specific NPS method, containing about 100 compounds, and particularly those recommended by SFTA [6], the new molecules, identified by ^{1}H NMR, were added. NPS were extracted from 1 mL of blood, or urine, in presence of 200 ng of MDMA-d_5 used as internal standard, with 1 mL borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5). After 15 min of agitation at room temperature, centrifugation (3000 rpm for 15 min) and evaporation to dryness at 45 °C, the residue was reconstituted in 50 μL of 5 mM ammonium formate buffer adjusted at pH 3. The whole hair strand (6 cm) was decontaminated twice with dichloromethane and cut into small pieces of less than 1 mm. Twenty milligrams were incubated overnight at 50 °C in 1 mL borate buffer pH 9.5, in presence of 2 ng of MDMA-d_5. NPS were extracted by 5 mL of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5).

Liquid chromatography was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained at 50 °C in a thermostatically controlled oven. A linear gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 13% organic phase B and the final gradient, at 15 min, was 95% organic phase B. An injection volume of 10 μL was used in all cases. A Xevo TQD triple quadrupole mass spectrometer was used for the detection. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis of NPS and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149 °C; desolvation gas nitrogen heated at 600 °C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple reaction monitoring conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation was performed. Cone voltage and collision energy were adjusted to optimize the signal for each compound. For the internal standard, the following transition was used: m/z 198.9 > 164.9 (22 V and 14 eV). MassLynx 4.1 software was used for quantification.

3.4. Validation method of 3-MeO-PCP

A validation was achieved for 3-MeO-PCP testing in blood and hair, by UPLC–MS/MS®, based on a previous application [18]. Linearity was observed in blood for 3-MeO-PCP concentrations ranging from 10 to 1000 ng/mL, with a correlation coefficient of 0.998. QC samples (50 ng/mL and 200 ng/mL), analysed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision and accuracy. These CVs were lower than 20%. Linearity was observed in hair for 3-MeO-PCP concentrations ranging from 10 to 20,000 pg/mg, with a correlation coefficient of 0.998. QC samples (50 pg/mg and 200 pg/mg), analysed in duplicate in six independent experimental assays, were used for determination of a coefficient of variation for precision and accuracy. These CVs were lower than 20%. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood or in hair. For both blood and hair, the matrix effects tested with 15 blank specimens was less than 20%. Recovery of 3-MeO-PCP in the two specimens was higher than 70%. Urine was tested after an appropriate dilution with water to fit to the blood calibration range.

Table 1
Identification of 7 powders, by ^{1}H NMR and purity.

Powder	Identification by ^{1}H NMR	Purity (%)
1	4-Chloro-alpha-pyrrolidinopentiophenone	81.2
2	Dibutylone	59.6
3	4-Chloroethcathinone	82.0
4	4-Alpha-pyrrolidinohexuiphenone	87.7
5	3,4-Methylenedioxy-*N*-benzylcathinone	50.8
6	3-Methoxy-phencyclidine	72.9
7	Alpha-pyrrolidinopropiophenone	89.5

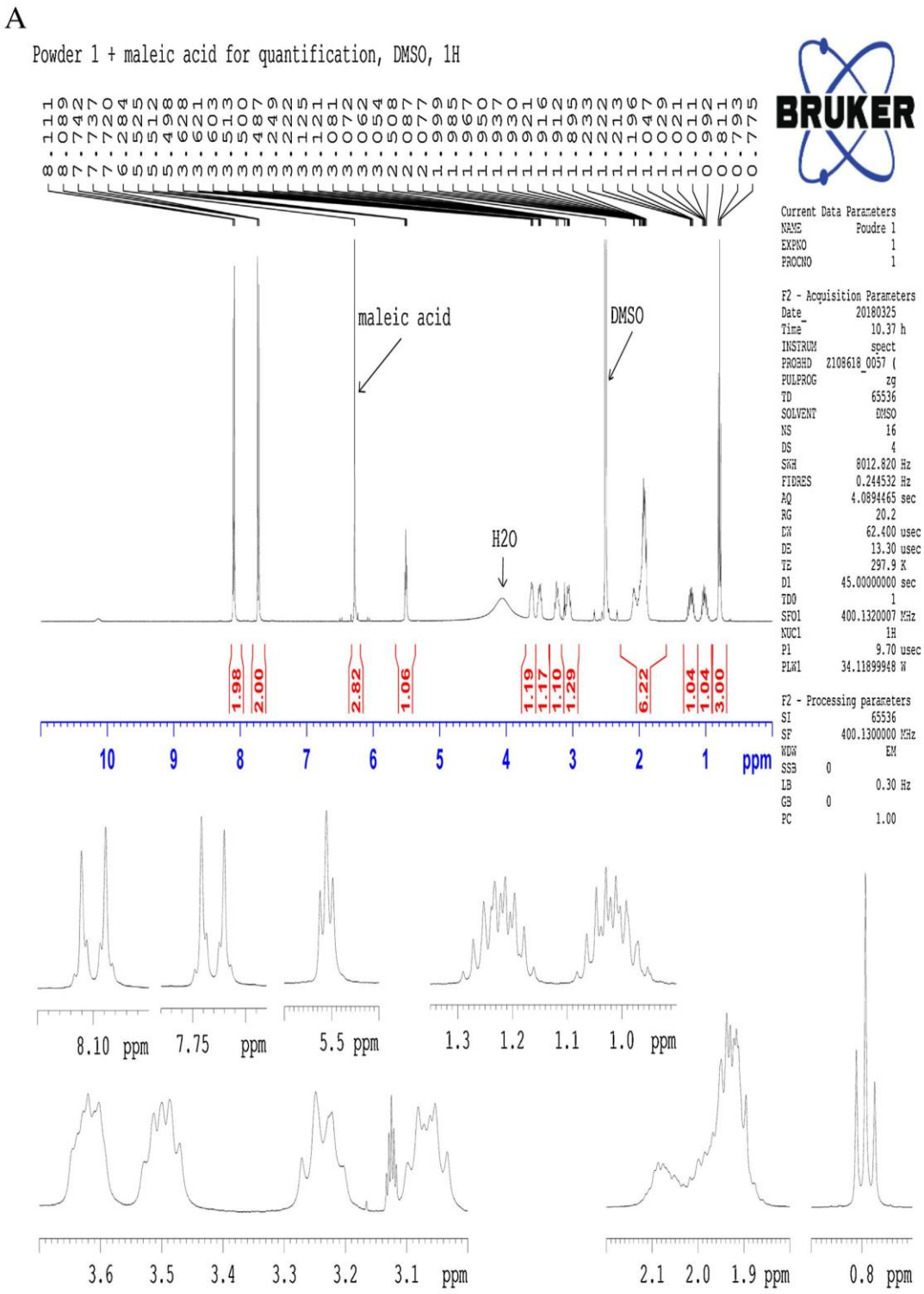

Fig. 2. (A–G) Responses of the 7 powders (1H spectra). (A) 4-Chloro-alpha-pyrrolidinopentiophenone, (B) dibutylone, (C) 4-chloroethcathinone, (D) 4-alpha-pyrrolidinohexuiphenone, (E) 3,4-methylenedioxy-*N*-benzylcathinone, (F) 3-methoxy-p.

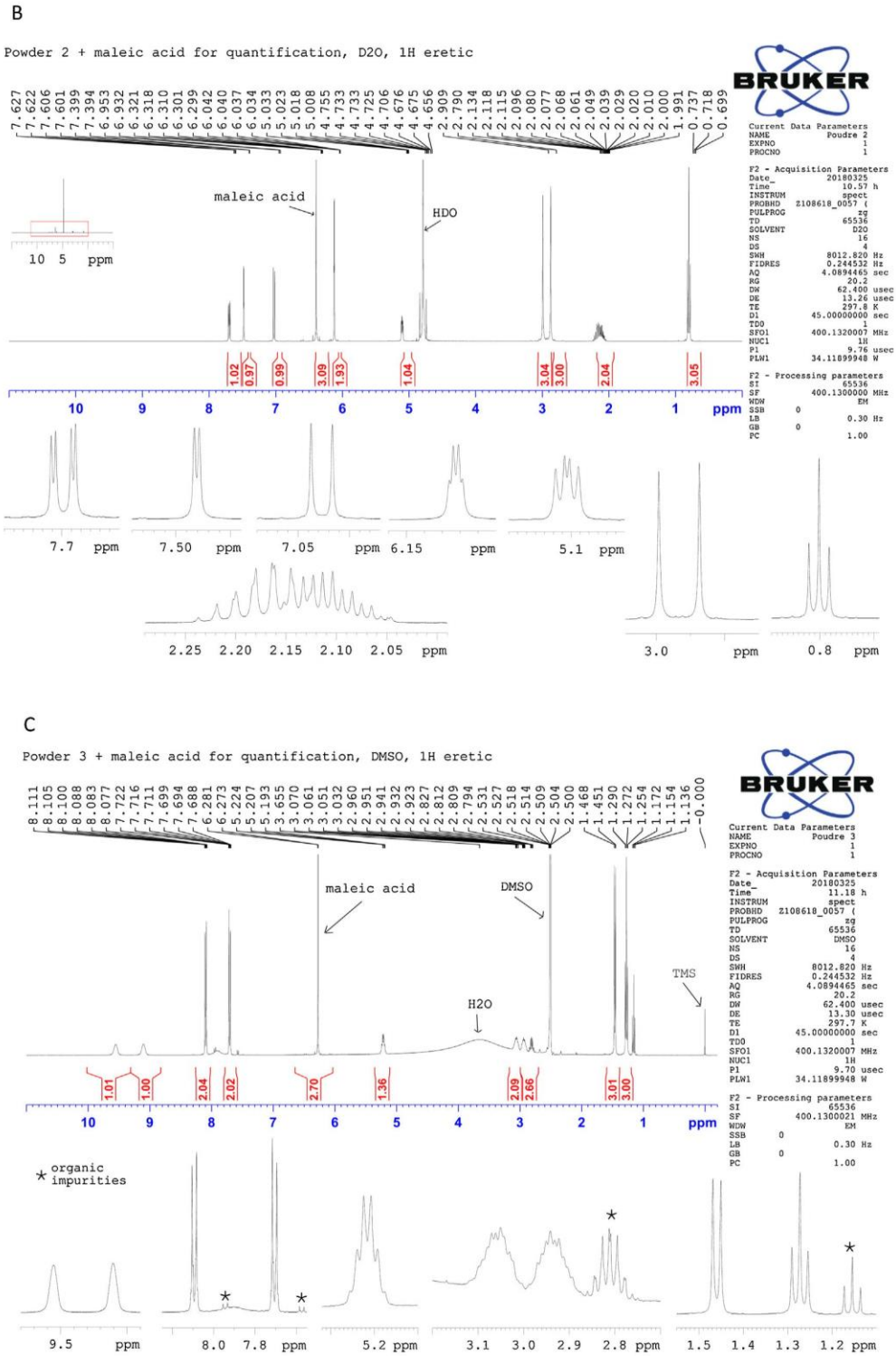

Fig. 2. (Continued)

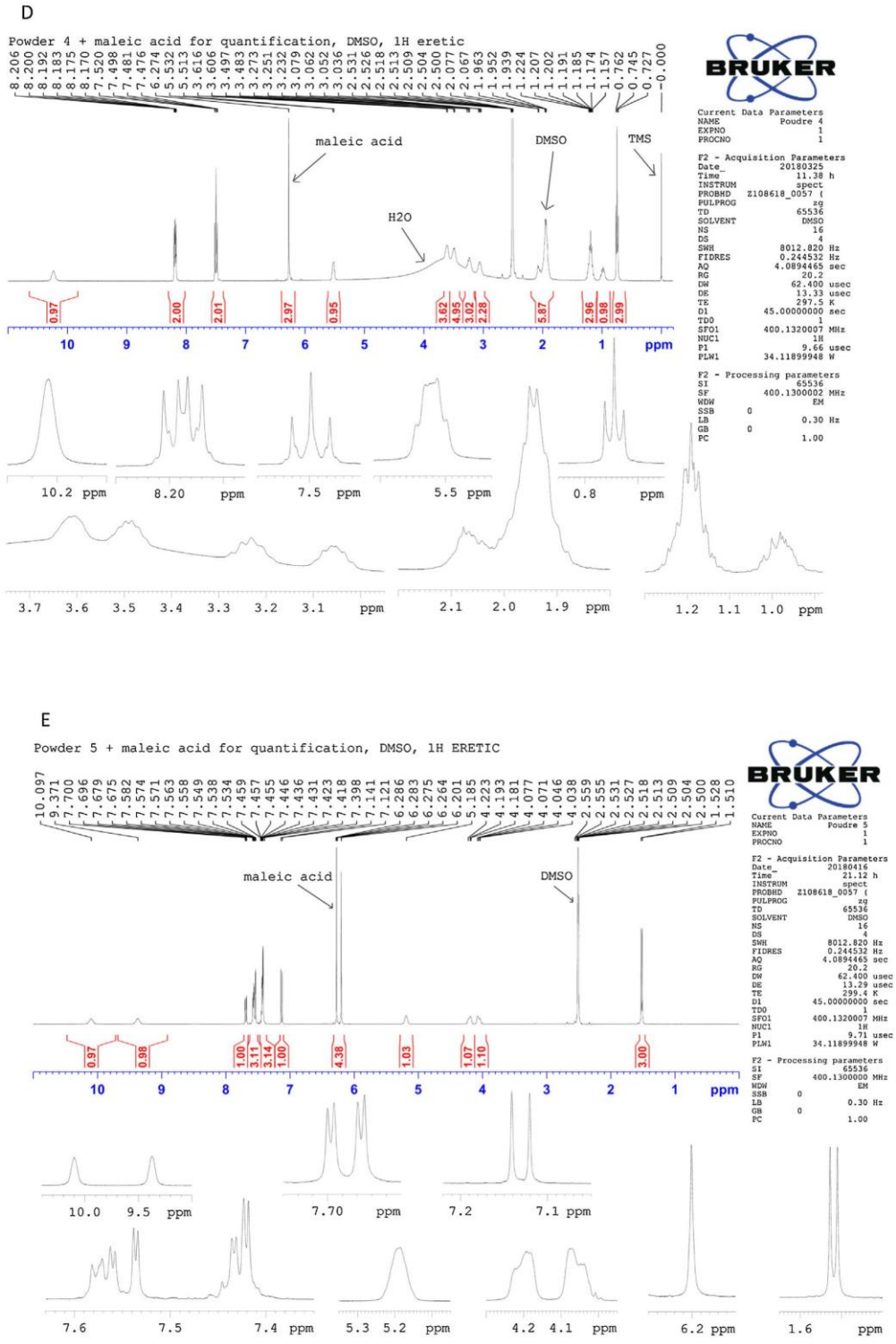

Fig. 2. (Continued)

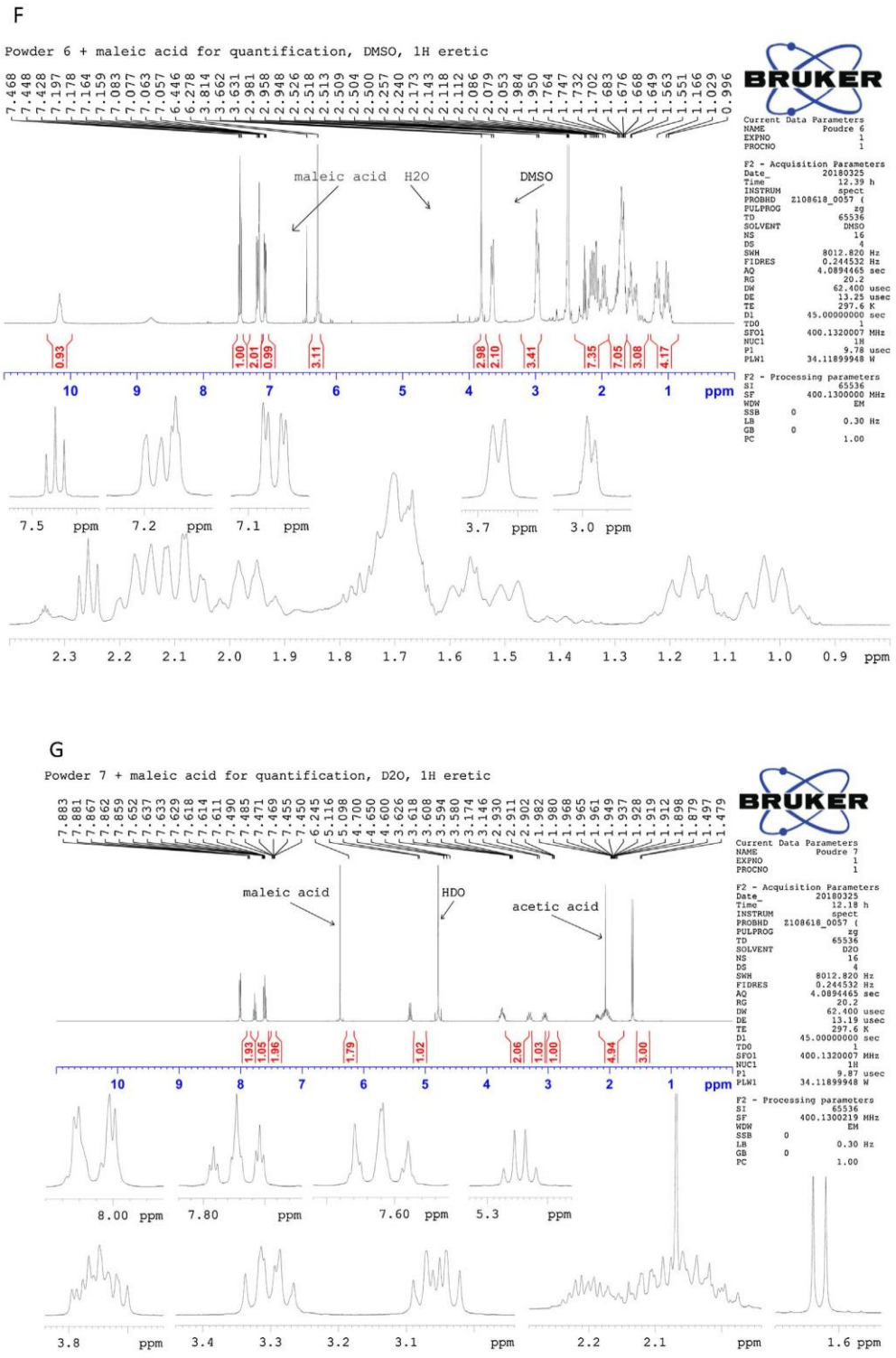

Fig. 2. (Continued)

Table 2
Retention times and specific transitions of 7 compounds (CV: cone voltage; CE: collision energy).

Compounds	RT (min)	Transition 1	CV1 (V)/CE1 (eV)	Transition 2	CV2 (V)/CE2 (eV)
4-Chloro-alpha-PVP	5.60	266.0 > 124.8	25/50	266.0 > 138.8	25/70
Dibutylone	2.62	236.0 > 148.8	20/65	236.0 > 85.8	20/80
4-Chloroethcathinone	3.50	211.9 > 193.9	20/50	211.9 > 158.9	20/65
4-Alpha-PHP	5.30	264.1 > 108.8	25/65	264.1 > 122.9	25/75
BMDP	4.35	284.0 > 90.9	20/50	284.0 > 166.1	20/50
3-MeO-PCP	5.95	274.0 > 120.9	10/28	274.0 > 188.9	10/35
Alpha-PPP	2.20	203.9 > 132.9	25/50	203.9 > 104.9	25/56

4. Results and discussion

The 7 powders and crystals were identified by 1H-NMR and data are presented in Table 1. As an example, the detailed interpretation of the powder 6 is available as supplement file. The compounds were 4-chloro-alpha-pyrrolidinopentiophenone, dibutylone, 4-chloroethcathinone, 4-alpha-pyrrolidinohexuiphenone, 3,4-methylenedioxy-*N*-benzylcathinone, 3-methoxy-phencyclidine and alpha-pyrrolidinopropiophenone. There is no matching between the label and the active ingredient contained in the dispatched bags. This clearly demonstrates the danger in consuming substances bought on the internet, as both identity of the substance and its purity are missing. The ^{1}H NMR spectra of the 7 compounds are presented in Fig. 2A–G. The purity of each powder (Table 1) was measured by qNMR method using maleic acid as internal standard and ranged from 50.8 to 89.5%, which are in the normal range of what is generally reported [1–3]. The compounds were added in the NPS library, and cone voltage and collision energy were adjusted to optimise their signals. The retention times and specific transitions are presented in Table 2.

Initial toxicological screening tests revealed only methadone, EDDP and oxazepam at therapeutic levels (351.0, 8.0 and 315.0 ng/mL, respectively). Using UPLC–PDA®, a peak was observed which did not match with the library of the manufacturer. Since these findings did not explain the death, further NPS screening was performed with the addition of the seven compounds identified by ^{1}H NMR to the library.

A NPS screening was realized using UPLC–MS/MS® in cardiac blood, peripheral blood, urine and hair. Only 3-MeO-PCP, or 4-MeO-PCP, were identified. In UPLC–MS/MS® 3-MeO-PCP and its positional isomer, 4-MeO-PCP, have the same chromatographic behaviour (same retention time and same MRM transition) under the used chromatographic conditions. Discrimination of the positional isomer was obtained by GC/MS after separation on an Elite-5MS (30 m × 0.25 mm i.d, 0.25 μm) on a Perkin Elmer system (Clarus 680 + Clarus SQ 8 T) as their retention times differ (8.58 min for 3-MeO-PCP and 8.74 min for 4-MeO-PCP). Using GC–MS, the results were consistent with the presence of 3-MeO-PCP in the biological fluids of the victim (1 single chromatographic peak at 8.58 min), which confirms the ^{1}H sNMR findings.

Peripheral blood concentration was 498 ng/mL, cardiac blood concentration was 743 ng/mL and urine concentration was 16.7 mg/L. The concentration difference between cardiac (743 ng/mL) and peripheral blood (498 ng/mL) may be attributed to post-mortem redistribution. Cardiac blood is more influenced by this phenomenon, which takes into account several endogenous factors (release, physicochemical, pharmacokinetics, etc.) and exogenous factors (type of sampling and delay between death and sampling). The cardiac to peripheral blood ratio was 1.49. The peripheral blood concentration was higher than the toxic values (ranging from 49 to 350 ng/mL) reported in intoxicated patients [19–21], or within the fatal values (ranging from 50 to 3200 ng/mL) reported in fatal cases [22,23]. It is therefore possible to anticipate that the victim was deceased from 3-MeO-PCP acute intoxication. The psychoactive effects of the drug were probably enhanced by the simultaneous consumption of other CNS depressants (methadone and oxazepam), found in the blood of the victim. Among the phencyclidine family, 3-MeO-PCP seems be the most active agent to produce vivid hallucinations, typed auditory, visual and tactile, but there is a paucity of data on 3-MeO-PCP and its clinical effects [24–28].

The hair test result for 3-MeO-PCP was 15,600 pg/mg. This concentration was in the range of those measured in case of PCP abuse [29]. Combined washes were tested and only revealed traces of 3-MeO-PCP, which can highly soften the possibility of external contamination. It is not possible to totally rule out contamination by drug handling, but it is more likely that the findings correspond to repetitive abuse. By providing information on exposure to drugs over time, hair analysis may be useful in verifying self-reported histories of drug use in any situation in which a history of past rather than recent drug use is desired. The toxicological significance of the measured concentrations is difficult to establish because this is only the second case describing hair analysis for 3-MeO-PCP in the medical literature (731–893 pg/mg [18]). Also, there is no controlled study about pharmacokinetic parameters of 3-MeO-PCP incorporation into hair. It is therefore not possible to interpret the data in terms of dosage and frequency of abuse, but given the concentration in the ng/mg range, one can anticipate repetitive exposures. In a recent article, Salomone et al. [30] have highlighted the difficulties in interpreting results of hair analysis for NPS. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between dosage frequency of use and hair concentrations. Due to these actual limitations, the authors concluded that 3-MeO-PCP hair results should be interpreted with caution by experienced forensic toxicologists.

5. Conclusion

The use of several technologies, including ^{1}H NMR, GC–MS and UPLC–MS/MS®, was necessary to bring enough data to document the submitted case, due to its complex nature. The simultaneous combination of these methods was found suitable, as (1) ^{1}H NMR allowed to identify the 7 powders unambiguously and to measure their purity, (2) UPLC–MS/MS® allowed to quantify 3-MeO-PCP in biological fluids and hair, and (3) ^{1}H NMR and GC/MS allowed to discriminate the structural isomers. This long and tedious practice was the only possible analytical strategy. In a series of unknown powders, bought on the internet, there is always a risk of obtaining a wrong substance. Only ^{1}H NMR allows to unambiguously identify the supposed parent compound, possible remains of chemical reagents, determine the purity when the reference standard is missing and establish with high degree of certainty the chemical structure. All these items are not possible by GC–MS. This approach allowed to list the cause of death as an acute 3-MeO-PCP poisoning, and the manner of the death was considered as accidental in a context of polydrug abuse.

Conflicts of interest

The authors declare no conflict of interest

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.forsciint.2019.03.003.

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Article 10: Murdered while under the influence of 3-MeO-PCP

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CASE REPORT

Murdered while under the influence of 3-MeO-PCP

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Abstract
The abuse of new psychoactive substances (NPS) has been dramatically increasing all around the world since the late 2000s. The availability of hundreds of NPS in the past decade is challenging for both public health and global drug policies. A 39-year-old woman, known as a multidrug addict, was murdered by her partner by ligature strangulation. A comprehensive toxicological screening by gas chromatography and ultra-high performance liquid chromatography with tandem mass spectrometry revealed the simultaneous presence of ethanol (1.37 g/L), diazepam (157 ng/mL) and nordiazepam (204 ng/mL), cocaine (25 ng/mL) and benzoylecgonine (544 ng/mL), and (3-methoxy-(1-(1-phenylcyclohexyl)piperidine) or 3-MeO-PCP, a dissociative hallucinogen anesthetic drug. Concentrations of 3-MeO-PCP were 63, 64, and 94 ng/mL in femoral blood, bile, and urine, respectively. Hair tested also positive for 3-MeO-PCP on 3 × 2-cm segments at 731, 893, and 846 pg/mg, indicating long-term abuse of the drug. This seems to be the first ever reported hair concentrations. Major impairment of the victim, including visual hallucinations and alteration of behavior, was attributed to the mixture of all the drugs, with a major contribution of 3-MeO-PCP. The toxicological findings were compared to the few reports available in the medical literature.

Keywords New psychoactive substances · 3-MeO-PCP · UPLC-MS/MS · Severe hallucinations

Introduction

For several years, new synthetic drugs appeared on the Internet and constituted a wide group called new psychoactive substances (NPS), which designate a wide range of molecules having the same pharmacological effects as various illegal substances such as ecstasy, amphetamines, benzodiazepines, opiates, or cannabis. Their chemical structures can be close to the original compound but can be also very far from it. These substances can skirt the narcotic laws because they are not listed as controlled drugs. Generally sold on the Internet, NPS are differentiated by their chemical names or trade names such as "bath salts," "plant for nutrients," and "incense." According to a survey of the *French Observatory Drugs and Drug Addiction* [1], more than 382 substances circulated freely in the European Union between 2008 and 2015, including 176 in France. NPS are becoming more prevalent in the world of teenagers and parties, given the ease of purchase and use.

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Among NPS, a group derived from phencyclidine (1-(1-phenylcyclohexyl)piperidine or PCP) has gained importance in the recent period because it was proposed as legally available alternatives, although in some European countries (e.g., France or Germany), it is banned by the narcotics act. This group includes 2-MeO-PCP, 3-MeO-PCP, 4-MeO-PCP, and 3-methoxyeticyclidine. 2- and 4-MeO-PCP were first synthetized in 1965 [2], while 3-MeO-PCP was first described in 1979 [3]. Although there is limited information available on these compounds, their behavioral effects are closely related to those produced by dissociative anesthetics, such as ketamine, methoxetamine, and PCP with high affinity for the PCP site on the glutamate *N*-methyl-D-aspartate (NMDA) receptor. In addition, they present appreciable affinities for the serotonin transporter and high affinities for sigma receptors [4].

The compounds have numerous psychedelic effects which are characteristic of dissociative anesthetics and include dissociation from the physical body, visual and auditive hallucinations, altered time and space, and change of consciousness. They also have stimulating effects like euphoria or empathy. 3-MeO-PCP, like PCP, is active via oral, inhalation, and injection routes in the low milligram range (3–5 to 10–20 mg) and therefore can be hard to accurately measure, being prone to overdosage [5]. It produces many adverse effects, including psychosis, confusion, violent behavior, hypertension, tachycardia, suicidal impulses,

and coma up to death. Dosage of 4-MeO-PCP is much higher, as the usual oral dose can be up to 250 mg. This difference in drug activity between isomers can be responsible of acute and severe intoxication in case of confusion by addicts [6]. Both drugs are available as powder or tablets.

To date, there are very few published reports on 3-MeO-PCP intoxications. Zidkova et al. [7] detected the drug in the serum of two subjects admitted to hospital for disorientation and hallucinations at 49 and 66 ng/mL. In Italy, Bertol et al. [8] found 180 and 350 ng/mL in the blood from two patients admitted to hospital in a comatose state. A nonfatal intoxication with a 3-MeO-PCP blood concentration at 140 ng/g at admission and seven deaths with femoral blood concentrations ranging from 50 to 380 ng/g were described by Johansson et al. [9]. Based on a multi collection of blood specimens from the nonfatal intoxication, the half-life of 3-MeO-PCP was estimated to 11 h. Finally, in two other papers [10, 11], 3-MeO-PCP peripheral blood concentrations were 3200, 630, and 139 ± 41 ng/mL from three postmortem cases. This demonstrates that there are no clear toxic and fatal concentrations established for 3-MeO-PCP, mainly because the drug is often associated to other recreative substances.

The objective of this publication is to present the analytical procedure established for assaying 3-MeO-PCP in postmortem specimens obtained from the victim of a murder while under the influence of the drug and to discuss possible aggressive behavior due to impairment.

Case history

A 39-year-old white woman (1.58 m tall, 61 kg) with a history of illicit drug use (heroin, cannabis, cocaine, GHB) was found dead at home after being assaulted by her partner. To the police, he immediately reported an altercation while under the influence of various drugs and he admitted having strangled her with a rope in order to constrict her neck. Various paraphernalia were also found at the scene, including spoons and plastic pipettes. External examination of the victim confirmed the presence of parched, horizontal ligature mark encircling the neck. A broad band-like red area, due to intense subcutaneous hemorrhage, and a clear ligature mark consisting of abrasions were visible. Autopsy showed fractures with associated hemorrhage of the superior horn of the thyroid cartilage and of the right greater horns of the hyoid bone, respectively. Intense multi viscera congestion was noticed. During the autopsy, femoral blood, bile, urine, and hair (10 cm, brown) were collected for toxicological investigations.

Blood from the partner, preserved with sodium fluoride and collected about 1 h after the event, was also tested for alcohol, pharmaceuticals, and drugs of abuse. The test revealed no alcohol, diazepam (2120 ng/mL), nordiazepam (2540 ng/mL), oxazepam (830 ng/mL), methadone (290 ng/mL), EDDP (54 ng/mL), Δ9-tetrahydrocannabinol (8.1 ng/mL), 11-OH-THC (2.8 ng/mL), and THC-COOH (190 ng/mL). All these drugs should have markedly impaired the subject. No 3-MeO-PCP was detected in his blood.

Toxicological analyses

Ethanol was tested by head space GC/FID on a Perkin Elmer system (TurboMatrix 40 & Clarus 580) using a standard validated procedure. Volatiles were tested by head space GC/MS on a Thermo system (Focus GC & DSQII) using a standard validated procedure. ELISA tests for illegal drugs were achieved using Alere Microplate kits using the recommendations of the manufacturer. Pharmaceuticals and drugs of abuse (including NPS) screenings were performed with ultra-performance liquid chromatography-diode array detection (Waters UPLC-PDA) and ultra-performance liquid chromatography tandem mass spectrometry (Waters UPLC-MS/MS).

During the initial screenings, 3-MeO-PCP or 4-MeO-PCP, which have the same chromatographic behavior (same retention time, same MRM transitions), was identified in the femoral blood. Discrimination of the positional isomer was obtained by GC/MS on a Perkin Elmer system (Clarus 680 and Clarus SQ 8T) as their retention times differ, i.e., 8.58 min for 3-MeO-PCP and 8.74 min for 4-MeO-PCP, while both have the same mass spectrum (*m/z* 230, 272, 273, 121, 216). The results were consistent with the presence of 3-MeO-PCP in the blood of the victim (one single chromatographic peak at 8.58 min).

As a consequence, a specific MRM method for 3-MeO-PCP testing in postmortem fluids and hair was developed.

3-MeO-PCP was extracted from 1 mL of fluid (blood, urine, bile) in presence of 2 ng of MDMA-d_5 used as internal standard, with 1 mL borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50/30/20/0.5). After extraction, centrifugation, and evaporation to dryness, the residue was reconstituted in 50 μL of ammonium formate buffer adjusted at pH 3.

Hair analysis of 3-MeO-PCP was performed after decontamination with dichloromethane and segmentation into 3 × 2-cm segments to cover the last 6 months before death, assuming a growth rate of 1 cm/month. Twenty milligrams was incubated overnight at 50 °C in 1 mL borate buffer pH 9.5, in presence of MDMA-d_5, and then, the mixture was extracted as blood.

Chromatography was achieved using a Waters HSS C18 column (150 × 2.1 mm × 1.8 μm) maintained at 50 °C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile LC-MS (mobile phase B) as mobile phases at flow rate of 0.4 mL/min. The initial gradient was 87% mobile phase A and the final gradient, at 7 min, was 5% mobile phase A. An injection volume of 10 μL was used in all cases. A Xevo triple quadrupole mass

spectrometer was used for the detection of the drugs. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of 3-MeO-PCP and the internal standard: capillary voltage at 1.5 kV, source block temperature at 149 °C, desolvation gas nitrogen heated at 600 °C and delivered at a flow rate of 1000 L/h. In order to establish appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion, and collision-induced dissociation of both species was performed. Collision energy was adjusted to optimize the signal for the two most abundant product ions of 3-MeO-PCP, i.e., *m/z* 274.0 > 120.9 (CV, 10 V; CE, 28 eV) and 274.0 > 188.9 (CV, 10 V; CE, 35 eV), and the most abundant product ion of internal standard *m/z* 198.9 > 164.9 (CV, 22 V; CE, 14 eV). Transition *m/z* 274.0 to 120.9 was used for quantification of 3-MeO-PCP. These transitions have already been described in the literature [9, 11]. MassLynx 4.1 software was used for quantification.

Linearity was observed in blood for 3-MeO-PCP concentrations ranging from 10 to 1000 ng/mL, with a correlation coefficient of 0.998. QC samples (50 and 200 ng/mL), analyzed in duplicate in six independent experimental assays, were used for determination of a coefficient of variation for precision and accuracy. These CVs were lower than 20%. Linearity was observed in hair for 3-MeO-PCP concentrations ranging from 10 to 1000 pg/mg, with a correlation coefficient of 0.999. QC samples (50 and 200 pg/mg), analyzed in duplicate in six independent experimental assays, were used for determination of a coefficient of variation for precision and accuracy. These CVs were lower than 20%. Under the used chromatographic conditions, there was no interference with the analytes by chemicals or any extractable endogenous materials present in blood or in hair.

Results and discussion

The analysis by gas chromatography coupled to mass spectrometry allowed the unambiguous identification of 3-MeO-PCP (Fig. 1) in the femoral blood of the victim and ruled out a possible contribution of its positional isomer 4-MeO-PCP. Using a dedicated UPLC-MS/MS method, the concentration of 3-MeO-PCP in the femoral blood was 63 ng/mL. The drug tested also positive in other biological matrices (Table 1). The blood concentration is close to the toxic values reported in intoxicated patients [7]. It is therefore possible to anticipate that the victim was impaired due to 3-MeO-PCP abuse. The psychoactive effects of the drug were even enhanced by simultaneous consumption of other CNS depressants. Indeed, blood alcohol concentration was 1.37 g/L and diazepam (157 ng/mL) and its main metabolite, nordiazepam (204 ng/mL), and cocaine (25 ng/mL) and its metabolites, benzoylecgonine (544 ng/mL) and cocaethylene (43 ng/mL), were identified in blood. Other authors [6–11] have also reported combination of 3-MeO-PCP with other drugs in both fatal and nonfatal intoxications. Although metabolites of 3-MeO-PCP, including hydroxylated, carboxylated, and O-demethylated metabolites, have been identified in human liver preparations [12], there was no attempt to characterize them in urine, mostly because the reference standards are not commercially available.

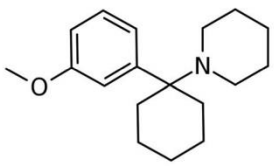

Fig. 1 Structural formula of 3-MeO-PCP

Segmental hair analysis is used to verify both previous drug history and recent enforced abstinence. Hair analysis is mostly suitable for accurately monitoring relative changes in drug intake by the same individual [13]. The hair test result for 3-MeO-PCP was the following: 731 (0–2 cm), 893 (2–4 cm), and 846 pg/mg (4–6 cm), supporting repetitive consumption of the drug over the last 6 months. Methadone (+ EDDP), cocaine and metabolites, and 6-acetylmorphine as specific marker of heroin were also identified in hair, clearly confirming the addiction to drugs of the donor. The toxicological significance of the measured concentrations is difficult to establish because this is the first case describing hair analysis for 3-MeO-PCP in the medical literature. Also, there is no controlled study about pharmacokinetic parameters of 3-MeO-PCP incorporation into hair. It is therefore not possible to interpret the data in terms of dosage and frequency of abuse. In a recent article, Salomone et al. [14] have highlighted the difficulties in interpreting results of hair analysis for NPS. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between dosage frequency of use and hair concentrations. The authors also considered different scenarios such as passive exposure vs. active consumption, mindful vs. unaware intake, and sporadic vs. chronic use. Due to these actual limitations, the authors

Table 1 Distribution of 3-MEO-PCP in the biological specimens collected during autopsy

Specimen	3-MeO-PCP concentration
Femoral blood	63 ng/mL
Bile	64 ng/mL
Urine	94 ng/mL
Hair, 0–2 cm	731 pg/mg
Hair, 2–4 cm	893 pg/mg
Hair, 4–6 cm	846 pg/mg

concluded that NPS hair results should be interpreted with caution by experienced forensic toxicologists.

The key point of this case report is the involvement of 3-MeO-PCP in the behavior of the victim. Blood and hair findings have demonstrated long-term abuse of several drugs. Among them, 3-MeO-PCP seems the most active to produce vivid hallucinations, typed auditory, visual, and tactile. There is a paucity of data on 3-MeO-PCP and its clinical effects and only one paper [15] reports an attempted murder by a subject under the influence of the drug. Unfortunately, no biological specimen was collected at the time of the event. In comparison, aggressive behavior, violence, and physical assault have been frequently described for PCP since 1979 [16–19]. It has been shown that PCP users have different characteristics from other drug users and that female PCP use is more common than use among males. Furthermore, there is evidence that those who respond to PCP with violence may differ from those who do not. In particular, female PCP using subjects reported more dysphoria and aggressiveness when not using PCP, while male subjects were more likely to report aggressive behavior and dysphoria under the influence [19]. However, the assumption that PCP provokes violent behavior in humans with predictable regularity has been challenged by some authors [20] who concluded after examination of hundreds of patients that clinical and forensic assumptions about PCP and violence are not warranted. Based on limited literature [6–9] on analytically confirmed nonfatal intoxications, there is little doubt that the measured 3-MeO-PCP concentration in the blood of the victim was responsible of strong behavior alteration. Thus, this, in combination with other drugs, should be considered as possible enhancers of the aggressive situation which has promoted the homicide.

Conclusion

3-MeO-PCP is a highly attractive drug for abusers seeking an alternative to PCP or for those who want to experiment new sensations (drug testers). It is one of the most potent known NMDA antagonists. The drug is easily accessible via the Internet at affordable prices. According to users, abuse dosages are usually in the range 3 to 20 mg and the duration of the effects has been reported to be 4 to 5 h. It is not detected by most routine drug screening tests and can be mixed up with the less potent 4-MeO-PCP. Confusion between both positional isomers can have serious health consequences and there is no way for users to know exactly what they are consuming. This work has presented a murder case where the victim was under the influence of 3-MeO-PCP and, according our knowledge, for the first time hair concentrations from a repetitive user.

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Partie 4 : Stratégies analytiques dédiées à l'identification des métabolites de NPS

L'identification des métabolites a un intérêt majeur pour les analystes. Elle devrait permettre d'augmenter la fenêtre de détection d'une consommation de NPS. En effet, pour la plupart des xénobiotiques, la demi-vie d'élimination des métabolites est très supérieure à celle de la molécule mère. Cependant, l'identification des métabolites n'est pas facile. Pour la grande majorité des NPS, très peu de données sont disponibles dans la littérature. Pour d'autres, ces données sont inexistantes. Une fois identifiés, les métabolites ne sont pas toujours disponibles à l'achat, et les standards existants sont très couteux et longs à recevoir (parfois plus de 6 mois). Il est donc nécessaire de mettre en place des stratégies directes d'identification, permettant au mieux de documenter les observations.
Par ailleurs, la présence de métabolite(s) renforce la spécificité des analyses.

Stratégie *in vitro*

In vitro, la production de métabolites peut être réalisée à partir d'incubations utilisant des microsomes hépatiques humains (HLMs). Le choix du modèle et des co-substrats permet l'identification de différentes voies métaboliques (phase I ou phase II).
Dans un premier temps, deux benzodiazépines de synthèses ont été étudiées en collaboration avec le Centre Hospitalier Universitaire de Lille, qui a développé le protocole. Pour ces deux molécules, le métizolam et le flunitrazolam, seule la voie métabolique de phase I a été étudiée. Cette approche *in vitro*, couplée à la spectrométrie de masse haute résolution, a permis l'identification de 3 métabolites mono-hydroxylés pour le métizolam, ainsi que d'un métabolite aminé pour le flunitrazolam. Une fois identifiées, ces métabolites ont été recherchés et détectés *in vivo*, dans des urines de consommateurs. L'étude du flunitrazolam chez un volontaire sain a permis de caractériser au moins 2 métabolites additionnels, le désalkyl-flunitrazolam et le 7-acétamido-flunitrazolam. Ceux-ci n'ont probablement pas été générés par les microsomes du fait d'une voie métabolique différente.
D'après nos résultats, il apparait qu'en ciblant les métabolites, la fenêtre de détection de la consommation de ces deux benzodiazépines de synthèse est très augmentée (au-delà de 48 heures).
Les résultats obtenus ont permis la rédaction de 2 publications *[Article 11 et 12].*

Compte tenu du potentiel de cette approche, le protocole a été mis en place et optimisé dans notre laboratoire à Strasbourg. Depuis, deux autres études ont été réalisées sur des NPS pour lesquelles aucune information n'existait dans la littérature. Il s'agit de la 3-MeO-PCP, un analogue de la phéncyclidine, et du deschloroétizolam, une autre benzodiazépine de synthèse. Ces études ont également donné lieu à la rédaction de 2 publications *[Article 13 et 14].*

Étude d'administration contrôlée à un sujet

Une étude post administration à un sujet permettrait d'obtenir des informations de détectabilité dans des matrices alternatives, telles que la salive, la sueur, l'air expiré, les cheveux et les poils. Systématiquement, la première étape de cette étude consiste à vérifier l'identité et le dosage de la substance avant de l'administrer au volontaire. Pour cela, la spectroscopie par RMN est une méthode largement utilisée puisqu'elle permet de valider la nature chimique de la substance, son degré de pureté et l'absence d'autre composé actif.

Trois études ont été réalisées au laboratoire, au cours de ma thèse. Ces études ont porté sur les mêmes benzodiazépines de synthèses que précédemment (le flunitrazolam, le deschloroétizolam et le métizolam). Les résultats obtenus ont donné lieu à 3 publications, portant sur la caractérisation et l'excrétion dans les matrices alternatives *[Article 15, 16 et 17].*

Nota : le volontaire participant à chacune de ces études a été informé des risques et des conséquences de l'exposition à des molécules achetées sur Internet. Il n'existe aucun lien de subordination vis-à-vis du volontaire et sa participation n'a pas donné lieu à une rémunération.

Résonance magnétique nucléaire

La résonance magnétique nucléaire (RMN) est fondée sur la mesure de l'absorption de la radiation de radiofréquence (RF) par un noyau atomique dans un champ magnétique fort. La spectroscopie par RMN constitue l'un des plus puissants instruments de détermination de la structure des espèces organiques aussi bien qu'inorganiques. Cette technique se montre également utile dans la détermination quantitative des espèces absorbantes.

Dans le cadre d'une saisie, le laboratoire a reçu plusieurs sachets de poudres, supposés être des NPS. L'analyse de ces poudres par spectroscopie RMN a permis l'identification de leurs structures chimiques, ainsi que leurs degrés de pureté (variable, de 50,8 à 89,5 %). Il n'y avait aucune correspondance entre les étiquettes des produits et les ingrédients actifs contenus dans les sachets. Cela démontre clairement le danger de consommer des substances achetées sur internet pour lesquelles l'identité et la pureté font défaut. La stratégie d'identification mise en place a été détaillée dans une publication *[Article 18].*

Article 11: Detection of the designer benzodiazepine metizolam in urine and preliminary data on its metabolism

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Detection of the designer benzodiazepine metizolam in urine and preliminary data on its metabolism

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Designer benzodiazepines provide an attractive alternative to prescribed benzodiazepines for abuse purposes as they are readily available *via* the Internet without control. Metizolam was ordered *via* the Internet and a 2 mg blue tablet was orally administered to a 54-year-old man. Urine samples were collected over 6 days in polypropylene tubes. After liquid/liquid extraction at pH 9.5, metizolam was analyzed by ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) using a standard method devoted to benzodiazepines, and ions transitions, at *m/z 328.9 > 275.0 and 328.9 > 300.0*. Metizolam was detectable in hydrolyzed urine during the 46-h period, with concentrations always lower than 11 ng/mL. About 0.3% of the initial dose was excreted in urines as total unchanged metizolam during the first 24 h. The most relevant potential CYP- and UGT-dependent metabolites of metizolam were investigated *in vitro* using human liver microsome incubation and, subsequently, liquid chromatography coupled with quadrupole-time of flight mass spectrometry (UHPLC-Q-TOF-MS) analysis. Three mono-hydroxylated metabolites were produced including a hydroxylation compound at the 2-ethyl moiety of metizolam (M1) as quantitatively main metabolite, and a *N*-hydroxymetiazolam (M2). The structure of the third metabolite (M3) could not be elucidated because of a too low experimental production rate. Two authentic urine samples were analyzed using the same analytical method to search for metabolites of metizolam. M1, together with its glucuronide (M1-Glu), and M2 were observed in urine at the 8 h mark, whereas only M1 and M1-Glu were still detected in urine at 30 h post administration. **Copyright © 2016 John Wiley & Sons, Ltd.**

Keywords: benzodiazepines; metizolam; urine; metabolism; HLMs

Introduction

Benzodiazepines are central nervous system (CNS) depressants commonly prescribed to treat anxiety, insomnia, and seizures. They are considered safer alternatives to barbiturates because they have fewer side effects and have a reduced risk of causing an overdose. However, benzodiazepine abuse is often associated with people taking benzodiazepines in combination with other controlled substances such as opiates, methadone, and cocaine. Benzodiazepines are taken in combination with opiates in particular because they enhance the euphoric effects of opiates. Moreover, benzodiazepines may temper the negative side effects (e.g. restlessness, agitation) of other drugs, such as stimulants (amphetamine, ecstasy). Although not approved for medical use, new benzodiazepines have been observed in several forensic cases (death, acute intoxication, drug-facilitated crime, driving under the influence of drug). These new benzodiazepines include phenazepam[1,2]; etizolam and pyrazolam[3]; flubromazolam[4]; and clonazolam, meclonazepam, and nifoxipam.[5]

The detection of designer benzodiazepines in biological fluids is challenging due to the lack of reference material, unawareness of their existence and few available data. Some of these drugs have been detected using immunoassays with high cross-reactivity,[3,6] demonstrating that designer benzodiazepines can be sometimes detected in standard blood or urine immunoassay drug screenings. Some authors have developed liquid chromatography-mass spectrometry (LC-MS) methods, mostly to study the metabolism of specific drugs, including phenazepam[1,2]; clonazolam, meclonazepam, and nifoxipam[5]; pyrazolam[7]; flubromazepam[8]; diclazepam[9]; and clonazolam, deschloroetizolam, flubromazolam, and meclonazepam.[10] Among the different designer benzodiazepine classes, the thienodiazepines, obtained by the replacement of the benzene ring by a thiophene ring, are represented by etizolam (licenced for use in Japan, Italy, and India), and more recently by metizolam (Figure 1), also known as desmethyletizolam. Metizolam differs structurally from etizolam through removal of the methyl group on the triazole ring. Although etizolam has already been described in overdose[11] and death cases,[12] there is no citation for

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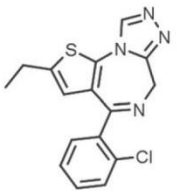

Figure 1. Chemical structure of metizolam.

metizolam in the case report literature. For instance, there are no data about metizolam analysis in biological specimens, its distribution, and metabolism. A Japanese company patented this compound in 1995 as an anti-anxiety medication, although metizolam has been developed far earlier 1995.[13,14] It has no history of human use prior to its availability by online vendors world-wide in September 2015.

Data on the metabolism of such designer benzodiazepines are of interest in the challenging field of analytical detection,[15] especially when the delay between consumption and biological sampling is substantial. The identification of their metabolites, which allows the efficiency of the toxicological screening libraries to be increased, could then improve the detection window in biological matrices.

In vitro studies, involving mephedrone, STS-135, 4-MEC, SF-AK-48, benzo[c]phenanthridine or quercetin and taxifolin galloyl esters have been already carried out in order to elucidate the main metabolic pathways of new psychoactive substances by using several approaches, such as drug incubation with human liver microsomes (HLMs)[16–19] or with human hepatocytes.[20,21] Furthermore, the combination of liquid chromatography system with high-resolution mass spectrometry (LC-HRMS) and powerful software proved helpful for the identification of the metabolites.[22,23]

In this study, standard ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and ultra-high performance liquid chromatography-quadrupole time of flight-mass spectrometry (UHPLC-Q-TOF-MS) techniques were used to detect metizolam and its metabolites in urine after administration of an oral dose of 2 mg to a human volunteer. In addition, metizolam metabolism was studied *in vitro* after drug incubation with human liver microsomes.

Material and method

Self-administration study

To gain preliminary data on elimination parameters of metizolam in urine and to investigate its metabolism, one of the authors (male, 54-year-old, 85 kg) ingested one blue tablet (declared as containing 2 mg of metizolam on the webshop) with 200 mL of tap water. Urine specimens were collected over 6 days in plastic vials without preservative and stored at −20 °C until analysis. During the first 24 h, the volume of each urine sample was recorded. Urine was collected at various times, with no fixed interval.

In France, approval by an ethics committee is not required for self-experiments (except for drugs of abuse, which is not the case for metizolam).

No blood sample was collected, as this required specific authorization due to the invasive nature of this specimen.

Chemicals and reagents

Metizolam (4-(2-chlorophenyl)-2-ethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine) was obtained from Chemicalwire (London, UK). β-OH-ethyltheophyllin, methyl-clonazepam, alamethicin (*Trichoderma viride*), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P), 5-sulfosalicylic acid, β- glucuronidase from bovine liver, magnesium chloride ($MgCl_2$), ammonium formate and formic acid, were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), while tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL, prepared from livers of 36 donors, including 11 women and 25 men, aged from 31 to 80 years, was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at -80 °C until use. LC-MS grade water and acetonitrile were purchased from Biosolve (Dieuze, France), while methanol HPLC grade and 30% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Leucine enkephalin, which was used as the MS calibration solution, was obtained from Waters (Milford, MA, USA). 25% ammonia was purchased from Merck (Fontenay-sous-Bois, France) and tris(hydroxymethyl)amino-methane hydrochloride (Tris-HCl) solution from Promega (Madison, WI, USA).

Urine extraction and standard UPLC-MS/MS analysis

Before analysis, urine specimens were hydrolyzed using 250 µL of β-glucuronidase at pH 5.2 (0.5 mL of 1 M ammonium acetate buffer) for 12 h at 40 °C. Metizolam was extracted from 1 mL hydrolyzed urine in presence of 10 ng of diazepam-d_5, used as an internal standard (IS), by 1 mL saturated ammonium chloride pH 9.5 buffer (adjusted with ammonia water) and 5 mL of dichloromethane/*n*-heptane/isopropanol (25/65/10, *v/v*). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 50 µL of acetonitrile/formic acid 0.1% (50/50, *v/v*).

LC was performed using a Waters (Milford, MA, USA) Acquity system. Chromatography was achieved using an Acquity BEH C18 column (100 x 2.1 mm, 1.7 µm) eluted with a gradient delivered at a flow rate of 0.5 mL/min (from 5% acetonitrile – 95% formic acid 0.1% to a ratio 95–5% at 5.5 min). An injection volume of 10 µL was used in all cases. A Quattro Premier XE triple-quadrupole mass spectrometer (Micromass, Waters, Milford, MA, USA) was used for analyses. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of metizolam and the IS: capillary voltage, 1.0 kV; source block temperature, 120 °C; and desolvation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 800 L/h. In order to establish appropriate multiple reaction monitoring (MRM) conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation (CID) of both species was performed. Collision gas (argon) pressure was maintained at 3.0×10^{-3} Bar and the collision energy (eV) was adjusted to optimize the signal response for the 2 most abundant product ions, and transitions at *m/z 328.9* to *275.0* and *m/z 328.9* to *300.0*. The transition *m/z 328.9* to *275.0* was used for quantitation. The transition *m/z 290.0* to *198.1* was used for diazepam-d_5. MassLynx 4.1 software was used for quantitation.

In urine, linearity was observed for metizolam concentrations ranging from 0.05 to 50 ng/mL with a correlation coefficient of

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0.999. Within-batch precisions at 1 and 10 ng/mL were better than 20 %. The limit of detection was estimated to be 25 pg/mL, with an S/N ratio of 3. Under the chromatographic conditions used, there was no interference with the analytes by chemicals or any extractable endogenous materials present in urines (matrix effect < 20 %).

In vitro incubation with human liver microsomes

Briefly, once thawed, HLMs (2 mg protein/mL for a final volume of 100 µL) were pre-activated by alamethicin on ice in an intermediate volume of 50 µL in 0.1 M Tris-HCl-$MgCl_2$ (10 mM $MgCl_2$ and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50 µL-dried residue of three different concentrations of a metizolam methanolic solution (90 µM, 450 µM or 900 µM). Fifty µL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37 °C for 60 min, and stopped by the addition of 100 µL of methanol-containing methyl-clonazepam at 1.25 mg/L and β-OH-ethyltheophyllin at 16 mg/L, used as internal standards for the chromatographic analysis. Samples were centrifuged at 4 °C for 14 min at 32 000 g and supernatants were diluted in 400 µL of 3 % 5-sulfosalicylic acid before injection of 75 µL into the chromatographic system.

Liquid chromatography with quadrupole-time of flight mass spectrometry detection

The Waters ultra-performance liquid chromatography system consisted of two binary solvent manager LC pumps, a sample manager autosampler and a column manager oven Acquity™. Mass spectrometry data were acquired using an XEVO G2-XS QTOF (Waters, Milford, MA, USA) instrument controlled with MassLynx™ 4.1 software. For mass accuracy, a LockSpray™ interface was used with a Leucine Enkephalin ($[M+H]^+$ *m/z* 556.2771) solution (200 pg/µL) infused at 10 µL/min as the lock mass, providing exact mass measurement within 1 ppm RMS mass accuracy.

Extractions of samples (75 µL of diluted supernatants or urine samples) were performed using an OASIS HLB on-line column (30 x 2.1 mm, 20 µm) (Waters, Milford, MA, USA). The mobile phases consisted in 0.2 % ammonia solution (A1) and 100 % methanol (B1) and the elution profile was as follows: 0.5 min, 0% B1, flow rate 2.0 mL/min; 1.0 min, 0% B1, flow rate 0.2 mL/min; 15 min, 100% B1, flow rate 0.2 mL/min; 30 min, 100% B1, flow rate 2.0 mL/min. The chromatographic separation was performed using an Acquity HSS C18 column (150 x 2 mm, 1.8 µm) (Waters, Milford, MA, USA) in an oven temperature of 50 °C, and mobile phases including ammonium formate buffer 5 mM, pH 3 (A2) and acetonitrile in 1% formic acid (B2); flow rate of 0.4 mL/min was used. Initial concentration of mobile phase B2 (3%) held until 0.5 min, increased to 40 % at 25 min, then to 100% at 26 min, and finally held until 30 min.

For detection, mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), 20 V as ion spray voltage, source temperature at 140 °C and desolvatation temperature at 500 °C with a desolvation gas flow rate of 900 L/h, nitrogen as desolvation gas and argon as collision gas. Conditions for the time of flight mass spectrometer (TOF MS^e) scan mode were as follows: scan range 100–1000 *m/z* for function 1 and 50–1000 *m/z*, with a collision energy ramp from 10–40 eV, for function 2.

Data processing and evaluation of metizolam metabolites were performed using the Metabolynx™ 4.1 software (Waters, Milford, MA, USA). Theoretically possible biotransformations of metizolam were simulated, including possible hydroxylation, *O*- or *N*-dealkylation, deamination and glucuronidation transformations. The automated metabolite profiling process was completed using the fragmentation interpretation software tool MassFragment™ 4.1 (Waters, Milford, MA, USA), to allow software driven assignment of metabolite structures from fragmentation patterns.

As previously reported, a control sample containing propafenone was analyzed using the incubation and chromatographic conditions as for the metizolam in order to assess the efficacy of the enzymatic system.[21] The efficacy was considered to be correct if the production of both main CYP- and UGT-derived metabolites of

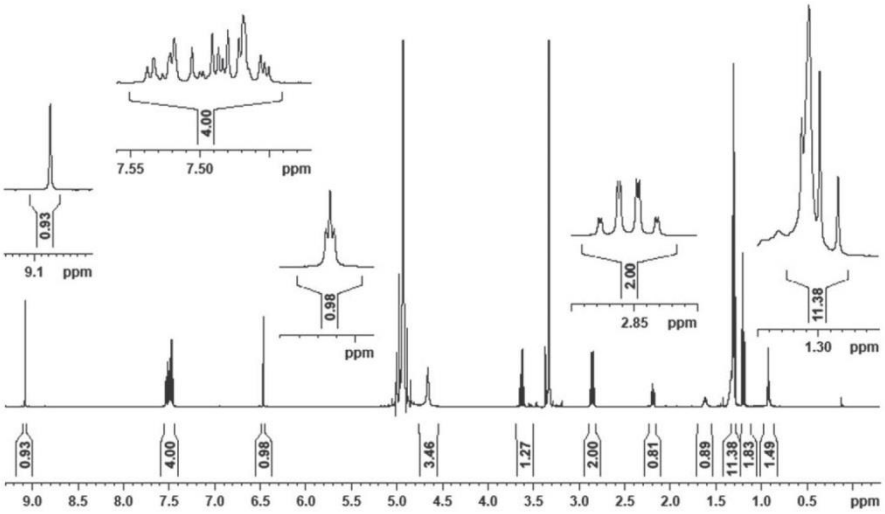

Figure 2. ^{1}H-NMR spectrum of the metizolam tablet including signals related to metizolam (δ 9.08 (1H, s, *N* = CH-*N*), 7.54-7.45 (4H, m, Ar-H), 6.46 (1H, t, S-C = CH, *J* = 1.1 Hz), 5.01 (2H, s, =*N*-CH_2), 2.85 (2H, qd, *N*-$\underline{CH_2}CH_3$, *J* = 7.4, 1.1 Hz), 1.30 (3H, *t*, *N*-$CH_2\underline{CH_3}$, *J* = 7.4 Hz) together with other signals, presumably related to tablet excipients.

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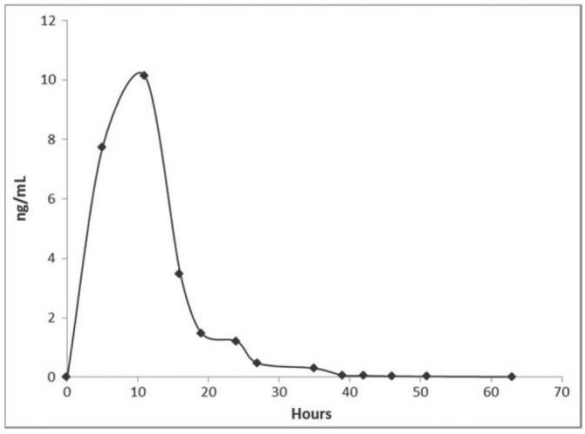

Figure 3. Concentration time profile of metizolam excretion in urines after administration of a single 2 mg tablet

propafenone (i.e., hydroxypropafenone and hydroxypropafenone glucuronide) was observed.

Metizolam tablet analysis

In order to confirm absence of other compounds such as synthesis impurities in tablet together to check its 2 mg metizolam announced dosage, a UHPLC-Q-TOF-MS analysis (using the previous described method) was completed by a nuclear magnetic resonance analysis (NMR). The NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 MHz equipped with a 5 mm quadruple nucleus probe (QNP) probe at 295 K. The ^{1}H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 s, a relaxation delay of 2 s and a flip angle of 30°. The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. For quantification and after thin manual grinding using a mortar, one tablet was dissolved in 600 µL of methanol-D_4 and the ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations. ERETIC consisted of a digitally generated Gaussian peak (on single point calibration using 15 mg of caffeine in 600 µL of $CDCl_3$ and a control point using 10 mg of the same substance in 600 µL of $CDCl_3$) inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the peak area integrals in the spectrum provide information about the sample purity.[24]

Results and discussion

When phenazepam and etizolam became scheduled in several countries, some laboratories turned their attention to investigational drugs that were never marketed as pharmaceuticals. This led to the appearance of designer benzodiazepines on webshops, sold for recreational purposes. A large series of drugs, including pyrazolam, flubromazepam, diclazepam, nifoxipam, meclonazepam, and flubromazolam, are available online via the Internet. Among these new designer benzodiazepines, not much attention was paid to metizolam. There is a real dearth of literature on this drug. It was postulated the likely effects based on structural similarity to classic benzodiazepines, but the only available source of information is the one found on drug discussion forums.

For identification and characterization of metizolam, NMR spectroscopy was applied (Figure 2). No significant impurity was detected in the metizolam tablet using UHPLC-Q-TOF-MS or NMR. In addition, the dosage of one tablet, using the ERETIC method, revealed a dosage of 1.8 mg of metizolam, in agreement with the announced 2 mg dosage (10 % of tolerance is an admitted value in a pharmaceutical laboratory).

After oral administration of one tablet of metizolam, the overall behaviour of the subject was not modified. A slight sensation of weakness was noticed, but no physical or behavioural impairment was observed.

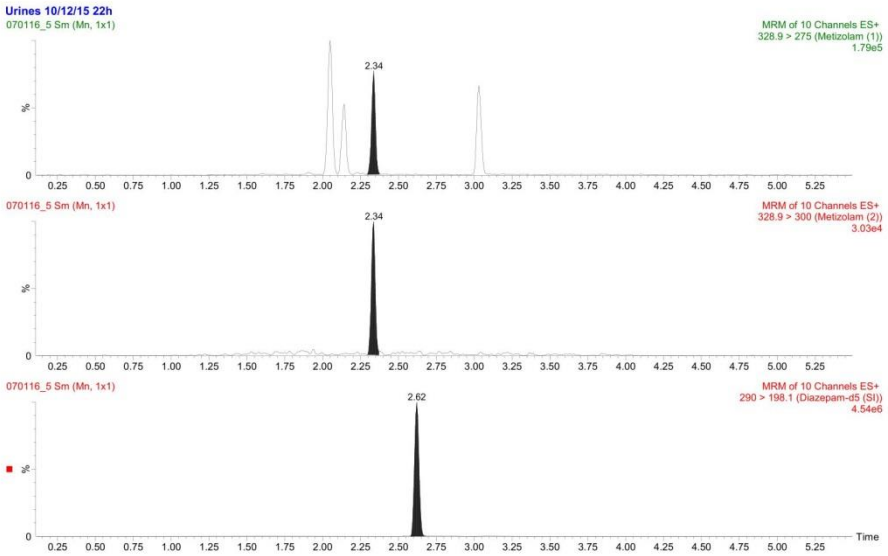

Figure 4. MRM chromatogram obtained after the extraction of urines collected 27 hours after administration of 2 mg of metizolam. Metizolam concentration was 0.47 ng/mL (from top to bottom: 2 transitions for metizolam and the transition of the IS). [Colour figure can be viewed at wileyonlinelibrary.com]

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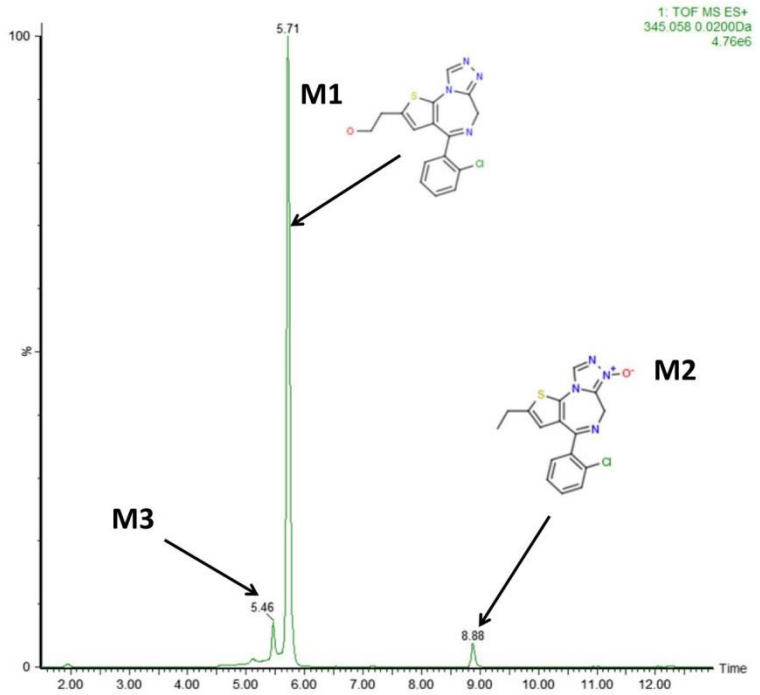

Figure 5. Identification of 3 metizolam metabolites by ToF MS. [Colour figure can be viewed at wileyonlinelibrary.com]

Unmetabolized metizolam could be detected in urine specimens up to 46 h. The concentration time profile of metizolam excretion in urines is presented in Figure 3. A typical chromatogram is presented in Figure 4. The drug was present at low concentrations, always lower than 11 ng/mL. Due to the fact that certified reference material for metizolam was unavailable, the quantitative data should be considered as rough estimates. During the first 35 h, all the urine specimens tested positive using the ELISA Alere Microplate kit for benzodiazepines (calibrated with oxazepam). At H35, the metizolam urine concentration was 0.24 ng/mL. Less than 0.3% of the ingested dose was excreted in urines as unchanged metizolam during the first 24 h.

In vitro experiments using HLM incubation and UHPLC-Q-TOF-MS analysis were performed in order to investigate the potential CYP- and UGT-dependent metabolites of metizolam. Regarding the three concentrations of metizolam (90 μM, 450 μM, or

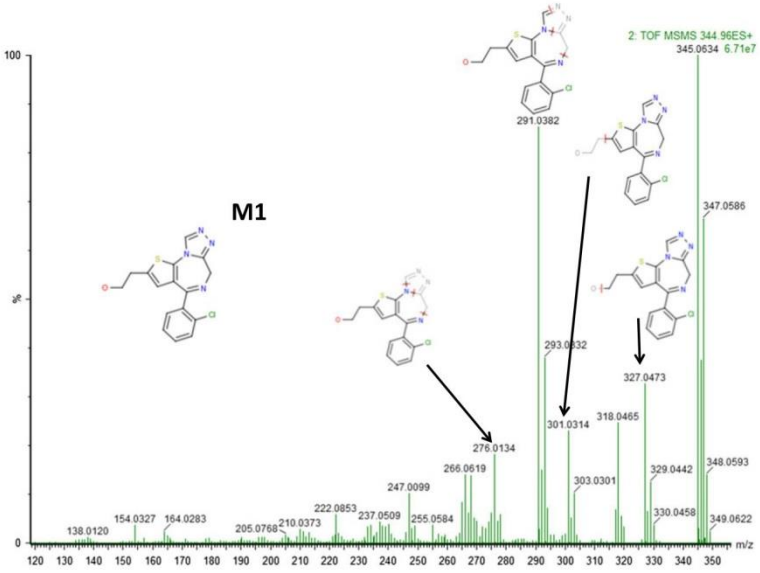

Figure 6. Mass spectrum of alpha-hydroxymetizolam (M1). [Colour figure can be viewed at wileyonlinelibrary.com]

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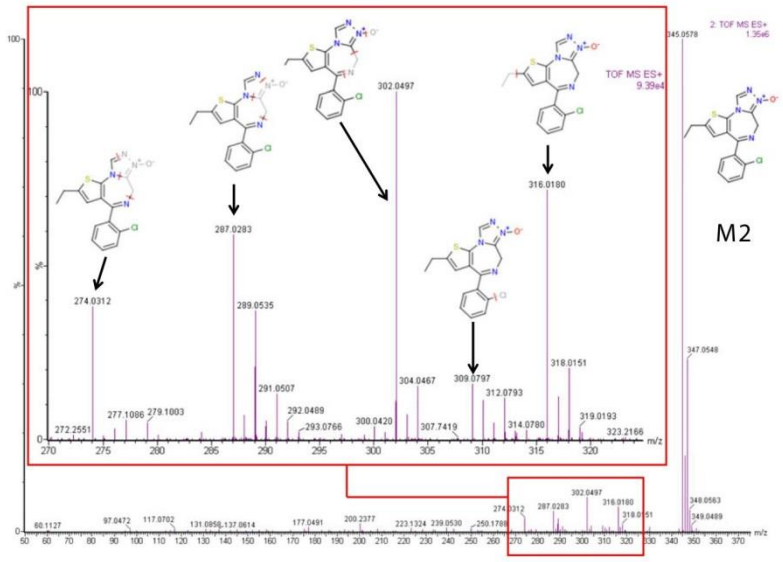

Figure 7. Mass spectrum of an *N*-hydroxylated metabolite of metizolam (M2). [Colour figure can be viewed at wileyonlinelibrary.com]

900 μM) tested for incubation experiments, a satisfactory metabolite production was observed after incubation of metizolam at 90 μM. Accordingly, the following results are those observed for this concentration. In addition, the analysis of the control sample containing propafenone with identical experimental conditions confirmed the efficacy of the enzymatic system through the achievement of expected metabolites (i.e., hydroxy-propafenone and hydroxy-propafenone glucuronide).

In addition to metizolam (observed = theoretical *m/z* $[M+H]^+$ 329.0628) with a retention time of 8.49 min, three monohydroxylated metabolites (theoretical *m/z* $[M+H]^+$ 345.0577) were detected at 5.46 (M3), 5.72 (M1) and 8.88 min (M2) after incubation

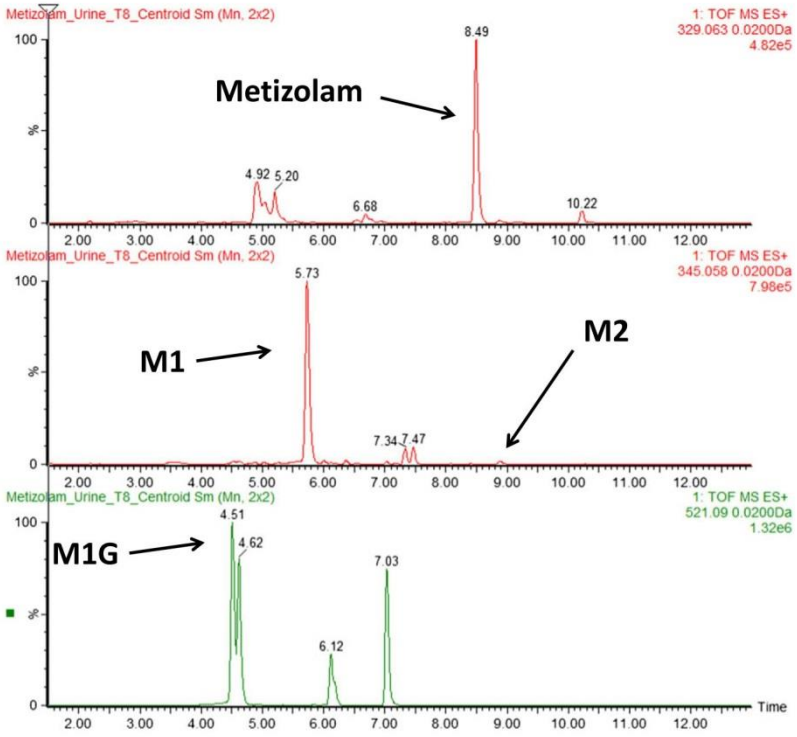

Figure 8. Chromatogram obtained after extraction of the urine of a subject administered with 2 mg of metizolam 8 hours before collection. [Colour figure can be viewed at wileyonlinelibrary.com]

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Conclusion

After designer amphetamines, cathinones, synthetic cannabinoids, designer opioids (including fentanyl derivatives), the emergence of designer benzodiazepines is in the logical step on the market for 'legal highs'. The recent addition of phenazepam and etizolam to the list of controlled substances opened the space for new candidates originally derived from pharmaceutical research. One of these new candidates is metizolam (also known as desmethyletizolam), a psychoactive drug of the thienodiazepine class (the benzene ring has been replaced by a thiophene ring). Anecdotal reports have documented the drug to produce depressant, anxiolytic, sedative, myo-relaxant and amnestic effects. The main findings of the present work suggest that metizolam is highly metabolized in humans, as very low concentrations (<0.5%) of the parent drug were observed in urines of a healthy volunteer administered with a single dose of 2 mg. Accordingly, the risk of missing metizolam use or abuse during conventional toxicological screening is important, irrespective of the analytical technique used. The identification of metizolam metabolites, such as the hydroxylated and glucuronidated metabolites, should improve the detection window of the drug. As it is possible to detect this new designer benzodiazepine with traditional ELISA tools, it also appears relevant to include this type of immunoassays in toxicological investigations. At this time, the authors did not encounter any real patient samples with metizolam in their case works and the prevalence of designer benzodiazepines cannot therefore be estimated. Since metizolam appears to be uncontrolled in many countries, one major concern about this drug regarding public health remains the lack of data on its potential acute toxicity and long-term side effects in humans.

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Article 12: Detection of the designer benzodiazepine flunitrazolam in urine and preliminary data on its metabolism

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RESEARCH ARTICLE

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Detection of the designer benzodiazepine flunitrazolam in urine and preliminary data on its metabolism

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Abstract

Designer benzodiazepines have emerged as recreational drugs. They are available via the Internet without control and are found in the form of falsified (fake) medicines. For some of them, limited information concerning their effects, their toxicity, and their detection in bio fluids is available in the literature. For others, nothing has been published, as in the case of flunitrazolam (FNTZ). To gain preliminary data on its elimination parameters in urine and to investigate its metabolism, one of the authors ingested one pink tablet bought on the Internet, after confirming the absence of other compounds and agreement with the labeled dosage (0.25 mg) by nuclear magnetic resonance (NMR). A software algorithm (MetaboLynx, Waters, Milford, MA, USA) was used to predict FNTZ biotransformation and four potential metabolites were proposed: 4-hydroxy-FNTZ, desnitro-FNTZ, 7-amino-FNTZ, and 7-acetamido-FNTZ. Urine samples were collected over 72 hours following oral administration of one tablet. After liquid/liquid extraction at pH 9.5, FNTZ concentrations were determined using ultra performance liquid chromatography–triple quadrupole–mass spectrometry (UPLC–QqQ–MS/MS). FNTZ remained detectable in hydrolyzed urine for 21 hours after ingestion, with concentrations ranging between 1 and 18 ng/mL. About 3% of the initial dose was excreted in urine as total unchanged FNTZ during this period. In vitro experiments (HLM incubations) were performed using ultra performance liquid chromatography–quadrupole time of flight-mass spectrometry (UPLC–QTOF–MS) in order to investigate the potential CYP- and UGT-dependent metabolites where only 7-amino-FNTZ was detected as the only metabolite. However, in the urine specimens, desnitro-FNTZ, 7-acetamido-FNTZ and 7-amino-FNTZ were the main detected compounds. The identification of FNTZ metabolites dramatically improves the detection windows of the drug up to 37 hours.

KEYWORDS

designer benzodiazepine, flunitrazolam, HLMs, metabolism, urine

1 | INTRODUCTION

New psychoactive substances (NPS) are comprised of various narcotic or psychotropic drugs (i) that are not controlled by the 1961 United Nations Single Convention on Narcotic Drugs or the 1971 United Nations Convention on Psychotropic Substances, and (ii) that usually (but not exclusively) replicate chemical structures and/or pharmacological effects of controlled substance groups, such as opiates, ecstasy, amphetamine, cocaine or cannabis. Currently, NPS represent a significant analytical and interpretative challenge for forensic and clinical

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toxicologists.[1-3] The online availability of NPS on specialized websites has rapidly increased over the last decade. For instance, 66 new substances were detected for the first time in European countries in 2016, bringing the number of new substances monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) to more than 670.[4]

Benzodiazepines are a class of drugs widely prescribed for the treatment of anxiety, insomnia, epilepsy, muscle spasms, or alcohol withdrawal. This class of drugs can also be found diverted from medical use by various substance user groups. Indeed, the extensive research around their chemical structures resulted in a wide variety of active compounds that did not obtain marketing authorization and which are now being rediscovered and offered for sale by online e-merchants of NPS and sold as, of referred to as "designer benzodiazepines."[5] The first example of illegal use of non-prescribed benzodiazepine was phenazepam, that was developed in the Soviet Union in the 1970s. Designer benzodiazepines began to appear as recreational drugs in about 2010 and it has been suggested that more than 20 designer benzodiazepines have been detected on the NPS market.[6] The effects of designer benzodiazepines depend on the dose consumed and most common side effects observed at low doses include drowsiness, fatigue, and lethargy. At higher doses, motor coordination disorders, dizziness, mood swings, and euphoria may occur. The slow elimination of some benzodiazepines causes their accumulation in lipid-based tissues, which can lead to a delayed overdose in case of repeated consumption. Prolonged periods of consumption can develop tolerance and dependence[7] with sudden drug discontinuation leading to withdrawal symptoms.Severe adverse effects can occur when designer benzodiazepines are consumed in combination with opioids due to synergistic effects that can increase the likelihood of inducing respiratory depression.[8] Substance use is widespread among poly-drug-consumers, because designer benzodiazepines are thought to balance the euphoria that may be induced by some opioids. Some benzodiazepines have a marked amnesic effect and the consumer loses memory for a few hours after administration.[9,10]

Among this family of compounds, there are different molecules for which no controlled studies have been reported. This is the case of flunitrazolam (FNTZ) that was discovered in the 1960s but that has never been marketed. Its chemical structure (Figure 1) is close to flunitrazepam, although FNTZ has a triazolo cycle. No information about doses, effects, and tolerability is currently available.

This study focused on the detection of FNTZ and its metabolites in urine following administration of an oral dose of 0.25 mg to a human volunteer, using standard ultra-performance liquid chromatography–triple quadrupole - mass spectrometry (UPLC–QqQ–MS/MS) and UPLC–quadrupole time of flight–mass spectrometry (UPLC–QTOF–MS). In addition, FNTZ metabolism was studied in vitro after FNTZ incubation with human liver microsomes.

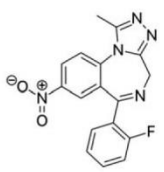

FIGURE 1 Chemical structure of flunitrazolam (C17H12FN5O2)

2 | MATERIALS AND METHODS

2.1 | Self-administration study

In order (a) to gain preliminary data on the elimination parameters of FNTZ in urine, (b) to investigate its metabolism, and (c) to be able to interpret analytical data, a male subject (56 years old, 76 kg) ingested one pink tablet bought on the Internet with a declared amount of 0.25 mg of active material. In France, given the subject was one of the authors, it was not necessary to obtain a written consent as FNTZ is not listed as a controlled substance. Urine specimens were collected over 72 hours in plastic vials without preservative and stored at +4°C until analysis. Urine was collected at various times, with no fixed interval. Volume of each urine was measured to establish the total urine FNTZ excretion. No blood sample was collected, as this required specific authorization due to the invasive nature of this procedure and specific oxygenotherapy, which was not available at the laboratory.

2.2 | Chemicals and reagents

FNTZ tablets were obtained from GR8 Research Chemicals - EU (Holland). β-OH-ethyltheophyllin (internal standard), methyl-clonazepam (internal standard), ammonium formate, formic acid, β- glucuronidase (*Helix pomatia*), alamethicin (*Trichoderma viride*), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P), were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL, prepared from livers of 36 donors, including 11 women and 25 men, aged from 31 to 80 years, was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at −80°C until use. LC–MS grade water, ethanol, methanol and acetonitrile were purchased from Biosolve (Dieuze, France) and all other chemicals were of analytical grade and obtained from common commercial sources.

2.3 | Nuclear magnetic resonance analysis

In order to confirm the purity and the amount of the FNTZ tablet (0.25 mg), a liquid chromatography high-resolution mass spectrometry detection (LC–HRMS) analysis (see section 2.6) was complemented by nuclear magnetic resonance (NMR) analysis, according to previous applications.[11,12] Briefly, the NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 MHz equipped with a 5-mm quadruple nucleus probe (QNP) probe at 295 K. The 1H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 seconds, a relaxation delay of 2 seconds and a flip angle of 30°. The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. After thin manual grinding of the powder using a

mortar for 5 minutes to obtain an homogenous item, 7.5 mg was dissolved in 600 µL of methanol-D_4 and the ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations of FNTZ. ERETIC consisted of a digitally generated Gaussian peak (on single-point calibration calibrated using 15 mg caffeine as a reference sample in 600 µL $CDCl_3$ and a control point using 10 mg of the same substance in 600 µL $CDCl_3$) inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the value peak area integrals in the spectrum give directly information about the concentration of protons sample purity.[13]

2.4 | Liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis

All urine samples were submitted to UPLC–QqQ–MS/MS analysis on an Acquity class I ultra-high performance liquid chromatography coupled to an Xevo TQD tandem mass spectrometer from Waters (Milford, MA, USA), using a screening method devoted to the identification and quantification of designer benzodiazepines.

Before analysis, urine specimens were hydrolyzed using 250 µL β-glucuronidase (10 000 U) at pH 5.2 for 12 hours at 40°C. FNTZ was extracted from 1 mL hydrolyzed urine in presence of 1 ng of diazepam-d_5 used as internal standard, 1 mL saturated borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5). After 15 minutes of agitation at room temperature, centrifugation (3000 rpm for 15 minutes) and evaporation to dryness at 45°C, the residue was reconstituted in 50 µL of 5mM ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters Acquity HSS C18 column (150 x 2.1 mm x 1.8 µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial percentage was 13% organic phase B and the final percentage, at 15 minutes, was 95% organic phase B. An injection volume of 10 µL was used in all cases. An Xevo TQD triple quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis of FNTZ and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000 L/h. To establish an appropriate multiple reaction monitoring (MRM) condition, the cone voltage was adjusted to maximize the intensity of the protonated molecule and collision induced dissociated of both species was performed. Cone voltage and collision energy were adjusted to optimize the signal for the two most abundant product ions of FNTZ: m/z 337.9 > 263.9 (60 V and 34 eV) and 337.9 > 102.8 (60 V and 40 eV), and the most abundant product ion of internal standard m/z 291.9 > 197.8 (56 V and 32 eV). MassLynx 4.1 software was used for quantification.

Linearity was observed for FNTZ concentrations ranging from 0.1 to 100 ng/mL, with a correlation coefficient of $r^2 = 0.992$. QC samples (1 and 10 ng/mL), analyzed in duplicate in ten independent experimental assays, were used for determination a coefficient of variation for precisions and accuracy. These CVs were lower than 20%. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were 0.1 ng/mL. Under the used chromatographic conditions, there was no interference with the analyses by chemicals or any extractable endogenous materials present in urine. The matrix effect (< 20%) was investigated with spiked FNTZ at 20 ng/mL in 10 blank mixed urine samples.

2.5 | In vitro metabolism

The formation of FNTZ metabolites were investigated by in vitro incubation with human liver (HLMs) and retrospective analysis of urine samples by LC–HRMS using a method previously reported.[11,12] Once thawed, HLMs (2 mg protein/mL for a final volume of 100 µL) were pre-activated by alamethicin (at 0.1 mmol/L) on ice in an intermediate volume of 50 µL in 0.1 M Tris–HCl-$MgCl_2$ (10mM $MgCl_2$ and 100mM Tris–HCl solution) at pH 7.4. This mixture was added to a 50 µL-dried residue of two different concentrations (100 µM or 500 µM) of a FNTZ methanolic solution. Fifty µL of a cofactor mixture (5mM UDPGA, 1.3mM NADPH, 3.3mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris–HCl was then added. The enzymatic reaction was performed at 37°C for 60 or 120 minutes and stopped by the addition of 200 µL of methanol and subsequently centrifuged at 4°C for 14 minutes at 32 000 g. Supernatants were frozen at −20°C until analysis. Ten µL methanol-containing methylclonazepam (0.125 mg/L) and β-OH-ethyltheophyllin at 1.6 mg/L (internal standards) together with 100 µL of ammonium formate buffer 5mM, pH 3, were added to 50 µL supernatant.

For urine samples, 10 µL of the same internals standards solutions used for the metabolite investigations were added to 1 mL hydrolyzed urine with 500 µL phosphate buffer pH 9.2, 1 M, and 3 mL of dichloromethane/ether/hexane/isoamyl alcohol (30/50/20/0.5; v/v/v/v). After centrifugation, supernatants were evaporated and 100 µL of ammonium formate buffer 5mM, pH 3/acetonitrile in 0.1 v/v % formic acid (87/13; v/v) were added.

2.6 | LC–HRMS analysis

The chromatographic separation of samples obtained from incubations with HLMs incubate or urine extracts (10 µL injected) was performed using an ACQUITY HSS C18 column (150 x 2 mm, 1.8 µm, Waters, Milford, MA, USA) placed in a column oven at 50°C. The mobile phases consisted of ammonium formate buffer 5mM, pH 3 (A) and acetonitrile in 0.1% (v/v) formic acid (B); the flow rate was 0.4 mL/min. Elution profile: initial concentration of mobile phase B (13%) held until 0.5 minutes, increased to 50% at 10 minutes, then to 95% at 10.25 minutes, and finally held until 12.25 minutes. Mass spectrometry data were acquired using an XEVO G2-XS QTOF (Waters, Manchester, UK) instrument controlled with MassLynx 4.1 software. Mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), ion spray voltage of 20 V, nitrogen as desolvation gas and argon as collision gas, source temperature at 140°C and desolvation temperature at 500°C with a desolvation gas (nitrogen) flow rate of 900 L/h. Conditions for the time of flight mass spectrometer scan mode were as follows: scan range 100–1000 m/z at 6 eV

(low energy) and 50–1000 m/z with a collision energy ramp from 10–40 eV (high energy).

2.7 | Metabolite identification

Data processing was performed using ChromaLynx, TargetLynx, MassFragment and MetaboLynx associated software packages (Waters, Manchester, UK). A software algorithm (MetaboLynx™) was used for in silico prediction of FNTZ biotransformations. Briefly, taking into account available data on the metabolism of related substances, sets of theoretically possible biotransformations of FNTZ were created based on the structure of the drug. The automated metabolite profiling process was completed through the use of the fragmentation interpretation software tool MassFragmentTM 4.1 (Waters, Manchester, UK), to enable software driven assignment of metabolite structures from fragmentation patterns. The LC–HRMS acquired data of

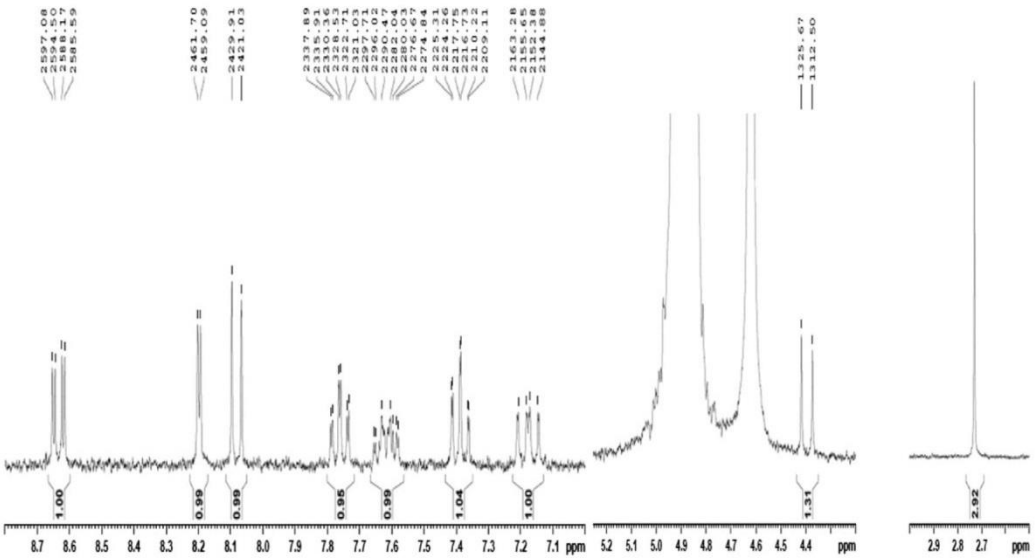

FIGURE 2 1H-NMR (MeOD) analysis of the flunitrazolam tablet including signals related to flunitrazolam [1H-NMR (MeOD): 8.63 (1H, dd, J 8.9, 2.8 Hz), 8.20 (1H, d, J 2.6 Hz), 8.08 (1H, d, J 8.9 Hz), 7.76 (1H, td, J 7.5, 1.9 Hz), 7.62 (1H, m), 7.39 (1H, td, J 7.5, 1.0 Hz), 7.18 (1H, m), 5.42 (1H, d, J 13.2 Hz), 4.39 (1H, d, J 13.2 Hz), 2.73 (3H, s)] together with other signals presumably related to powder excipients

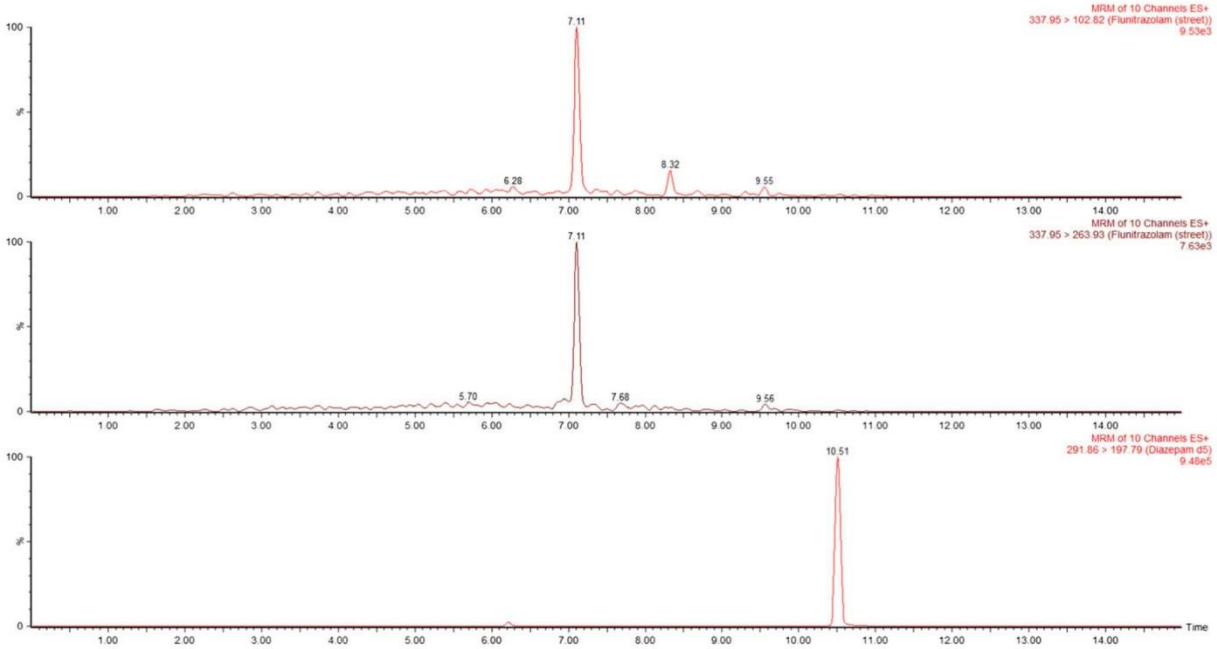

FIGURE 3 Chromatogram obtained after extraction of urine at H + 15. Flunitrazolam concentration was 2.1 ng/mL. A, B, two transitions for flunitrazolam (m/z: 337.95 > 102.82 and 337.95 > 263.93) and C, one transition for diazepam-d5 (m/z: 291.86 > 197.79) [Colour figure can be viewed at wileyonlinelibrary.com]

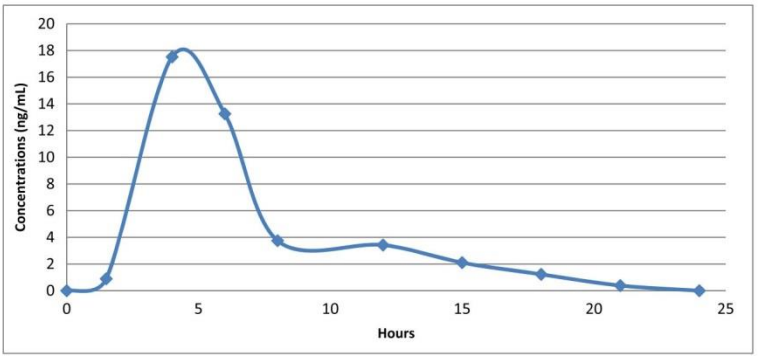

FIGURE 4 Concentration time profile of flunitrazolam excretion in urines after administration of a single 0.25 mg tablet [Colour figure can be viewed at wileyonlinelibrary.com]

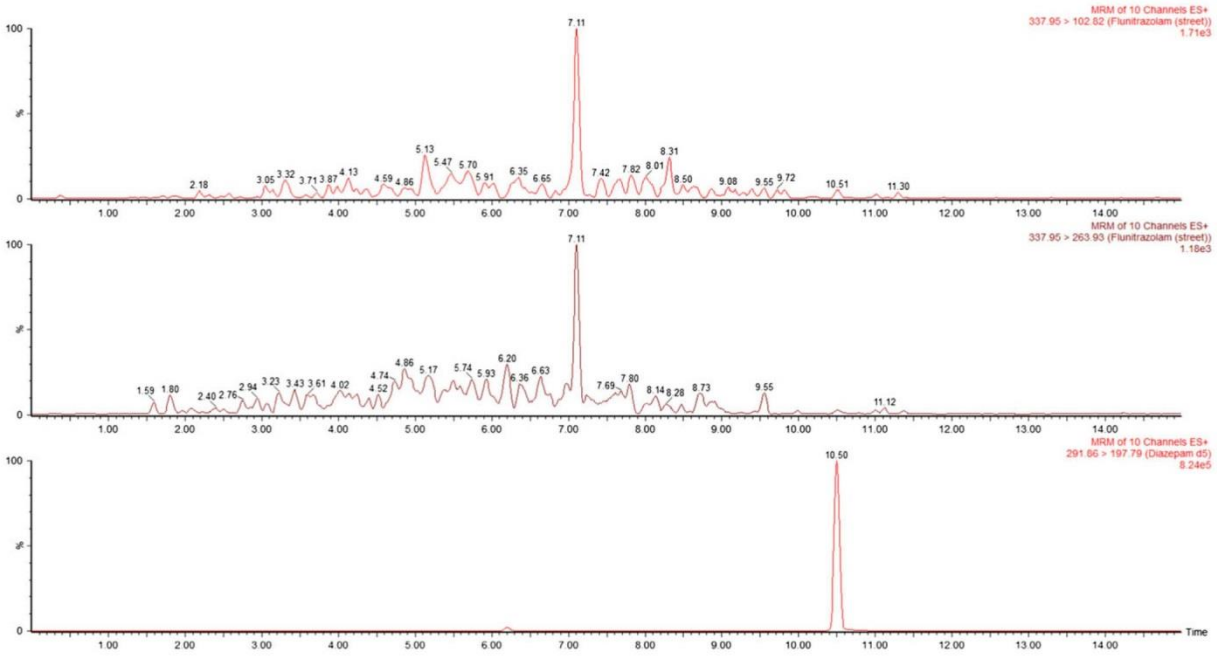

FIGURE 5 Chromatogram obtained after extraction of urine at H+21. Flunitrazolam concentration was 0.3 ng/mL. A, B, two transitions for flunitrazolam (m/z: 337.95 > 102.82 and 337.95 > 263.93) and C, one transition for diazepam-d5 (m/z: 291.86 > 197.79) [Colour figure can be viewed at wileyonlinelibrary.com]

HLM incubates are subsequently cross-checked to in silico predicted biotransformations: Criteria used for identification of the targeted compounds included 0.003 ppm as exact mass error of the precursor ion as well as product ions.

3 | RESULTS AND DISCUSSION

For identification and characterization of the FNTZ tablet, LC–HRMS and NMR spectroscopy (Figure 2) were applied. No organic impurity was detected in the FNTZ tablet using LC–HRMS or NMR. In particular, the presence of other benzodiazepines-related compounds or benzodiazepines precursors could not be confirmed. In addition, using the ERETIC method, NMR analysis revealed a 0.23 mg content in the tablet in agreement with the labeled amount of 0.25 mg. Five tablets were tested with comparable results although one cannot exclude that the ingested tablet was different.

After oral administration of one FNTZ tablet, the overall behavior of the subject was not modified. A slight sensation of weakness was noticed (feeling that it was time to go to bed for sleeping), but no physical or behavioral impairment was observed.

Unmetabolized FNTZ could be detected in urine specimens up to 21 hours using LC–MS/MS. A typical chromatogram is presented in Figure 3. The concentration time profile of FNTZ excretion in urine samples is presented in Figure 4. The drug was present at low concentrations and always below 18 ng/mL. Figure 5 shows a representative LC–MS/MS trace at an FNTZ concentration of 0.3 ng/mL. About 3% of the initially ingested dose was excreted in urine as unchanged FNTZ, during the first 21 hours. All urine specimens tested negative using the ELISA Alere Microplate kit for benzodiazepines, which was expected given the very low concentrations and the lack of cross-reactivity of the kit with nitrobenzodiazepines.

In vitro HLMs incubations and LC–HRMS analyses were performed in order to investigate the appearance of CYP- and UGT-

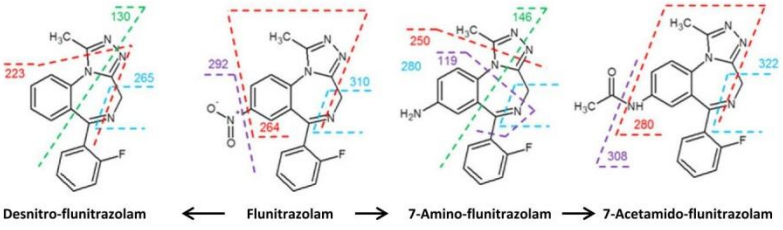

FIGURE 6 Metabolites of flunitrazolam: Proposed metabolic pathway and postulated main fragmentation pattern [Colour figure can be viewed at wileyonlinelibrary.com]

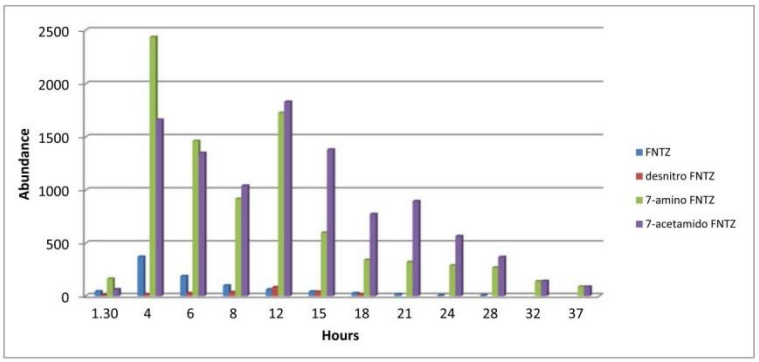

FIGURE 7 Metabolite chromatographic peak area in urine samples, observed using LC–HRMS [Colour figure can be viewed at wileyonlinelibrary.com]

dependent FNTZ metabolites.[14] Using a software algorithm, four potential FNTZ metabolites were predicted: 4-hydroxy-FNTZ, desnitro-FNTZ, 7-amino-FNTZ and 7-acetamido-FNTZ which is in agreement with observed in urine from subjects undergoing flunitrazepam treatment.[15] Regarding the two tested FNTZ concentrations (100 µM or 500 µM) and the two incubation times (60 and 120 minutes), a satisfactory metabolite production (largest chromatographic peak area related to metabolite) was observed after 2 hours at 500 µM. 7-amino-FNTZ ($C_{17}H_{14}FN_5$; m/z $[M + H]^+$ 308.1306) was detected as the only metabolite produced in vitro using HLMs under the investigated conditions. In the urine of the volunteer, 7-amino-FNTZ was detectable for a period of up to 37 hours. Desnitro- FNTZ ($C_{17}H_{13}FN_4$; m/z $[M + H]^+$ 293.1179) and 7-acetamido-FNTZ ($C_{19}H_{16}FN_5O$; m/z $[M + H]^+$ 350.1411) were also identified in urine samples, along the excretion time (Figure 6). The area responses of desnitro-FNTZ were always lower than compared to the two other metabolites and re-analysis showed chemical instability. There was no attempt to characterize the degradation product(s) in this study, it was only observed the lack of a chromatographic signal at the retention time of desnitro -FNTZ three weeks after the initial test. Hydroxylated forms of FNTZ were neither detected in HLMs incubates nor in the urine samples.

Based on the chromatographic peak areas determined from the urine samples using LC–HRMS (Figure 7), 7-acetamido-FNTZ, and mainly 7-amino-FNTZ were deemed the main urinary metabolites which remained detectable for up 37 hours after FNTZ intake. These observations are consistent with flunitrazepam[15] and meclonazepam[16] metabolic pattern. However, the window of detection of FNTZ is largely lower that the one observed for the two other benzodiazepines. Even though the data collected from this self-experiment are limited to one subject, both 7-acetamido-FNTZ, and above all 7-amino-FNTZ, could be proposed as metabolites to be recorded in HRMS libraries.

The protocol to obtain all these data has already been described for other designer benzodiazepines by Scandinavian and German groups, including flubromazolam[17,18] pyrazolam,[17] diclazepam,[19] and some designer nitrobenzodiazepines clonazolam, meclonazepam, and nifoxipam.[20]

4 | CONCLUSION

So-called benzodiazepine designer drugs belong to a class of NPS that has expanded in recent years. A typical response to legislative control is the appearance of new drug analogs, which means that people who use these substances are exposed to designer benzodiazepines where limited information exists concerning their effects, their toxicity as well as methods of analysis. The main findings of this study suggest that FNTZ is metabolized in humans into 7-acetamido-FNTZ and 7-amino-FNTZ and that the identification of FNTZ metabolites improves the detection windows of this drug.

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Article 13: Metabolism to parent 3-MeO-PCP ratio in human urine collected in two fatal cases

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Short Communication

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Metabolites to parent 3-MeO-PCP ratio in human urine collected in two fatal cases

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Abstract

In this article, two fatal cases related to the use of 3-methoxyphencyclidine (3-MeO-PCP) are described. This compound is a new psychoactive substance that belongs to the phencyclidine family. In the recent period, this dissociative drug has gained interest because of its proposal as a legally available alternative to phencyclidine in some countries. The scientific literature related to 3-MeO-PCP is very poor. Using standard ultra-performance liquid chromatography-mass spectrometry and ultra-performance liquid chromatography-tandem mass spectrometry, the authors focused on the detection of 3-MeO-PCP and its metabolites in human urine. 3-MeO-PCP metabolism was studied *in vitro* after drug incubation with human liver microsomes and the identified metabolites were investigated in the urine of the two forensic cases. 3-MeO-PCP metabolites, including *O*-demethyl-3-MeO-PCP, piperidine-hydroxy-3-MeO-PCP, *O*-demethyl-piperidine-di-hydroxy-3-MeO-PCP and piperidine-di-hydroxy-3-MeO-PCP, were detectable in the urine from both cases and the ratio between metabolites and parent 3-MeO-PCP, always lower than 1, were calculated to estimate the proportionality of metabolites. At this stage, one can conclude that testing for 3-MeO-PCP metabolites does not increase the window of detection of the drug.

Introduction

3-Methoxyphencyclidine (3-MeO-PCP) is a new psychoactive substance belonging to the family of phencyclidine (1-(1-phencyclohexyl)piperidine or PCP) which has gained renewal of interest in the recent period. 3-MeO-PCP was described for the first time in 1979 (1) and proposed at that time as legally available alternative to phencyclidine. This compound has a high affinity for the PCP-site of the glutamatergic *N*-methyl-D-aspartate (NMDA) ionotropic receptor and produces numerous psychedelic effects which are characteristic of so-called dissociative anesthetics, such as ketamine. Clinical effects include dissociation from the physical body, visual and auditive hallucinations, altered time and space, and change of consciousness (2). 3-MeO-PCP is usually snorted, smoked, ingested as a tablet, or injected intravenously or subcutaneously, with an abuse dosage estimated between 3 and 20 mg (3). It produces many side effects including psychosis, confusion, violent behavior, hypertension, tachycardia and suicidal impulses, but little information is available on this compound. The authors have recently worked on two fatal cases related to 3-MeO-PCP use (4)

Case 1 was a 39-year-old woman with a history of illicit drug use who was found dead at home after being assaulted by her partner. External examination of the victim confirmed the presence of parched, horizontal ligature mark encircling the neck. A broad band-like red area, due to intense subcutaneous hemorrhage and a clear ligature mark consisting of abrasions were visible. Autopsy showed fractures with associated hemorrhage of superior horn of the thyroid cartilage and of the right greater horns of hyoid bone, respectively. Intense multi viscera congestion was noticed. During the autopsy, femoral blood, bile, urine and hair were collected for toxicological investigations (4). Case 2 was a 41-year-old man who was found dead at home. His body was lying on the floor with several traces of needle marks and a large amount of dried blood

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around his mouth. There was no trace of violence or traumatic lesions on the body that could explain his death. Only an external body examination was performed where cardiac blood, peripheral blood, urine and hair specimens were collected for toxicological investigations. At the time, the authors presented the results to the legal authorities, it was frustrating to not being able to test for 3-MeO-PCP metabolites in urine, due to a lack of available methodology.

In the scientific literature, there is only one *in vitro* study characterizing 3-MeO-PCP metabolites (5). This study was performed on human liver microsomes followed by *in vivo* tests based on rat urine. It revealed the existence of about 30 metabolites, but nothing was done on human specimens. More recently, a second study proposed the identification of 3-MeO-PCP metabolites in human urine but did not interpret the results (6). The authors found the presence of putative hydroxylated metabolites *cis* and trans-1-[1-(3-methoxyphenyl)−4-hydroxycyclohexyl]-piperidine (*cis*-trans-3-MeOP-PCP) and 1-[1-(3-methoxyphenyl)cyclohexyl]4-hydroxypiperidine (3-MeO-PCHP). These two studies did not discuss the interest of testing for 3-MeO-PCP metabolites in the case of intoxication.

In this paper, the authors focused on the detection of 3-MeO-PCP and its metabolites in human urine collected in the two fatal cases previously described, using standard ultra-performance liquid chromatography-mass spectrometry (UPLC–MS) and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS-MS). 3-MeO-PCP metabolism was studied *in vitro* after drug incubation with human liver microsomes which allowed the production of several metabolites. In a second time, they were compared to the responses obtained in the two authentic human specimens.

Materials and Methods

Chemical

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99–100%, AnalaR NORMAPUR and 36% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). 3-MeO-PCP, β-glucuronidase (Helix pomatia), alamethicin (Trichoderma viride), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P) were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at −80°C until use. HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany). MDMA-d5 was purchased from Lipomed (Arlersheim, Switzerland) and was diluted to appropriate concentrations using methanol, according to the manufacturer's recommendations.

3-MeO-PCP analysis by UPLC–MS-MS

Biological specimens were submitted to UPLC–MS-MS analysis using a specific method devoted to the identification and quantification of 3-MeO-PCP (4). Briefly, the drug was extracted from 1 mL of fluid (blood, hydrolyzed urine) in presence of MDMA-d5 used as internal standard, with saturated borate buffer pH 9.5 and a mixture of ether/dichloromethane/hexane/isoamylalcohol (50/30/20/0.5). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in ammonium formate buffer adjusted at pH 3. Liquid chromatography was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile LC–MS (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 13% organic phase B and the final gradient, at 15 min, was 95% organic phase B. An injection volume of 10 µL was used in all cases. A Xevo TQD triple quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ES+). The following conditions were found to be optimal for the analysis: capillary voltage at 1.5 kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1,000 L/h. Collision energy was adjusted to optimize the signal for the two most abundant product ions of 3-MeO-PCP: m/z 274.0 > 120.9 and 274.0 > 188.9, and the most abundant product ion of internal standard m/z 198.9 > 164.9. Transition m/z 274.0 > 120.9 was used for quantification of 3-MeO-PCP. MassLynx 4.1 software was used for quantification.

Linearity was observed in blood for 3-MeO-PCP concentrations ranging from 10 to 1,000 ng/mL, with a correlation coefficient of 0.998. QC samples (50 ng/mL and 200 ng/mL), analyzed in duplicate in six independent experimental assays, were used for determination a coefficient of variation for precision and accuracy. These CVs were lower than 20%.

In vitro metabolism

The formation of 3-MeO-PCP metabolites were investigated by *in vitro* incubation with human liver microsomes (HLMs). Once thawed, HLMs (2 mg protein/mL for a final volume of 100 µL) were pre-activated by alamethicin (at 0.1 mmol/L) on ice in an intermediate volume of 50 µL in 0.1 M Tris-HCl-MgCl2 (10 mM MgCl2 and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50-µL dried residue of 100 mg/L of a 3-MeO-PCP methanolic solution. Fifty µL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37°C for 120 min and stopped by the addition of 200 µL of methanol, subsequently centrifuged at 4°C for 10 min at 14,000 tr/min. Ten µL of supernatant was immediately injected and analyzed by ULPC–MS.

3-MeO-PCP and metabolites analysis by UPLC–MS

For urine samples, 3-MeO-PCP was extracted from 1 mL hydrolyzed urine in presence of 100 ng of MDMA-d5, with 1 mL saturated borate buffer pH 9.5 and 5 mL of a mixture of dichloromethane/ether/hexane/isoamylalcohol (30/50/20/0.5). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 50 µL of ammonium formate buffer adjusted at pH 3 and 10 µL was injected and analyzed by UPLC–MS. Chromatography was achieved using a Waters HSS C18 column (150 × 2.1 mm × 1.8 µm) maintained at 50°C in a thermostatically controlled enclosure. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile LC–MS (mobile phase B) as mobile phases at flow rate of 0.4 mL/min. The initial gradient was 87% mobile phase A and the final gradient, at 7 min, was 5% mobile phase A. An injection volume of 10 µL was used in all cases. A Xevo triple quadrupole mass spectrometer was used, but operating in the single MS

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Table I. Metabolites observed *in vitro* using human liver microsomes (name, elemental composition, mass, characteristic ions and retention time)

Metabolites	Elemental composition	Mass (m/z) [M+H$^+$]	Characteristic ions	RT (min)
3-MeO-PCP	$C_{18}H_{28}NO$	274.2	274.2–86.1–118.1–122.1–189.1	6.6
O-demethyl-3-MeO-PCP	$C_{17}H_{26}NO$	260.2	260.2–86.1–122.0–175.2	4.4
Piperidine-HO-3-MeO-PCP	$C_{18}H_{28}NO_2$	290.3	290.3–104.4–118.3–122.1–189.1	5.3
O-demethyl-piperidine-di-HO-3-MeO-PCP	$C_{17}H_{26}NO_3$	292.4	292.4–104.1–118.2–122.1–189.1	5.9
Piperidine-di-HO-3-MeO-PCP	$C_{18}H_{28}NO_3$	306.4	306.4–104.1–118.2–122.1–189.1	5.9
Cyclohexyl-HO-piperidine-di-HO-3-MeO-PCP	$C_{18}H_{28}NO_4$	322.3	322.3–118.3–122.1–189.1	5.1

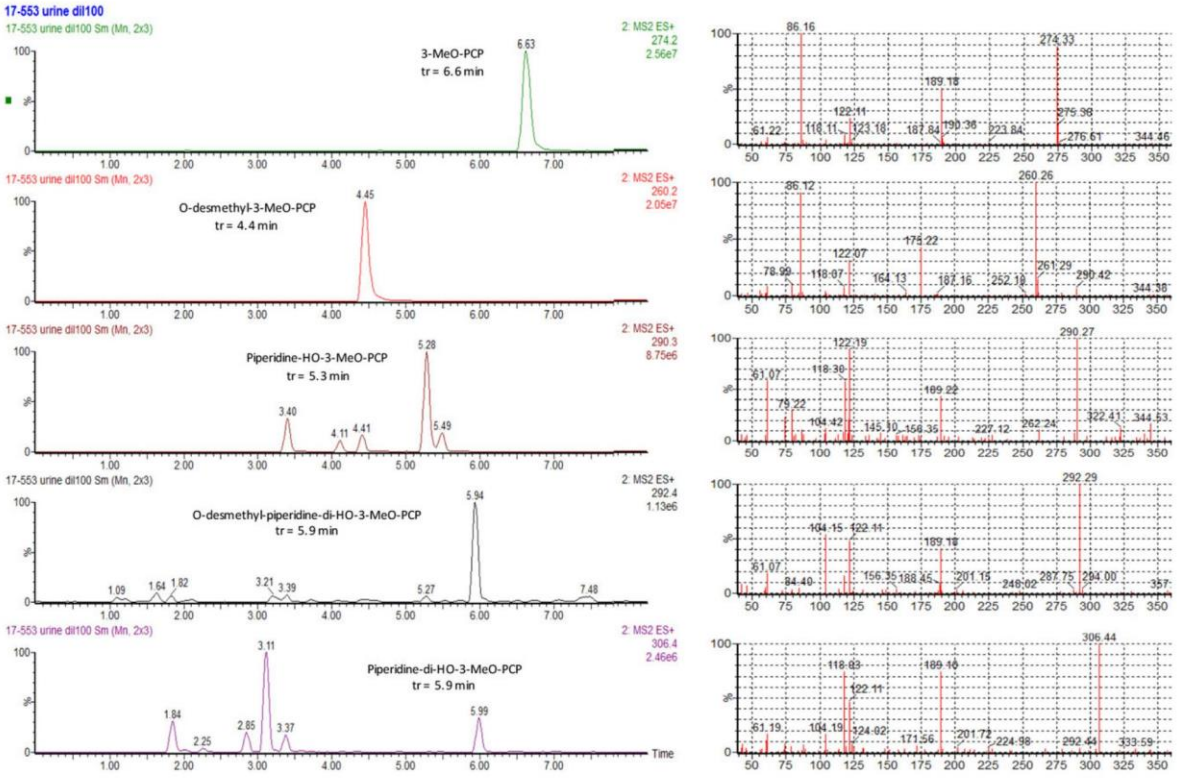

Figure 1. The mass spectral data for each identified metabolites and parent 3-MeO-PCP in Case 2. From top to bottom: 3-MeO-PCP ($C_{18}H_{27}NO_2$; m/z [M+H$^+$] 274.2), *O*-demethyl-3-MeO-PCP ($C_{17}H_{26}NO$; m/z [M+H$^+$] 260.2), Piperidine-hydroxy-3-MeO-PCP ($C_{18}H_{28}NO_2$; m/z [M+H$^+$] 290.3), *O*-demethyl-piperidine-di-hydroxy-3-MeO-PCP ($C_{17}H_{26}NO_3$, m/z [M+H$^+$] 292.4) and Piperidine-di-hydroxy-3-MeO-PCP ($C_{18}H_{28}NO_3$; m/z [M+H$^+$] 306.4).

mode. Ionization was achieved using electrospray in the positive ionization mode (ES+). The following conditions were found to be optimal for the analysis of 3-MeO-PCP, metabolites and the internal standard: capillary voltage at 3 kV; source block temperature at 149°C; desolvation gas nitrogen heated at 400°C and delivered at a flow rate of 800 L/h. In order to obtain appropriate condition, the cone voltage was tested at different values (from 20 to 90 V) to maximize the intensity of the protonated molecular ion of each compound. The range of m/z was between 40 and 680. MassLynx 4.1 software was used for identification.

Results and Discussion

In Case 1, 3-MeO-PCP was identified in femoral blood and urine at 63 and 94 ng/mL, respectively. In Case 2, 3-MeO-PCP was identified in femoral blood and urine at 498 and 16,700 ng/mL, respectively. The femoral blood concentrations were included in the fatal values reported in the literature (range 50–3,200 ng/mL) (7–9).

In vitro HLMs incubation was performed in order to investigate the appearance of CYP- and UGT-dependent 3-MeO-PCP metabolites. A satisfactory metabolite production was observed after 2 h incubation at 100 mg/L of the drug. *O*-demethyl-3-MeO-PCP ($C_{17}H_{26}NO$; m/z [M+H$^+$] 260.2), piperidine-hydroxy-3-MeO-PCP ($C_{18}H_{28}NO_2$; m/z [M+H$^+$] 290.3), *O*-demethyl-piperidine-di-hydroxy-3-MeO-PCP ($C_{17}H_{26}NO_3$, m/z [M+H$^+$] 292.4), piperidine-di-hydroxy-3-MeO-PCP ($C_{18}H_{28}NO_3$; m/z [M+H$^+$] 306.4) and cyclohexyl-hydroxy-piperidine-di-hydroxy-3-MeO-PCP ($C_{18}H_{28}NO_4$; m/z [M+H$^+$] 322.3) were detected *in vitro* using HLMs under the investigated conditions. The chromatographic and spectral data for the metabolites are described in Table I. The mass spectral data from Case 2 are presented in Figure 1. These metabolites were also reported by Michely *et al.* (5). These authors reported some other minor species, along with several isomers,

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Table II. Metabolites detected in urines of the two described cases and ratio (peak area) between metabolites and parent drug (ND: no detected). Area of each compound was acquired at 35 V (cone voltage) and the protonated ion was recorded

Metabolites	Case 1 (ratio %)	Case 2 (ratio %)
3-MeO-PCP	100 (94 ng/mL)	100 (16,700 ng/mL)
O-demethyl-3-MeO-PCP	69	72
Piperidine-HO-3-MeO-PCP	25	25
O-demethyl-piperidine-di-HO-3-MeO-PCP	7	3
Piperidine-di-HO-3-MeO-PCP	7	2
Cyclohexyl-HO-piperidine-di-HO-3-MeO-PCP	ND	ND

using liquid chromatography coupled to high resolution mass spectrometry.

In the urine of both fatal cases, four from five metabolites obtained after *in vitro* HLMs incubation were identified and presented in Table II. 3-MeO-PCP metabolites reference standards are not available for purchase making impossible any quantification. However, the ratio between each metabolites and parent 3-MeO-PCP (based on peak area) allows to estimate its analytical response in the full spectrum mode. The identification of metabolites strengthens the specific analysis of 3-MeO-PCP in these two fatal cases, but does not seem to increase the window of detection in case of intoxication, as the ratio metabolites to parent drug was always lower than 1. However, it is possible that some ratios would increase at some points of the parent drug excretion curve. Due to a lack of controlled studies, it is not possible to firmly conclude that one or several metabolite(s) would be present while 3-MeO-PCP detection would be negative, for example at the end of drug excretion. This point will have to be clarified by extending the number of cases analyzed.

Conclusion

3-MeO-PCP metabolism was studied *in vitro* using human liver microsomes and allowed the identification of *O*-demethyl-3-MeO-PCP, piperidine-hydroxy-3-MeO-PCP, *O*-demethyl-piperidine-di-hydroxy-3-MeO-PCP, piperidine-di-hydroxy-3-MeO-PCP and cyclohexyl-hydroxy-piperidine-di-hydroxy-3-MeO-PCP. For the first time, these metabolites have been identified in human urine specimens obtained in two fatal cases. The ratio between each metabolite and parent 3-MeO-PCP, always lower than 1, shows, at this time, that there is lack of evidence for testing 3-MeO-PCP metabolites in case of acute intoxication.

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Article 14: Simple strategy to identify drug metabolites by UPLC-MS. Demonstration with deschloroetizolam

Alice Ameline, Camille Richeval, Jean-Michel Gaulier, Jean-Sébastien Raul, Pascal Kintz

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SIMPLE STRATEGY TO IDENTIFY DRUG METABOLITES BY UPLC-MS

Demonstration with deschloroetizolam

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Introduction

The fate of xenobiotics in the body is based on the phases of absorption, distribution, metabolism and excretion. After absorption and distribution into the body, xenobiotics can be eliminated totally or partially in its unchanged form, accumulate in the body or undergo chemical transformation. The chemical transformation is the most frequent process and is most often hepatic. These transformation reactions, also called biotransformation or metabolism, modify the structure of xenobiotics to form metabolites. Depending on the nature of the metabolite, toxic or not, this new form is called biodetoxication or biotoxication [1]. Metabolites identification has a major interest to analysts as they are specific biomarkers of any drug consumption and generally have a longer identification time period in the urine versus the parent compound. A metabolite is usually more polar than its parent molecule to facilitate its elimination in the urine. However, the identification of metabolites of a new drug, and particularly of a new psychoactive substance (NPS) is not easy. For most substances from this group, very little information is available in the literature. For most NPS, scientists have little knowledge about their pharmacokinetic and production of identifiable metabolites. Unfortunately, even once identified, most of these metabolites are not available for purchase. To identify potential metabolites, a common approach is to use human liver microsomes that can imitate the production of metabolites by the liver. Then, after production, it is possible to identify the candidates by chromatography coupled to high resolution mass spectrometry. However, this technology is very expensive and not available to all laboratories. As an alternative at lower costs, the use of liquid chromatography coupled with mass spectrometry can be a possibility, in combination with literature review and mass spectra interpretation.

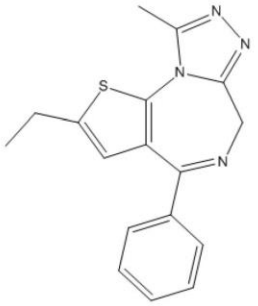

Figure 1. Chemical structure of Deschloroetizolam

To illustrate this analytical strategy, the authors used deschloroetizolam, a molecule from the family of designer benzodiazepines, as test molecule (Figure 1). This molecule is a psychoactive drug of the thienodiazepines class that, as do benzodiazepines, binds to modulatory sites on the GABA receptors [2]. Deschloroetizolam is closely related to etizolam, triazolam, and alprazolam, and is not recognized as a controlled substance in many parts of the world. This compound is supposed to have a rapid onset of action (between 1 and 5 minutes) and is half as potent as etizolam, with a duration of effects twice as long (between 8 and 10 hours). About 2 mg of deschloroetizolam are thought to have a similar potency of 10 mg of diazepam according to some "unofficial" websites. Deschloroetizolam presents physical effects (sedation, respiratory depression, muscle relaxation, and dizziness)

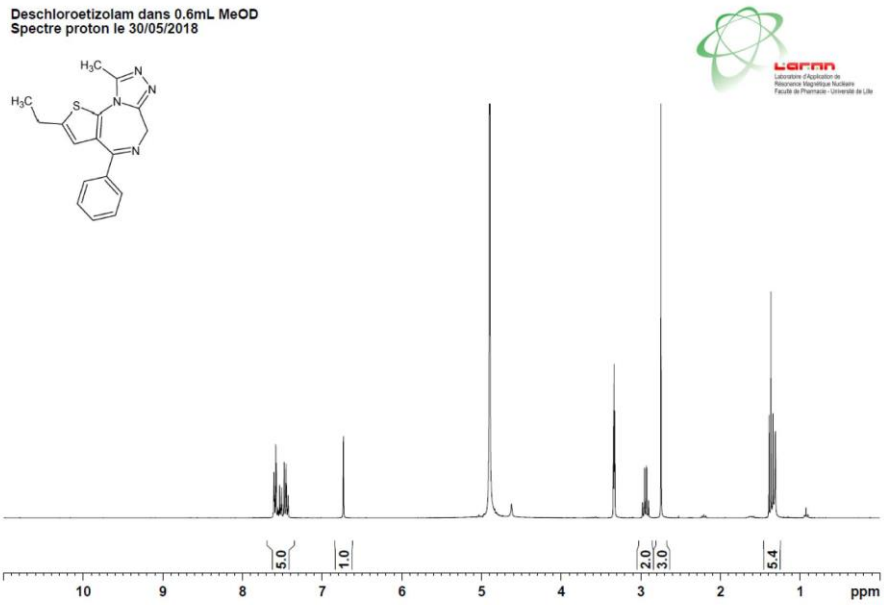

FIGURE 2. 1H-NMR (MEOD) ANALYSIS OF THE DESCHLOROETIZOLAM TABLET

and cognitive effects (amnesia, anxiety suppression, thought deceleration, analysis suppression, disinhibition, euphoria, delusion of sobriety, and dream potentiation). All these effects have been reported based upon personal experiences of abusers [3].

Papers dealing with deschloroetizolam are seldom available in the scientific literature. A comprehensive review only identified one paper, presenting several metabolites after an in vitro study [4]. It seems that there is no data dealing with authentic human specimens. It is therefore of interest to add new data to the existing literature.

In this paper, the authors focused on the detection of deschloroetizolam metabolites in human urine after controlled administration and its interest to increase the window of detection of the drug.

Materials and methods

Chemical

Deschloroetizolam tablets were obtained from GR8 Research Chemicals-EU (Holland). Ammonium formate 99 % was provided by Alfa Aesar (Schiltigheim, France). Formic acid 99-100 %, AnalaR NORMAPUR and 36 % hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). β-glucuronidase (Helix pomatia), alamethicin (Trichoderma viride), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase (G6PD), glucose-6- phosphate (G6P) were all purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Tetra-sodium salt of a reduced form of nicotinic acid adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany). A pool of human liver microsomes (HLMs) at a concentration of 20 mg of microsomal protein/mL was purchased from Biopredic (La Bretèche, Saint Grégoire, France) and preserved at -80°C until use. HPLC-grade acetonitrile (ACN), methanol (MeOH), dichloromethane, hexane and ethyl acetate were obtained from Merck (Darmstadt, Germany). MDMA-d5 was purchased from Lipomed (Arlersheim, Switzerland) and was diluted to appropriate concentrations using methanol, according to the manufacturer's recommendations.

Analytical protocol

The detection of deschloroetizolam metabolites was studied in human urine collected from a test subject (male, 57-year old, 78 kg) who ingested a tablet bought on the internet. This tablet contained 6 mg of active material according to the vendor. The first step was to check the purity and the concentration of the tablet by nuclear magnetic resonance (NMR) spectroscopy. Urine samples were collected over 72 hours in plastic vials with no preservative and stored at +4°C until analysis.

Urine samples were analysed using UPLC-MS/MS in the MRM mode to test for deschloroetizolam. It was possible to fix 2 specific ion transitions as deschloroetizolam was available as reference standard. Then, the same samples were analysed by full scan UPLC-MS in order to identify the metabolites, based on their retention time and their mass spectra. Finally, the unambiguous characterization of the metabolites was confirmed after production by HLMs and definitive identification by high resolution mass spectrometry (UPLC-HRMS).

Nuclear Magnetic Resonance analysis

In order to confirm the purity of the deschloroetizolam tablet and the absence of contamination products, a NMR spectroscopy analysis was realized, according to a previous application [5]. Briefly, the NMR spectra were recorded on AVANCE 300 (Bruker Biospin, France) operating at 300 MHz equipped with a 5 mm quadruple nucleus probe (QNP) probe at 295 K. The 1H spectra were recorded with 64 scans, a 32-K time-domain data points with 4800 Hz spectral width, an acquisition time of 3.42 s, a relaxation delay of 2 s and a flip angle of 30°.

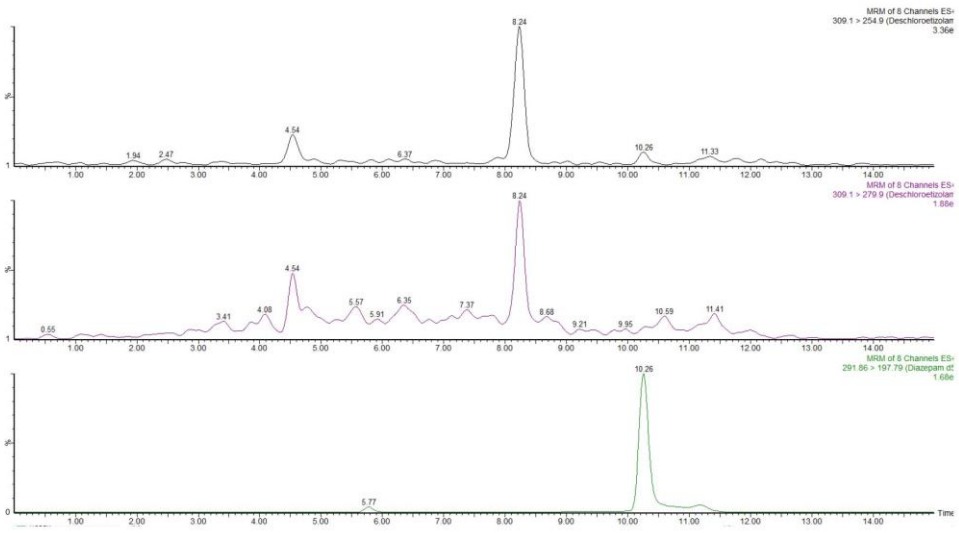

Figure 3. Chromatograms obtained after extraction of urine at H+30. Deschloroetizolam concentration was 1.9 ng/mL. From top to bottom: 2 transitions of deschloroetizolam (m/z: 309.1 > 254.9 and 309.1 > 279.9) and 1 transition of diazepam-d5 (m/z: 291.9 > 197.8)

The Free Induction Decays (FIDs) were transformed (0.3 Hz broadening) and the baseline was corrected. After thin manual grinding of the powder using a mortar for 5 min to obtain an homogeneous item, 7.5 mg was dissolved in 600 µL of methanol-D4 and the ERETIC (Electronic REference To access In vivo Concentrations) method was used for the determination of absolute concentrations of deschloroetizolam. ERETIC consisted of a digitally generated Gaussian peak (on single point calibration calibrated using 15 mg caffeine as a reference sample in 600 µL CDCl3 and a control point using 10 mg of the same substance in 600 µL CDCl3) inserted into the spectrum of a sample by the software after processing the FID. With ERETIC, the value peak area integrals in the spectrum give directly information about the concentration of protons sample purity [6].

Analysis of deschloroetizolam by UPLC-MS/MS

All urine samples were submitted to UPLC-MS/MS analysis on an Acquity class I ultra-high performance liquid chromatography coupled to a Xevo TQD tandem mass spectrometer from Waters (Milford, MA, USA), using a screening method devoted to the identification and quantification of designer benzodiazepines. Before analysis, urine specimens were hydrolyzed using 250 µL β-glucuronidase (10000 U) at pH 5.2 for 12 h at 40 °C. Deschloroetizolam was extracted from 1 mL hydrolyzed urine in presence of 1 ng of diazepam-d5 used as internal standard, 1 mL saturated borate buffer pH 9.5 and 5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5). After 15 min of agitation at room temperature, centrifugation (3000 rpm for 15 min) and evaporation to dryness at 45 °C, the residue was reconstituted in 50 µL of 5 mM ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters Acquity HSS C18 column (150 x 2.1 mm x 1.8µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial percentage was 13% organic phase B and the final percentage, at 15 min, was 95% organic phase B. An injection volume of 10 µL was used in all cases. A Xevo TQD triple

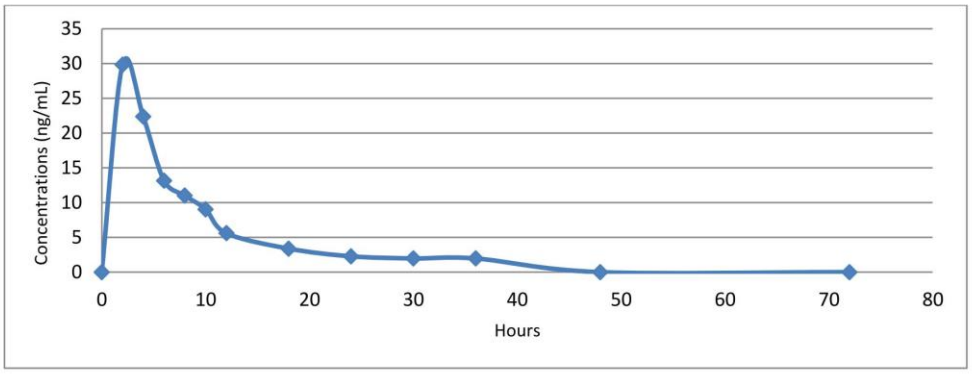

Figure 4. Concentration time profile of deschloroetizolam excreted in urine after administration of a single 5.3 mg tablet

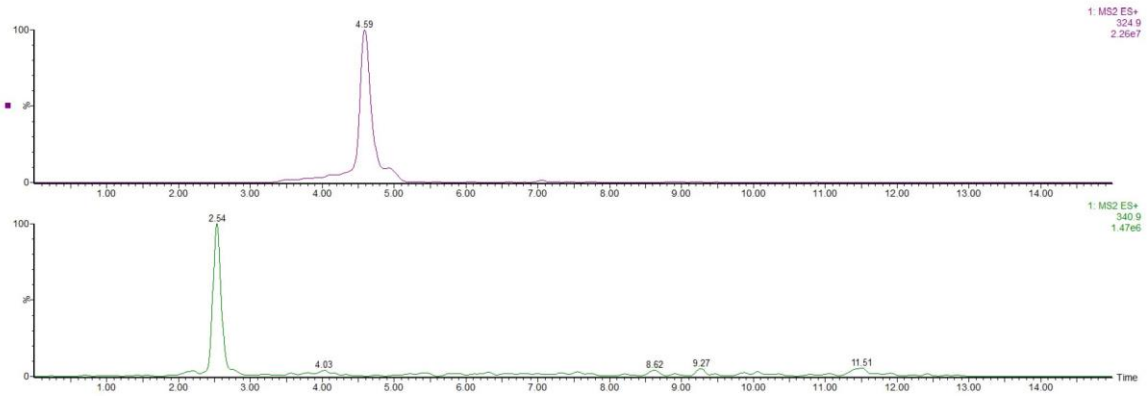

Figure 5. Chromatograms obtained after analyzed of supernatants in vitro study. From top to bottom: peak of monohydroxy-deschloroetizolam (MH+=324.9 g/mol, retention time at 4.59 min) and peak of dihydroxy-deschloroetizolam (MH+=340.9 g/mol, retention time at 2.54 min)

quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ESI+). The following conditions were found to be optimal for the analysis of deschloroetizolam and the internal standard: capillary voltage at 1.5kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000L/h. In order to establish appropriate multiple reaction monitoring condition, the cone voltage was adjusted to maximize the intensity of the protonated molecule and collision induced dissociated of both species was performed. Cone voltage and collision energy were adjusted to optimize the signal for the 2 most abundant product ions of deschloroetizolam: m/z 309.1 > 254.9 (56 V and 26 eV) and 309.1 > 279.9 (56 V and 24 eV), and the most abundant product ion of internal standard m/z 291.9 > 197.8 (56 V and 32 eV). These conditions were already published (Ameline and Arbouche, 2018). MassLynx 4.1 software was used for quantification.

Linearity was observed in urine for deschloroetizolam concentrations ranging from 1 to 100 ng/mL, with a correlation coefficient of $r^2 = 0.998$. QC samples (1 and 10 ng/mL), analysed in duplicate in ten independent experimental assays, were used for determination a coefficient of variation for precisions and accuracy. These CVs were lower than 20 %. The limit of detection and the lower limit of quantification were 0.01 and 1 ng/mL, respectively. Under the used chromatographic conditions, there was no interference with the analyses by chemicals or any extractable endogenous materials present in urine. The matrix effect (< 20 %) was investigated with spiked deschloroetizolam at 10 ng/mL in 10 blank urine samples.

Analysis of deschloroetizolam metabolites by UPLC-MS

Urine samples were extracted by the same protocol as to identify deschloroetizolam (see paragraph Analysis of deschloroetizolam by UPLC-MS/MS). A Xevo triple quadrupole mass spectrometer was used, but operating in the single MS full scan mode, in the range of m/z between 40 and 680. Ionization was achieved using electrospray in the positive ionization mode (ES+). The following conditions were found to be optimal for the analysis of deschloroetizolam metabolites and the internal standard: capillary voltage at 3 kV; source block temperature at 149°C; desolvation gas nitrogen heated at 400°C and delivered at a flow rate of 800 L/h. In order to obtain appropriate condition, the cone voltage was tested at different values (from 20 to 90 V) to maximize the intensity of the protonated molecular ion of each compound. MassLynx 4.1 software was used for identification.

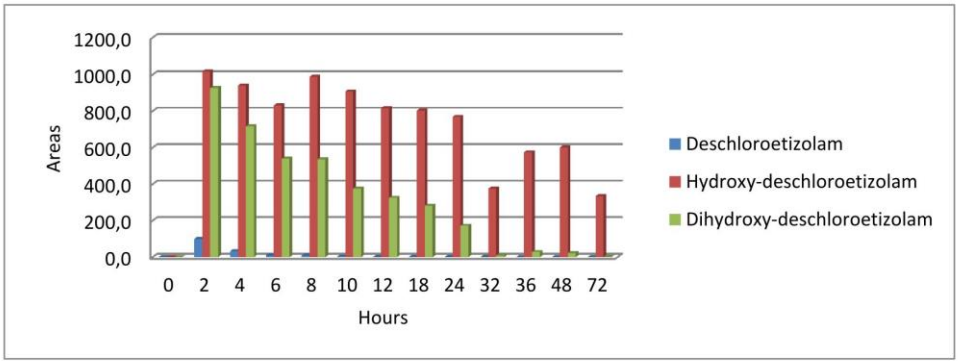

Figure 6. Concentration time profile of deschloroetizolam excreted in urine after administration of a single 5.3 mg table

Metabolites	Elemental composition	Mass (m/z) [M+H+]	Characteristic ions	RT (min)
Deschloroetizolam	$C_{17}H_{16}N_4S$	309.1	254.9 – 279.9	8.24
Hydroxy-deschloroetizolam	$C_{17}H_{17}N_4SO$	324.9	270.9 – 296.9	4.59
Dihydroxy-deschloroetizolam	$C_{17}H_{18}N_4SO_2$	340.9	287.0 – 95.9	2.54

Table 1. Chromatographic and spectra data, obtained after in vitro study, using HLMs (name, elemental composition, mass [MH+], characteristic ions and retention time)

In vitro metabolism

The formation of deschloroetizolam metabolites were verified and investigated by in vitro incubation with human liver microsomes (HLMs) [7]. Once thawed, HLMs (2 mg protein for a final volume of 100 µL) were pre-activated by alamethicin (at 0.1 mmol/L) on ice in an intermediate volume of 50 µL in 0.1 M Tris-HCl-MgCl2 (10 mM MgCl2 and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a 50 µL-dried residue of 100 mg/L of a deschloroetizolam methanolic solution. Fifty µL of a cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37 °C for 120 min and stopped by the addition of 200 µL of methanol, subsequently centrifuged at 4 °C for 10 min at 14000 tr/min. Ten µL of supernatant was immediately injected and analysed by ULPC-MS.

UPLC-HRMS analysis

The chromatographic separation of samples obtained from incubations with HLMs incubate or urine extracts (10 µL injected) was performed using an Waters (Milford, MA, USA) ACQUITY HSS C18 column (150 x 2 mm, 1.8 µm, Waters) placed in a column oven at 50 °C. The mobile phases consisted of ammonium formate buffer 5mM, pH 3 (A) and acetonitrile in 0.1% (v/v) formic acid (B); the flow rate was 0.4 mL/min. Elution profile: initial concentration of mobile phase B (13 %) held until 0.5 min, increased to 50 % at 10 min, then to 95 % at 10.25 min, and finally held until 12.25 min. Mass spectrometry data were acquired using a XEVO G2-XS QTOF (Waters, Manchester, UK) instrument controlled with MassLynx 4.1 software. Mass spectrometric conditions were as follows: positive electrospray ionization interface (ESI+), ion spray voltage of 20 V, nitrogen as desolvation gas and argon as collision gas, source temperature at 140 °C and desolvation temperature at 500 °C with a desolvation gas (nitrogen) flow rate of 900 L/h. Conditions for the time of flight mass spectrometer scan mode were as follows: scan range 100-1000 m/z at 6 eV (low energy) and 50-1000 m/z with a collision energy ramp from 10-40 eV (high energy).

Results and discussion

For identification and characterization of the deschloroetizolam tablet, NMR spectroscopy was applied. No organic impurity was detected in the deschloroetizolam tablet (Figure 2). In particular, the presence of other benzodiazepines-related compounds or benzodiazepines precursors could not be confirmed. In addition, using the ERETIC method, NMR analysis revealed a 5.3 mg content in the tablet in agreement with the labeled amount of 6.0 mg. Four tablets were tested with comparable results although one cannot exclude that the ingested tablet was different.

The first step of this study was to analyse all urine samples by UPLC-MS/MS to determine the concentrations of deschloroetizolam and the time of the last positive specimen after an oral consumption of 5.3 mg. In the urine of the volunteer, parent deschloroetizolam was detected up to 36 hours after inhalation. A typical chromatogram is presented in Figure 3. The concentration time profile of deschloroetizolam excretion in urine is presented in Figure 4. A very rapid increase was observed after the consumption of the tablet with a concentration peak at H+2, then a decrease in a few hours. From the 36th hour, deschloroetizolam is no longer identifiable. Drug concentrations were always below 30 ng/mL. The concentration profile of deschloroetizolam in urine was never described in the literature, as this is the first real case of demonstration of deschloroetizolam controlled excretion in the urine.

Afterwards, the urine samples were analysed by full scan UPLC-MS in order to test for the presence of metabolite(s). According to the available information in the literature, only one in vitro study was performed (Huppertz, 2015) and has identified four possible metabolites undergoing phase I (three monohydroxylated and one dihydroxylated), which were detected by QTOF analysis. According to the structural similarities with etizolam, the hydroxylation sites are conveniently 9-methyl, 2-ethyl and the 6-position (see Figure 1). The combination of hydroxylation which gives the dihydroxylated metabolite is not known to date [8]. This hypothesis has never been verified in human authentic specimens, but only after using HLMs. Based on the molar weight of deschloroetizolam, MM = 308.4 g/mol (C17H16N4S), the three monohydroxylated metabolites must have a molar weight of 324.4 g/mol (308.4 g/mol + 16 for the addition of one oxygen atom). For the dihydroxylated metabolite, its predictable weight must be 340.4 g/mol (308.4 g/mol + 2x16 g/mol, for two additional oxygen atoms). These three molar weights (308.4; 324.4 and 340.4 g/mol) are therefore those to be targeted by full scan LC/MS to demonstrate the presence of deschloroetizolam and its metabolites in urine. Two of the 4 metabolites described in the scientific literature (Huppertz, 2015) were identified in the urine specimens of the volunteer, including one monohydroxylated metabolite (C17H17N4SO; MH+ = 324.9 g/mol) and the dihydroxylated metabolite (C17H18N4SO2; MH+ = 340.9 g/mol). Chromatograms are presented in Figure 5. The two metabolites (2.54 and 4.59 min) were eluted from the chromatographic column earlier that the parent drug (8.24 min), which is consistent with the use of a reverse stationary phase (a C18 column), due to a higher polar chemical behaviour (Table 1).

Based on the chromatographic peak areas determined from the urine samples using UPLC-MS (Figure 6), the two metabolites were deemed the main urinary metabolites which remained detectable for up 72 hours after deschloroetizolam intake. The identification of deschloroetizolam metabolites strengthens the specific analysis of deschloroetizolam, and seems to greatly increase the window of detection in case of consumption.

The unambiguous confirmation of the metabolites identified by UPLC-MS was then realized by combination between HLMs and UPLC-HRMS. In vitro HLMs incubation was performed in order to investigate the appearance of CYP- and UGT-dependent deschloroetizolam metabolites. A satisfactory metabolite production was observed after 2 hours incubation at 100 mg/L of the drug. Monohydroxy-deschloroetizolam and dihydroxy-deschloroetizolam were detected in vitro under the investigated conditions. The analyses by UPLC-HRMS allowed identify the same two metabolites, up to 72 hours, as with the UPLC-MS, confirming the utility of alternative technology, less expensive than the reference high resolution mass spectrometry. This is an advantage from an academic point of view, for toxicological risk assessments, and development of urine screening approaches.

Conclusion

In toxicology, the identification of metabolites of a drug has a major interest to analysts, because they allow to increase the window of detection after consumption. Unfortunately, due to paucity of literature, particularly for NPS, there is little knowledge about the pharmacokinetics of these drugs. In most cases, the identity of the corresponding metabolites is unknown. Sometimes, the only studies which are available are those performed in vitro using HLMs or animal model(s). This was the case of deschloroetizolam, a designer benzodiazepine, which was never reported in authentic human urine. In this paper, it has been described that when only bench-top LC-MS is available, the identification of metabolites is not easy, but feasible. Two of the four metabolites already described in the literature after in vitro study have been identified by this simple method and were subsequently confirmed by in vitro method and UPLC-HRMS.

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TIAFT AWARDS

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Article 15: Characterization of flunitrazolam, a new designer benzodiazepine, in oral fluid after a controlled single administration

Alice Ameline, Camille Richeval, Jean-Michel Gaulier, Jean-Sébastien Raul, Pascal Kintz

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Letter to the Editor

Letter to the Editor

Characterization of Flunitrazolam, a New Designer Benzodiazepine, in Oral Fluid After a Controlled Single Administration

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To the Editor:

Benzodiazepines are a class of drugs widely prescribed for the treatment of anxiety, insomnia, epilepsy, muscle spasms or alcohol withdrawal. Reactive to GABA type A receptors, benzodiazepines have a rapid and weakly toxic symptomatic efficacy. This class of drugs is often diverted from the medical use both by opiates addicts or recreative abusers. Indeed, the extensive research around their chemical structures resulted in a wide variety of active compounds that never got a marketing authorization and which are now picked up by online e-merchants of new psychoactive substances and sold under the name "designer benzodiazepines". The first example of illegal use of non-prescribed benzodiazepine was phenazepam, developed in the Soviet Union in the 1970s. Since the end of year 2010, designer benzodiazepines were proposed as recreational drugs. These drugs were etizolam, metizolam, pyrazolam, flubromazepam, flubromazolam, clonazolam, meclonazepam and nifoxipam. These compounds are sold as tablets, powder or blotters, at very affordable prices.

The deleterious effects of designer benzodiazepines depend on the dose consumed. The most common side effects at low doses are drowsiness, fatigue and lethargy. At higher doses, motor coordination disorders, dizziness, mood swings and euphoria may occur. The slow elimination of some benzodiazepines causes their accumulation in lipid-based tissues, which can lead to a delayed overdose in case of repeated consumption. After a long period of consumption, the abuser can develop tolerance and dependence. Sudden drug discontinuation can cause a withdrawal symptom. Severe effects occur when designer benzodiazepines are consumed in combination with opioids. Both pharmacological classes induce a synergistic effect and a respiratory depression (1). Abuse is widespread among poly-consumers, because designer benzodiazepines balance the euphoria due to opioids. Some benzodiazepines have a marked amnesic effect and the consumer loses memory for a few hours after administration (2).

The detection of designer benzodiazepines in biological fluids is challenging owing to the very few available data. Some of these compounds have been detected using immunoassays such as ELISA with high cross-reactivity, demonstrating that designer benzodiazepines can be detected during standard blood or urine drug screening. A few authors have developed methods to study the elimination rate and the metabolism of specific drugs, including etizolam (3, 4), metizolam (5–7), meclonazepam (4, 8), diclazepam (4, 9, 10), flubromazolam (11, 12), pyrazolam (11), flubromazepam (13), adinazolam, cloniprazepma, fonazepam, 3-hydroxyphenazepam and nitrazolam (7). Currently, there is no citation and no data for flunitrazolam testing in blood, urine and alternative biological specimens, such as oral fluid (PubMed consultation on 8 November 2017). Flunitrazolam was discovered in the 1960s but has never been marketed for unknown reasons. Its chemical structure is close to flunitrazepam, although flunitrazolam has a triazolo cycle. Scientists have very little information about doses, effects and tolerability.

In order to be able to interpret data on flunitrazolam and to obtain information on its detectability in oral fluid, one of the authors (male, 56 years old, 85 kg) ingested one pink tablet bought on the internet and declared to contain 0.25 mg of active material. Oral fluid was collected over 8 h using the NeoSalTM (Neogen) device, used as recommended by the manufacturer by handing the pad in the mouth for 2 min. Immediately thereafter, the pad was placed into the buffer, present in a plastic tube. No blood sample was collected, as this requires specific authorizations due to the invasive nature of this specimen. All oral fluid samples were stored at +4°C until analysis.

For identification and characterization of flunitrazolam, nuclear magnetic resonance (NMR) spectroscopy was applied (5). No significant impurity was detected in the tablet. In addition, the dosage of one tablet, using the ERETIC (Electronic Reference To access In vivo Concentrations) method, revealed a dosage of 0.23 mg of flunitrazolam, in agreement with the announced 0.25 mg.

All oral fluid samples were submitted to UPLC–MS-MS analysis on an Acquity class I ultra-high performance liquid chromatography coupled to a Xevo TQD tandem mass spectrometer (UPLC–MS-MS)

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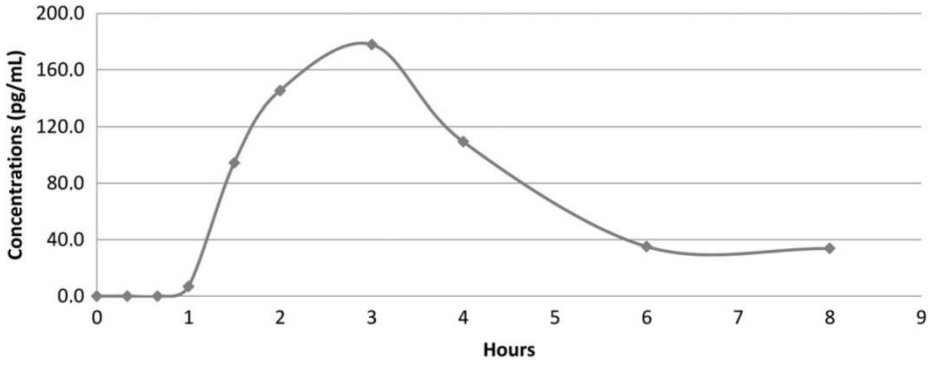

Figure 1. Concentration time profile of flunitrazolam excretion in saliva after administration of a single 0.25 mg tablet to a male volunteer.

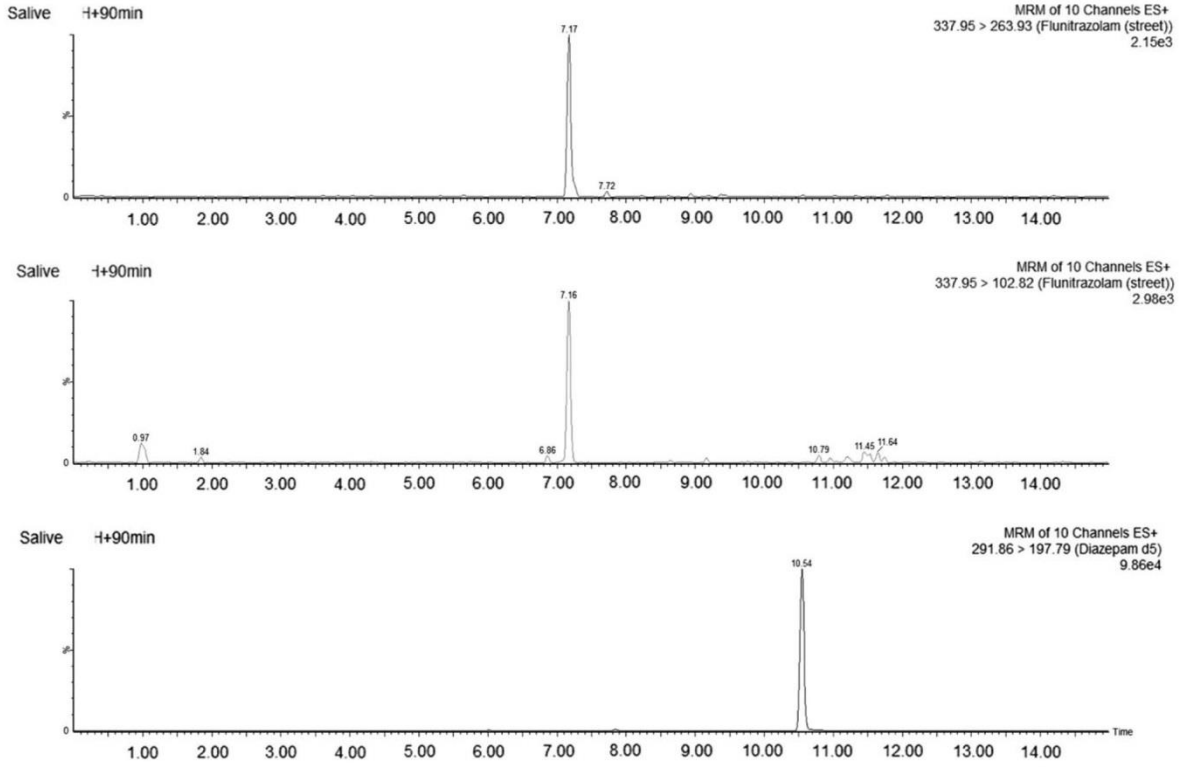

Figure 2. Chromatogram obtained after extraction of saliva at H+90 min. Flunitrazolam concentration was 94 pg/mL. From top to bottom: two transitions for flunitrazolam (*m/z*: 337.9 > 102.8 and 337.9 > 263.9) and one transition for diazepam-d5 (*m/z*: 291.9 > 197.8).

from Waters (Milford, MA, USA), using a screening method devoted to the identification and quantification of designer benzodiazepines. Flunitrazolam was extracted from 500 µL of mixed oral fluid and buffer in presence of 1 ng of diazepam-d5 used as internal standard, 500 µL saturated borate buffer pH 9.5 and 2.5 mL of a mixture of ether/dichloromethane/hexane/isoamyl alcohol (50:30:20:0.5). After 15 min of agitation at room temperature, centrifugation (3,000 rpm for 15 min) and evaporation to dryness at 45°C, the residue was reconstituted in 50 µL of 5 mM ammonium formate buffer adjusted at pH 3. Chromatography was achieved using a Waters Acquity HSS C18 column (150 × 2.1 mm × 1.8 µm) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.4 mL/min. The initial gradient was 87% phase A and the final gradient, at 15 min, was 5% phase A. An injection volume of 10 µL was used in all cases. A Xevo TQD triple quadrupole mass spectrometer was used for the detection of the molecule. Ionization was achieved using electrospray in the positive ionization mode (ES+). The following conditions were found to be optimal for the analysis of flunitrazolam and the internal standard: capillary voltage at 1.5 kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1,000 L/h. In order to establish appropriate multiple reaction monitoring condition, the

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cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociated of both species was performed. Cone voltage and collision energy were adjusted to optimize the signal for the two most abundant product ions of flunitrazolam: *m/z* 337.9 > 263.9 (60 V and 34 eV) and 337.9 > 102.8 (60 V and 40 eV), and the most abundant product ion of internal standard *m/z* 291.9 > 197.8 (56 V and 32 eV). MassLynx 4.1 software was used for quantification.

Because of the nature of the study (detectability of flunitrazolam in oral fluid), a simple analytical validation was achieved. Linearity was observed for flunitrazolam concentrations ranging from 10 to 1,000 pg/mL, with a correlation coefficient of 0.999 (y = 0.0003x – 0.0006). QC samples (100 and 200 pg/mL), analyzed in duplicate in five independent experimental assays, were used for determination a coefficient of variation for precision and accuracy. These CVs were lower than 20%. The limit of detection and the lower limit of quantification were 5 and 10 pg/mL, respectively. Under the used chromatographic conditions, there was no interference with the analyses by chemicals or any extractable endogenous materials present in oral fluid. The matrix effect (<20%) was investigated with spiked flunitrazolam at 200 pg/mL in 15 blank mixed oral fluid and buffer samples.

It is generally accepted that chemical testing of oral fluid is an objective mean of diagnosis of drug use and can demonstrate a recent exposure. Flunitrazolam was detectable in oral fluid between 1 and 8 h, with estimated concentrations ranging between 7 and 178 pg/mL. The excretion pattern of flunitrazolam is presented in Figure 1. T_{max} was obtained at 3 h. A typical oral fluid chromatogram is presented in Figure 2. As it was described for other benzodiazepines (14), the excretion of flunitrazolam in oral fluid is very weak. None of the oral fluid concentration was >200 pg/mL. This should prevent its detection during routine analyses. Furthermore, contamination of the oral cavity during the collection of the specimens cannot be ruled out, and effects of collection methods on drug concentrations in oral fluid are not well described in the scientific literature. It was not possible to evaluate the amount of collected oral fluid, even by weighing the test tube before and after the centrifugation, as the volume of the buffer varies from device to device.

The designer benzodiazepines are a growing class of NPS. With the introduction of new regulations concerning the first designer benzodiazepines (phenazepam and etizolam), users are turning to less known molecules, for which very little data have been published, including their effects, their toxicities and the analytical methods necessary to detect them (15). It appears that the use of alternative matrices, such as oral fluid, is steadily gaining recognition in forensic, occupational and traffic medicine. Although limited to a single administration to one volunteer, this article presents for the first time the detection of flunitrazolam in a human biological specimen. It can be used as a basis to develop a procedure in blood or urine.

There is still no case of flunitrazolam poisoning described in the literature and the risk of missing it during conventional screening is large, irrespective of the analytical technique used. Parent drug concentrations are very low in oral fluid, however, detection is possible in the early hours after administration.

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Article 16: Documentation of a little-studied designer benzodiazepine after a controlled single administration: II. Concentration profile of deschloroetizolam in saliva

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

Therapeutic Drug Monitoring. 2018;40(6):759-761

Documentation of a Little-Studied Designer Benzodiazepine After a Controlled Single Administration: II. Concentration Profile of Deschloroetizolam in Saliva

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To the Editor:

Benzodiazepines are a class of drugs widely prescribed for the treatment of anxiety, insomnia, epilepsy, muscle spasms, or alcohol withdrawal. Binding to GABA type A receptors, benzodiazepines have a weakly toxic efficacy in comparison with barbiturates. This class of drugs is often diverted from the medical use both by opiate addicts or recreative abusers. Indeed, the extensive research around their chemical structures resulted in a wide variety of active compounds that were never commercialized and are now picked up by online e-merchants of new psychoactive substances (NPSs) and sold under the name "designer benzodiazepines".[1] The first example of illegal use of a nonprescribed benzodiazepine was phenazepam, developed in the Soviet Union in the 1970s. Since the end of 2010, designer benzodiazepines have been marketed on the internet as recreational drugs. These drugs included etizolam, metizolam, pyrazolam, flubromazepam, flubromazolam, clonazolam, meclonazepam, and nifoxipam. They are available as tablets, powders, or blotters, at very affordable prices through the internet.

The deleterious effects of designer benzodiazepines depend on the dose consumed. The most common side effects at low doses are drowsiness, fatigue, and lethargy. At higher doses, motor coordination disorders, dizziness, mood swings, and euphoria may occur. The slow elimination of some benzodiazepines causes their accumulation in lipid-based tissues, which can lead to a delayed overdose in cases of repeated consumption. After a long period of consumption, the abuser can develop tolerance and dependence. Sudden drug discontinuation can cause a withdrawal syndrome. Severe effects occur when designer benzodiazepines are consumed in combination with opioids. Both pharmacological classes induce a synergistic effect in terms of respiratory depression. Abuse is widespread among polyconsumers because designer benzodiazepines enhanced the euphoria caused by opioids. Some benzodiazepines have a marked amnesic effect, and the consumer loses memory for a few hours after administration[1]

The detection of designer benzodiazepines in biological fluids is challenging because of minimal available information in the scientific literature. Several of these compounds have been detected using immunoassays such as ELISA with high cross-reactivity, demonstrating that some designer benzodiazepines can be detected during standard blood or urine drug screening.[2] A few authors have developed methods to study the detection, the elimination rate, or the metabolism of specific drugs, including etizolam,[3] metizolam,[4] meclonazepam,[5] diclazepam,[3] flubromazolam,[6] flubromazepam,[7] adinazolam, cloniprazepam, fonazepam, 3-hydroxyphenazepam, and nitrazolam.[8]

Currently, there are no data for deschloroetizolam testing in blood, urine, and alternative biological specimens, such as oral fluid. Deschloroetizolam is a psychoactive drug of the thienodiazepines class that, as do benzodiazepines, binds to modulatory sites on the GABA receptors. Deschloroetizolam is closely related to etizolam, triazolam, and alprazolam and is not recognized as a controlled substance in many parts of the world. This compound is supposed to have a rapid onset of action (between 1 and 5 minutes) and is half as potent as its parent compound etizolam with a duration of effects twice as long (between 8 and 10 hours).[9] About 2 mg of deschloroetizolam is believed to be of similar potency to 10 mg of diazepam according to some "unofficial" websites. Deschloroetizolam has physical effects (sedation, respiratory depression, muscle relaxation, and dizziness) and cognitive effects (amnesia, anxiety suppression, thought deceleration, analysis suppression, disinhibition, euphoria, delusion of sobriety, and dream potentiation). All these effects have been reported based on personal experiences of abusers.[9]

To be able to interpret data on deschloroetizolam and to obtain information on its detectability in oral fluid, one of the authors (male, 56 years old, 78 kg) ingested one-half pink tablet bought on the internet, which was claimed to contain 12 mg of active material according to the seller. In France, given the subject was one of the authors, it was not necessary to obtain a written consent because deschloroetizolam is not listed as a drug of abuse. Oral fluid was collected over 18 hours using the Neo-Sal (Neogen, Lansing, MI) device, used as recommended by the manufacturer by holding the pad in the mouth for 2 minutes. Immediately thereafter, the pad was placed into the buffer in a plastic tube. All oral fluid samples were stored at +4°C until analysis. No blood sample was collected because this requires specific oxygenotherapy, which was not available at the laboratory. The control of the identity of the active ingredient and its purity were determined by nuclear magnetic resonance. Testing for deschloroetizolam in the oral fluid specimens was achieved by ultra–high-performance liquid chromatography coupled to a Xevo TQD tandem mass spectrometer (UHPLC-MS/MS; Milford, MA). The complete procedures are presented in the Supplemental Digital Content 1 (see Table, http://links.lww.com/TDM/A261).

No significant impurity was detected in the deschloroetizolam tablet using nuclear magnetic resonance. In addition, the dosage of one-half tablet, using the Electronic REference To access In vivo Concentration (ERETIC) method, revealed a dosage of 6 mg of deschloroetizolam, in

The authors declare no conflict of interest.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.drug-monitoring.com).

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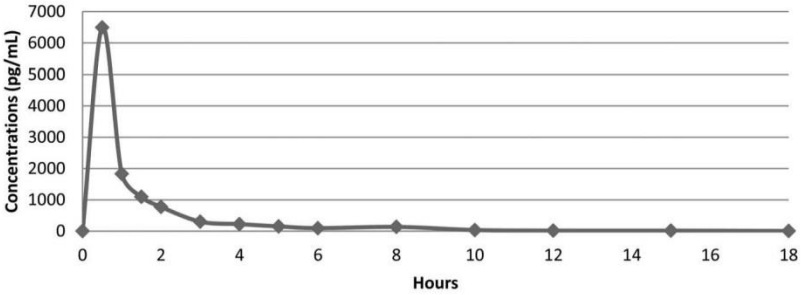

FIGURE 1. Concentration–time profile of deschloroetizolam excretion in saliva after administration of 6 mg to a male volunteer.

agreement with the amount claimed by the seller.

After oral administration of one-half tablet of deschloroetizolam, the overall behavior of the subject changed rapidly. After 15 minutes, immoderate effects were present, including fatigue, dizziness, language disorders, and concentration trouble, which lasted for the next consecutive 90 minutes. Deschloroetizolam was detectable in oral fluid between 30 minutes and 18 hours, with estimated concentrations ranging between 3 and 6491 pg/mL. The time–concentration data of deschloroetizolam are presented in Figure 1. T_{max} was obtained at 30 minutes. In comparison with what was described for other benzodiazepines, the excretion of deschloroetizolam in oral fluid is very rapid and markedly contrasts with the usual delayed pattern. After only 30 minutes, the saliva concentration of deschloroetizolam peaked at 6491 pg/mL, which then rapidly declined. This result is consistent with the effects felt by the volunteer. Although one cannot exclude the contribution of contamination of the oral cavity by the tablet, a major washout can be anticipated during the previous minutes. The washout, and therefore the authentic T_{max}, is consistent with the alteration of the subject's behavior.

The rapid decrease in drug concentrations should prevent its detection during routine analyses, particularly if oral fluid is collected several hours after exposure. Contamination of the oral cavity during the early collection of the specimens cannot be ruled out. The possible effects of the collection method on drug concentrations in oral fluid cannot be anticipated. It was not possible to evaluate the amount of collected oral fluid, even by weighing the test tube before and after the centrifugation, because the final volume of the buffer and oral fluid varies from device to device.

The authors are aware of the limitations of this short experimentation because a single dose was administrated to one single subject. However, the generated information should be considered sensitive enough for the forensic community. Indeed, the low concentrations measured in the oral fluid after administration prevent routine detection of the drug and more extensively to all benzodiazepines. The present article should be considered as a basis for the detection of this class of NPS in oral fluid, as very little, if any, information is available in the scientific literature.

The designer benzodiazepines are a growing class of NPS. With the introduction of regulations concerning the first designer benzodiazepines (phenazepam and etizolam), users are turning to lesser-known molecules, for which very little data have been published, including their effects, their toxicities, and the analytical methods necessary to detect them. It seems that the use of alternative matrices, such as oral fluid, is steadily gaining recognition in forensic, occupational, and traffic medicine. Although limited to a single administration to one volunteer, this article presents for the first time the detection of deschloroetizolam in a human biological specimen. It can be used as a basis to develop a procedure in blood or urine. This compound produces more rapid effects that other designer benzodiazepines already studied. There is still no case of deschloroetizolam poisoning described in the literature, and the risk of missing it during conventional screening is large, irrespective of the analytical technique used.

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metabolism of designer benzodiazepines–an update comprising adinazolam, cloniprazepam, fonazepam, 3-hydroxyphenazepam, metizolam and nitrazolam. *J Mass Spectrom.* 2016;51: 1080–1089.
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Article 17: Characterization of metizolam, a designer benzodiazepine in alternative biological specimens

Pascal Kintz, Carole Jamey, Alice Ameline, Camille Richeval, Jean-Sébastien Raul

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ORIGINAL ARTICLE

Characterization of metizolam, a designer benzodiazepine, in alternative biological specimens

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KEYWORDS
Designer benzodiazepines;
Metizolam;
Alternative specimens;
Saliva;
Hair;
Exhaled breath;
Sweat

Summary Designer benzodiazepines provide an attractive alternative to prescribed benzodiazepines for abuse purposes as they are readily available over the Internet at a low price. Although these compounds have been developed by pharmaceutical companies, at this time, they do not have a legal status. Their detection in biological fluids is challenging owing to the very few available data. For instance, there is no information about metizolam (also known as desmethyletizolam) analysis, distribution and metabolism in the scientific literature. Metizolam was ordered via Internet and a 2 mg blue tablet was orally administered to a 54-year-old healthy man (85 kg). Oral fluid (saliva) was collected over 8 hours using the NeoSal™ device. Sweat was collected with the PharmChek™ sweat patch technology over 72 hours. Exhaled breath was collected with the ExaBreath® DrugTrap device over 15 hours. Beard hair was collected 4 and 10 days after administration. Finally, head hair was collected 3 weeks after administration. Metizolam was detectable in saliva during 8 hours, with concentrations lower than 1 ng/mL (max at 0.77 ng/mL after 2 hours). Increasing concentrations up to 186 pg/patch (after 72 hours) of metizolam were detected in the sweat patches. Metizolam produced for 4 hours a very low but significant chromatographic signal in exhaled breath, with concentrations always lower than 10 pg/filter. Finally, metizolam tested positive in beard hair (0.73 and 0.28 pg/mg after 4 and 10 days, respectively) and head hair (0.27 pg/mg). From the data obtained from one subject,

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metizolam showed very low concentrations in various alternative matrices, attesting that the potential forensic implications (drug-facilitated crime, addiction, DUID...) of metizolam abuse must be considered as a new challenge for toxicologists.

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Introduction

Benzodiazepines are central nervous system depressants commonly prescribed to treat anxiety, insomnia, and seizures. Benzodiazepines are considered safer alternatives to barbiturates because they have fewer side effects and have a reduced risk of causing an overdose. However, benzodiazepine abuse is often associated with people taking benzodiazepines in combination with other controlled substances such as opiates, methadone, and cocaine. Benzodiazepines are taken in combination with opiates in particular because they enhance the euphoric effects of opiates; for other drugs, such as stimulants, benzodiazepines may temper the negative side effects (e.g., restlessness, agitation).

Although not approved for use, new benzodiazepines have been observed in forensic cases (death, acute intoxication, drug-facilitated crime, driving under the influence of drug). This includes phenazepam [1,2], etizolam and pyrazolam [3], flubromazolam [4], or clonazolam, meclonazepam and nifoxipam [5].

The detection of designer benzodiazepines in biological fluids is challenging owing to the very few available data. Some of these drugs have been detected using immunoassays with high cross-reactivity [3,6], demonstrating that designer benzodiazepines can be detected in standard blood or urine immunoassay drug screening. Other authors have developed liquid chromatography mass spectrometry methods, mostly to study the metabolism of specific drugs, including clonazolam, meclonazepam and nifoxipam [5], pyrazolam [7], flubromazepam [8], diclazepam [9], and clonazolam, deschloroetizolam, flubromazolam and meclonazepam [10]. Among the designer benzodiazepines group, the thienodiazepines, obtained after replacement of the benzene ring by a thiophene ring, are represented by etizolam (licenced for use in Japan, Italy and India), and more recently by metizolam (Fig. 1), also known as desmethyletizolam. Metizolam differs structurally from etizolam through removal of the methyl group on the triazole ring. Although etizolam has already been described in overdose [11] and death cases [12], there is no citation for metizolam (PubMed, consultation on 24 July 2016). For instance, there is no data about metizolam analysis in alternative biological specimens, such as hair, saliva or exhaled breath. This compound was patented by a Japanese company in 1995, as an anti-anxiety medication. It has no history of human usage prior to its availability by online vendors in September 2015.

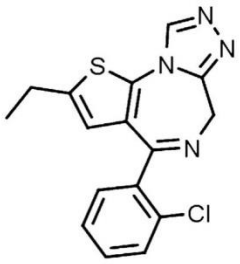

Figure 1. Chemical structure of metizolam.

A UPLC-MS/MS technique were used to detect metizolam in saliva, hair, sweat and exhaled breath after administration of an oral dose of 2 mg to a human volunteer.

Material and method

Self-administration study

To gain preliminary data on the detectability of metizolam in various alternative specimens, one of the authors (male, 54-year-old, 85 kg) ingested one blue tablet (declared 2 mg metizolam) with 200 mL of tap water. Oral fluid was collected over 8 hours using the NeoSal™ (Neogen) device and used as recommended by the manufacturer, by handling the pad in the mouth of the subject for 2 min. Immediately thereafter, the pad was placed into the diluent, present in a plastic tube. Sweat was collected with the PharmChek™ (Sudormed) sweat patch technology over 72 hours. Four patches were placed over the skin of the right arm at T0 and periodically removed. Exhaled breath was collected with the ExaBreath® DrugTrap Sensa Bues™ device (Sensa Bues™) over 15 hours. This device consists of a filter that traps the aerosols (that contain the drugs) from breath. The test was complete after 20 breaths through the device, which approximately corresponds to 20—30 litres of breath going through the filter (about 2 min). No mouth rinsing was performed prior to breath sampling. Beard hair was collected 4 and 10 days after administration. Finally, head hair was collected 3 weeks after administration. Except hair, all specimens were stored at +4 °C until analysis.

In France, approval by an ethics committee is not required for self-experiments (except for drugs of abuse, that is not the case for metizolam).

No blood sample was collected, as this requires specific authorizations due to the invasive nature of this specimen.

For identification and characterization of metizolam, nuclear magnetic resonance (NMR) spectroscopy was applied. No significant impurity was detected in the metizolam tablet using UHPLC-Q-TOF/MS or NMR (Fig. 2). In addition, the dosage of one tablet, using the ERETIC (Electronic REference To access In vivo Concentrations) method, revealed a dosage of 1.8 mg of metizolam, in agreement with the announced 2 mg dosage [13].

Chemicals and reagents

Metizolam (4-(2-chlorophenyl)-2-ethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a] [1,4] diazepine; CAS No. 40054-68-0) was obtained from Chemicalwire (London, UK).

Ammonium formate and formic acid were provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France). LC-MS grade water and acetonitrile were purchased from Biosolve (Dieuze, France), while methanol HPLC grade and 30% hydrochloric acid were purchased from VWR Prolabo (Fontenay-sous-Bois, France). Diazepam-d_5 was purchased from LGC Promochem (Molsheim, France) and diluted to appropriate concentration (variable according to the matrix) using methanol.

ExaBreath® DrugTrap (SensAbues®), NeoSal™ (Neogen), and PharmChek™ (Sudormed) devices were kindly provided by the respective companies.

Saliva extraction

Metizolam was extracted from 1 mL mixed saliva and diluent in presence of 1 ng of diazepam-d_5 used as internal standard by 1 mL saturated ammonium chloride pH 9.5 buffer (adjusted with ammonia water) and 5 mL of dichloromethane/*n*-heptane/isopropanol (25/65/10, v/v). After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

It was not possible to evaluate the amount of collected oral fluid, even by weighing the test tube before and after the centrifugation, as the volume of the buffer and oral fluid varies from device to device.

Sweat extraction

The sweat pad was extracted with 5 mL of methanol in the presence of 1 ng of diazepam-d_5 (internal standard) by horizontal shaking for 15 min. After centrifugation, the methanol was evaporated to dryness (gentile nitrogen stream) and finally, the residue was solubilized in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

Exhaled breath extraction

The extraction procedure was done by putting the device with outlet down on top of a disposable glass test tube with the filter still in the device. Ten millilitres of methanol were added by pipetting it carefully onto the filter avoiding the wall. This was repeated 10 times with the same methanolic solution, in order to extract maximum of the drug. 1 ng of diazepam-d_5 was added to the methanol at the last step, as internal standard. After centrifugation, the methanol was evaporated to dryness (gentle nitrogen stream) and finally, the residue was solubilized in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

Hair extraction

Metizolam was extracted from 20 mg of hair in presence of 1 ng of diazepam-d_5 used as internal standard by overnight incubation in 1 mL saturated ammonium chloride pH 9.5 buffer (adjusted with ammonia water). Then, 5 mL of dichloromethane/*n*-heptane/isopropanol (25/65/10, v/v) were added. After extraction, centrifugation and evaporation to dryness, the residue was reconstituted in 50 μL of acetonitrile/formic acid 0.1% (50/50, v/v).

UPLC-MS/MS analysis

LC was performed using a Waters Acquity system. Chromatography was achieved using a Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm) eluted with a gradient delivered at a flow rate of 0.5 mL/min (from 5% acetonitrile—95% formic acid 0.1% to a ratio 95—5% at 5.5 min). An injection volume of 10 μL was used in all cases. A Quattro Premier XE triple-quadrupole mass spectrometer (Micromass-Waters) was used for analyses. Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of metizolam and the IS: capillary voltage, 1.0 kV; source block temperature, 120 °C; and desolvatation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 800 L/h. In order to establish appropriate multiple reaction monitoring (MRM) conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociation (CID) of both species was performed. Collision gas (argon) pressure was maintained at 3.0×10^{-3} Bar and the collision energy (eV) was adjusted to optimize the signal for the 2 most abundant product ions: transitions m/z 328.9 to 275.0 and m/z 328.9 to 300.0 for metizolam. Transition m/z 328.9 to 275.0 was used for quantitation. The transition m/z 290.0 to 198.1 was used for diazepam-d_5. MassLynx 4.1 software was used for quantitation.

Because of the nature of the study (detectability of metizolam in alternative specimens), no full analytical validation was achieved. Linearity (6 points, correlation coefficient > 0.99) was observed for metizolam concentrations ranging from 50 to 1000 pg/mL, 0.1 to 10 pg/mg, 5 to 500 pg/patch and 5 to 50 pg/filter in saliva, hair, sweat and exhaled air, respectively. Within-batch precisions ($n = 6$) at 100 pg/mL (saliva), 1 pg/mg (hair), 50 pg/patch (sweat), and 10 pg/filter (exhaled breath) were better than 25%. The limits of quantitation were estimated to be 2 pg/mL, 0.1 pg/mg, 5 pg/patch and 5 pg/filter for saliva, hair, sweat and exhaled air, respectively, with a S/N ratio of 3. Under the chromatographic conditions used, there was no interference with the analytes by chemicals or any extractable endogenous materials present in the various matrices (matrix effect < 20%).

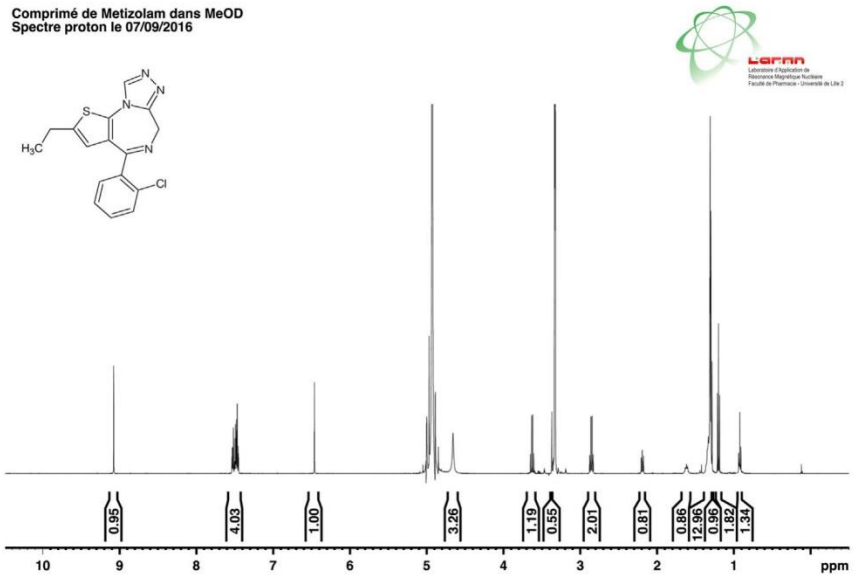

Figure 2. NMR analysis of the metizolam tablet.

Results and discussion

It is generally accepted that chemical testing of biological fluids is the most objective means of diagnosis of drug use. The presence of a drug analyte in a biological specimen can be used as evidence of recent exposure. The standard in drug testing is the immunoassay screening, followed by the gas or liquid chromatographic-mass spectrometric (GLC/MS or MS-MS) confirmation conducted on a urine sample. More recently, a variety of body specimens other than urine, such as oral fluid, sweat or hair have been proposed to document drug exposure.

When phenazepam and etizolam became scheduled in several countries, some laboratories turned their attention to investigational drugs, never before marketed as pharmaceuticals. This led to the appearance of designer benzodiazepines in Internet shops, sold for recreational purposes. A large series of drugs, including pyrazolam, flubromazepam, diclazepam, nifoxipam, meclonazepam or flubromazolam were available on the dark market. Among these new designer benzodiazepines, not much attention was given to metizolam. Besides the historical archives from Japan, there is a real dearth of literature on this drug. It was postulated the likely effects based on structural similarity to classic benzodiazepines, but the only available source of information is that which can be found on drug discussion forums.

After oral administration of 2 mg of metizolam to a 54-year-old healthy man (85 kg), which corresponds to a usual dose, the overall behaviour of the subject was not modified. A slight sensation of sedation was noticed, but impairment was not observed.

Metizolam was detectable in saliva during 8 hours, with concentrations always lower than 1 ng/mL. The Cmax was 0.77 ng/mL, obtained 2 hours after administration. The excretion pattern of metizolam is presented in Fig. 3. A typical saliva chromatogram is presented in Fig. 4. As it was described for other benzodiazepines [14], the excretion of metizolam in saliva was very weak. This should prevent its detection during routine analyses. Contamination of the oral cavity during the collection of the specimen after 30 min cannot be ruled out. The NeoSal™ (Neogen) was found suitable for saliva collection. The effect of collection methods on drug concentrations in oral fluid is not well described in the scientific literature. However, caution has to be taken. As the oral fluid flow rate increases (that can be the case when using a collection device), the concentration of bicarbonate ions increases. This may alter the saliva-plasma ratio in a pH-dependent manner, especially for weakly basic drugs with a pK_a approaching the oral fluid pH.

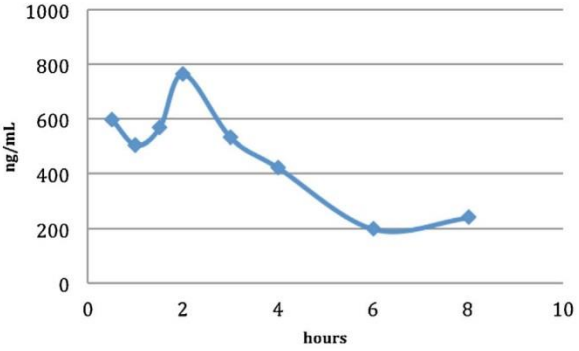

Figure 3. Concentration time profile of metizolam excretion in saliva after administration of a single 2 mg tablet.

Between 300 to 700 mL/day of *insensible* sweat is produced over the whole body, likely caused by diffusion through the skin, whereas 2 to 4 L/h of *sensible* sweat may be produced for short periods by extensive exercise. The amount of sweat that is produced is thus affected by body

putting both phenazepam and etizolam on the list of controlled substances, there is space for new candidates from pharmaceutical research. Metizolam (also known as desmethyletizolam) is a short-acting psychoactive drug of the thienodiazepine class (the benzene ring has been replaced by a thiophene ring). It has been shown by anecdotal reports to produce depressants, anxiolytic, sedative, muscle relaxant and amnestic effects. Parent drug concentrations are very low in alternative matrices but detection is possible in the early hours after administration. The risk of missing metizolam during conventional screening is large, irrespective of the analytical technique used.

Except in UK (26 May 2016) and Sweden (26 January 2016), metizolam is not known to be specifically illegal. One major concern about this drug regarding public health is the lack of data on acute human toxicity and potential long-term effects.

Disclosure of interest

The authors declare that they have no competing interest.

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Article 18: Use of nuclear magnetic resonance (NMR) spectroscopy to identify unknown powders

Alice Ameline, Delphine Garnier, Jean-sébastien Raul, Pascal Kintz

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Use of nuclear magnetic resonance (NMR) spectroscopy to identify unknown powders

--Projet de manuscrit--

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Ordre des auteurs:	Alice Ameline, MS
	Delphine Garnier
	Jean-sébastien Raul
	Pascal Kintz
Résumé:	The authors describe the identification of 7 powders, supposed to be NPS, involved in a complex toxicological fatal case. The identification of these substances requires the development of a specific, quality and comprehensive method. However, it is difficult to obtain and purchase the reference standards of all substances. Some are excessively expensive, and some, even, do not exist in the catalogue of referenced suppliers. In addition to the difficulties associated with molecule characterization (identification, quantification, purity), analytical methods used routinely, such as LC-MS/MS, are not always sufficient to firmly identify NPS, for example in case of positional isomers. It is therefore necessary to use another method, such as NMR spectroscopy, to discriminate the species. The analytical strategy and the performance of NMR are developed in this article. The 7 powders were 4-chloro-alpha-pyrrolidinopentiophenone, dibutylone, 4-chloroethcathinone, 4'-méthyl-α-pyrrolidinohexiophénone, 3,4-methylenedioxy-N-benzylcathinone, 3-methoxy-phencyclidine and alpha-pyrrolidinopropiophenone, with purity between 50.8 and 89.5%. There was no matching between the label and the active ingredient contained in the dispatched bags. This clearly demonstrates the danger in consuming substances bought on the internet, as both identity of the substance and its purity are missing.

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Article original

Use of nuclear magnetic resonance (NMR) spectroscopy to identify unknown powders

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Abstract

The authors describe the identification of 7 powders, supposed to be NPS, involved in a complex toxicological fatal case. The identification of these substances requires the development of a specific, quality and comprehensive method. However, it is difficult to obtain and purchase the reference standards of all substances. Some are excessively expensive, and some, even, do not exist in the catalogue of referenced suppliers. In addition to the difficulties associated with molecule characterization (identification, quantification, purity), analytical methods used routinely, such as LC-MS/MS, are not always sufficient to firmly identify NPS, for example in case of positional isomers. It is therefore necessary to use another method, such as NMR spectroscopy, to discriminate the species. The analytical strategy and the performance of NMR are developed in this article. The 7 powders were 4-chloro-alpha-pyrrolidinopentiophenone, dibutylone, 4-chloroethcathinone, 4'-méthyl-α-pyrrolidinohexiophénone, 3,4-methylenedioxy-N-benzylcathinone, 3-methoxy-phencyclidine and alpha-pyrrolidinopropiophenone, with purity between 50.8 and 89.5 %. There was no matching between the label and the active ingredient contained in the dispatched bags. This clearly demonstrates the danger in consuming substances bought on the internet, as both identity of the substance and its purity are missing.

Key words

NMR spectroscopy, powder, purity, NPS

Introduction

For the past ten years, there has been a significant increase in cases of intoxication with new psychoactive substances (NPS). These new substances are often sold on the internet, but also on the street, more often in festive contexts (rave parties, concerts, private parties). It is extremely easy to get them at a low price, but it is impossible to know what is actually bought. It is not uncommon that the active ingredient or the concentration of the product is not mentioned on the packaging. However, even when the active ingredient is indicated, its concentration may be inaccurate or misleading. Contamination with active substances, fake medicines or counterfeit drugs have been described since many years [1-3]. The lack of information leads to a large number of intoxication due to errors of dosage or the potentiation of several substances consumed simultaneously [4-5].

The identification of these substances requires the development of a specific and comprehensive method. However, it is difficult to obtain and purchase the reference standards of all substances. Some are excessively expensive, and some do not even exist in the catalogue of referenced suppliers. Toxicology laboratories are therefore limited and must adapt to the material they can purchase and try to achieve legal exchanges between colleagues. In addition to the difficulties associated with molecule characterization (identification, quantification, purity), analytical methods used routinely are not always sufficient to firmly identify NPS. For example, liquid chromatography, which is the most widely separative method proposed, may not discriminate positional isomers, such as mephedrone (4-MMC) and 3-methylmethcathinone (3-MMC), which are NPS very commonly abused [6-7]. In LC-MS/MS, their retention times may be identical as well as their fragmentation pattern. It is therefore necessary to use another method, such as nuclear magnetic resonance (NMR) spectroscopy, to discriminate the species.

NMR is a physical phenomenon in which spin bearing nuclei (most commonly ^{1}H, ^{13}C, ^{19}F, ^{31}P), placed in a strong magnetic field, may absorb and re-emit electromagnetic radiation. This method not only makes it possible to definitively identify a compound, but also to confirm the absence of other organic compounds and to determine the purity of a product [8-9].

This article focuses on the use of NMR spectroscopy to identify 7 unknown small bags of powders, involved in a complex toxicological fatal case [10].

Materials and methods

Chemical

Maleic acid (99.98 %) was provided by Merck (Darmstadt) as TraceCert® quality for quantitative NMR. Deuterated acetonitrile (ACN-d_3), deuterated methanol (MeOH-d_4), deuterated water (D_2O) and deuterated dimethylsulfoxide (DMSO-d_6) were purchased from Eurisotop (Saarbrücken).

Nuclear Magnetic Resonance analysis

5 to 10 mg of each powder was completely dissolved in deuterated acetonitrile and deuterated methanol (because of possible low solubility of compounds in acetonitrile). Several spectra were recorded: ^{1}H, $^{13}C\{^{1}H\}$, ^{13}C DEPT135, ^{1}H-^{1}H *g*COSY, edited ^{1}H-^{13}C *g*HSQC and ^{1}H-^{13}C *g*HMBC. In case of fluorinated compound, ^{19}F and $^{19}F\{^{1}H\}$ NMR spectra were also recorded. Based on the combined analysis of these 1D- and 2D- data and the molecular mass and fragmentation patterns found by LC-MS full scan, a structure was established by the operator. This approach has already been proposed in the literature for other NPS [11-12].

In order to confirm the absence of other organic compounds such as synthetic impurities and to evaluate the purity of each sample, a stock solution of defined concentration (5.0 mg / mL) of maleic acid (Certified Reference Material) in either D_2O or DMSO-d_6 was prepared and used to prepare a sample of known analyte concentration and a proton spectrum was recorded (10 mg/ mL). The sample purity was defined by the following equation:

$$P_{sample} = \frac{I_{analyte}}{I_{CRM}} \times \frac{N_{CRM}}{N_{analyte}} \times \frac{M_{analyte}}{M_{CRM}} \times \frac{m_{CRM}}{m_{sample}} \times P_{CRM}$$

with:
P_{sample}: purity of the sample as mass fraction (%)
P_{CRM}: purity of the CRM as mass fraction (%)
$I_{analyte}$: integral of the analyte signal
I_{CRM}: integral of the CRM signal
$N_{analyte}$: number of analyte protons
N_{CRM}: number of CRM protons
$M_{analyte}$: molecular mass of the analyte (g/mol)
M_{CRM}: molecular mass of the CRM (g/mol)
m_{sample}: mass of sample (mg)
m_{CRM}: mass of CRM (mg)

The NMR spectra were recorded on AVANCE 400 (Bruker Avance III, France) operating at a proton Larmor frequency of 400 MHz equipped with a 5 mm double resonance broad band probe head (BBO+F) probe at 295 K. For quantitative NMR, the ^{1}H spectra were recorded with 16 scans, a 64-K time-domain data points with 8012 Hz spectral width, an acquisition time of 3409 s, a relaxation

delay of 45 s and a flip angle of 90°. The Free Induction Decays (FIDs) were Fourier transformed (0.3 Hz broadening) and the phase and the baseline were corrected. In these measurement conditions, the value of the peak area integrals in the spectrum are directly proportional to the number of corresponding spins (i.e. nuclei) for each compound. This gives direct information about the respective concentration of protons for maleic acid and the compound of interest's sample purity and, using the above equation, direct access to sample purity, independently of the eventual presence of non-organic components [13]. The analyses of the spectra were performed using TopSpin 3.5 pl7.

Results and discussion

The 7 powders were identified by NMR and results are presented in Table 1. The compounds were 4-chloro-alpha-pyrrolidinopentiophenone, dibutylone, 4-chloroethcathinone, 4'-méthyl-α-pyrrolidinohexiophenone, 3,4-methylenedioxy-N-benzylcathinone, 3-methoxy-phencyclidine and alpha-pyrrolidinopropiophenone. There was no matching between the label and the active ingredient contained in the dispatched bags. This clearly demonstrates the danger in consuming substances bought on the internet, as both identity of the substance and its purity are missing.
The following lines describe for each drug the obtained data.

Powder 1

The integration of all proton resonances belonging to powder 1 in the spectrum in MeOD-d_4 summed to a total of 20 H (Figure 1 to Figure 4). The $^{13}C\{^1H\}$ spectrum led to 12 distinct signals (Figure 5). The presence of a signal at 196.4 ppm in the carbon spectrum also indicated the presence of at least 1 oxygen atom, while MS's nitrogen rule yielded to an uneven number of nitrogen atoms. Combined together, the most likely formula was $C_{15}H_{20}NOCl$ for powder 1. The insaturation degree for this formula is 6.
The CH or CH_3 nature of the 14.1 and 70.4 ppm ^{13}C signals were determined by the integration value of their corresponding protons (identified by HSQC, Figure 7 to Figure 9).
The protons attached to the two big sp^2 CH signals around 130 ppm strongly couple with each other (identified by COSY, Figure 10) forming two kinds of doublet that integrate for two protons each (^1H spectrum, Figure 2). This indicates a proton and carbon equivalency and is only compatible with our spectrum in case of a para-disubstituted benzene pattern. The high-order nature (at a Larmor frequency of 400 MHz) of these two aromatic proton multiplets (8.12 and 7.64 ppm) is compatible with the electronic effect expected from a chlorine atom directly attached to the ring.

The precise attribution between the {1,3} and the {4,6} sets of signals was possible using the HMBC spectrum (see Figure 8). The strong 3-bond correlation H{1,3} and C8 indicates that the ketone is likely directly correlated to the benzene ring. C8 also displays correlation peaks with the H9 and H11.

Starting from H9, the sequential attribution of the side chain could be obtained by analysis of the 1H, ^{1}H gCOSY spectrum (Figure 10), and confirmed by the ^{1}H, ^{13}C gHMBC (Figure 11). In contrary to the well-defined protons resonances ascribed to the side chain (their complexity arises from the molecule chirality at C5), the remaining proton resonances are broad, probably due to intramolecular impaired rotation. As a consequence, no correlation is seen in the ^{1}H, ^{13}C gHMBC spectrum between the protons and carbons of the positions 13, 14, 15 and 16. Nevertheless, 3-bond correlation peaks of weak intensity were detected in the ^{1}H, ^{1}H gCOSY spectrum, confirming those broad signals belonging to the same, separate spin system.

The proposed 4-chloro-alpha-pyrrolidinopentiophenone (4-Cl-α-PVP) structure (Figure 12) was confirmed by recording another proton spectrum of powder 1 in DMSO-d_6 and compared with the published NMR spectrum of structurally confirmed 4-Cl-α-PVP reference material published as a SWGDRUG monograph by the US Department of Justice – Drug Enforcement Administration's Special Testing and Research Laboratory (latest revision: 9/6/2016). The proton and carbon assignments to 4-Cl-PVP are summarized in Table 2.

Powder 2

The fine structure of the three proton signals at 6.96, 7.41 and 7.63 ppm (Figure 13 and Figure 14) features the typical pattern for ortho- para- trisubstitution of a benzene ring. The big coupling constant of 8.37 Hz arises from direct neighbours interactions (^{3}J), that is, interactions between H1 and H6 (Table 3). The smaller coupling constant J_{HH} = 1.86 Hz corresponds to long range coupling. While $^{4}J_{HH}$ coupling constants in aromatic rings may be observed in good resolved spectra, the observation at this magnetic field of $^{5}J_{HH}$ is much more unlikely, so that the observed coupling is ascribed to interaction between H1 and H3. Due to the double electronic inductive attractive effect of the two neighbouring oxygens, the protons of acetal moiety {H8, H8'} are detected at a high frequency for an aliphatic moiety. This signal displays an unresolved fine structure, probably due to diastereotopy (bent O-CH_2-O structure plus chiral center at C9, $J_{H8,H8'}$ <1 Hz). The chiral center in C9 also explains the doublet of doublet pattern of the H9 at 5.04 ppm, the coupling to diastereotopic H12 and H12' being angle dependant.

^{13}C resonances could also be ascribed to the structure, using only ^{13}C{^{1}H} and DEPT135 spectra (Figure 15 and Figure 16) and electronic considerations. Four resonances disappear in the DEPT135, which is in line with the four expected quaternary sp^2 carbons. The 192 ppm resonance is typical a

ketone moiety and is therefore ascribed to C10. Chemical shift above 150 ppm occur when sp^2 carbons bear heteroatoms, such as in phenols or O-phenyl esters. This allows to ascribe the 125. 5 ppm resonance to C2. Ketones have an unscreening effect (+4.5 ppm) when in para-position from the considered carbon but an almost no effect when in ortho- or meta-position. Similarly, -OMe moieties have strong screening effects when placed in ortho- or para-position (-14.4 and -7.8 ppm, respectively), but only a small unscreening one in meta (+1 ppm). Combined together, these effects lead to the attribution of the 151.2 ppm resonance to C5 and the 145.8 ppm resonance to C4. Such an analysis leads to a similar chemical shift prediction for C1 and C3: without an ^{1}H, ^{13}C-gHSQC spectrum, which was not performed due to the easy structure identification, it is not possible to precisely ascribe the 105.1 ppm and 105.9 ppm resonances to C1 and C3.

Only two resonances correspond to CH_2 moieties. As in the proton spectrum and from the same reason, the acetal is detected at a particularly high chemical shift for an aliphatic carbon, almost in the sp^2 region, at 100.0 ppm. The other CH_2 resonance, at 19.4 ppm can be therefore attributed to C12. Similarly, no doubt remains about the respective assignment of the 4.4 ppm and 67.3 ppm resonances to the screened C13 and the nitrogen-neighbouring C11.

The proposed dibutylone (Figure 17) structure was confirmed by recording another proton spectrum of powder 2 in DMSO-d_6. The proton and carbon assignments to dibutylone are summarized in Table 3. The only difference with the reference data (bk-DMBDB SWGDRUG monograph of the US Department of Justice – Drug Enforcement Administration's Special Testing and Research Laboratory) is the presence in our spectrum of a broad singlet integrating for 6 protons instead of two, sharp singlets integrating for three protons each. However, addition of maleic acid for quantification led to the modification of the spectrum, mirroring the reference spectrum. In the latter case, the chemical exchange between the 15 and 16 moieties has been slowed enough by protonation of the amine from the maleic acid so that both NCH_3 can be discriminated within the NMR timescale. This coalescence phenomenon also occurs in the $^{13}C\{^{1}H\}$ spectrum and is responsible for the ~41 ppm hump, where the C15 and C16 resonance(s) are expected.

Powder 3

The ^{1}H-NMR spectrum of powder 3 indicated that the powder is no pure product, but a mixture of compounds (Figure 18):

- Two species display a typical para-disubstituted phenyl doublet-like pattern.
- The large resonance at 7.68 ppm corresponds to labile protons, probably a rest of base or acid used during the last step of the synthesis.
- A CH_3-CH_2-X system at 1.29 ppm (triplet) and 2.97 ppm (quadruplet)

- Water

Since the integrals ratios of the last two match (10:7:10, so about 3:2:3) they may belong to the same $CH_3CH_2NH_3^+$ species, which may be one of the reagents for the synthesis of 4-chloroethcathinone (4-CEC).

Powder 3 led to sufficient signal for the recording of a proton spectrum and proton-detected experiments (COSY, HSQC, and, in some extend, HMBC) – Figure 19 to Figure 21). However, 256 scans were in this case not enough to record a decent $^{13}C\{^1H\}$ NMR spectrum (spectrum not shown). Except for the two quaternary C2 and C5 atoms, all carbon nuclei could be detected indirectly (by HSQC and for C8, using HMBC), so we could identify the main compound in Powder 3 unambiguously without recording another dataset using more spectrometer time.

The COSY spectrum of powder 3 (Figure 19) allows the identification 3 different spins systems for the main compound. The first one, displayed in green, corresponds to the already identified para-disubstituted pattern. The presence of higher order coupling at such a frequency between the two aromatic protons is in line with a chlorine atom directly attached to the phenyl moiety. The black one corresponds to a CH_3- moiety directly attached to a CH moiety. The very high field of H9 suggest that, as in powder 1, 2, 4,5 and 7, this CH is directly linked to a nitrogen atom. The red spin system corresponds to a CH_2-CH_3 moiety. It is noteworthy that the two protons H12 and H12' are diastereotopes (unambiguously identified as a single CH_2 using ^1H, ^{13}C edited HSQC – Figure 20), suggesting a chiral centre in the vicinity (in our case, C9).

Connectivity between the spin systems was achieved by analysis of the HMBC spectrum (Figure 21): three-bond correlation peaks between H{1,3} and C8 on the one hand and C8 and H11 on the other hand are in line with C8 being linked to C3 (through the not detected C5) and C11 (through C9). All taken together, 4-CEC was proposed as a candidate (Figure 22). Proton and carbon assignment to 4-CEC are summarized in Table 4.

The 4-CEC structure was confirmed by recording another proton spectrum of powder 3 in DMSO-d_6 and compared with the published NMR spectrum of structurally confirmed 4-CEC reference material published as a SWGDRUG monograph by the US Department of Justice – Drug Enforcement Administration's Special Testing and Research Laboratory.

<u>Powder 4</u>

The integration value of all compound's proton resonances in powder 4's proton NMR spectra amounts to 23 (Figure 23). The broad resonance located at 11.37 ppm is characteristic of a labile proton, in this case an ammonium ion as 4'-méthyl-α-pyrrolidinohexiophenone (4F-PHP) is probably isolated as an hydrochlorid.

The two 8.11 and 7.31 ppm aromatic resonances integrate for 2 protons each, which is in line with a para-disubstitution pattern of a benzene ring. However, the fine structure of these two proton resonances, hints towards the presence of an additional spin in the spin system, coupling with about 9 Hz with the 7.31 ppm proton and about 5.6 Hz with the 8.11 ppm proton (Figure 24). In the $^{13}C\{^{1}H\}$ NMR spectrum (Figure 25), only three aromatic resonances, out of four expected resonances, are detected: one being a quaternary carbon, the two others being sp^2 *C*H. However, in spite of the ^{1}H decoupling, these 3 signals at 132.4, 132.5 and 116.8 ppm all come as sharp doublets with decreasing coupling constants, which is in line with an I=1/2 heteroatom with 100% natural abundancy (either ^{19}F or ^{31}P, considering chemical stability) being directly bound to the benzene ring (Figure 26).

A ^{19}F atom (in para, or 4th position of the ring, which could explained the "4F-" in the bag label) was deemed more likely , so two ^{19}F NMR spectrum were recorded, one with and one without proton decoupling . The complex multiplet in the ^{19}F NMR spectrum (Figure 27 and Figure 28) confirmed the presence of fluorine in powder 4.

The chemical shift of the carbon bearing the fluorine atom was identified using the HMBC spectrum (Figure 29 and Figure 30). Indeed, C2 intensely correlates with H{4,6} and less intensely with H{3,7} (two-bond correlation). C8 is found to be a doublet, with a small coupling constant of J_{CF} = 1.4 Hz. This is in line with the ketone being directly attached to the phenyl ring, and in line with the HBMC cross peak between H{4,6} and C8. The sequential interpretation of the COSY spectrum (Figure 31) led to the identification of all resonances belonging to the pentyl sidechain. The corresponding carbon resonances were ascribed using the HSQC spectrum (Figure 32). The protons in position 11 and 17 show strong diastereotopic behaviours, as do H13/H16 and H14/H15. This is due to the stereogenic carbon C10, and, for the pyrrolidinic group, additional impaired intramolecular rotation.

As for powder 1, while no correlation is observed within the pyrrolidinic group in HMBC, cross peaks in the COSY spectrum confirm H13/H16 and H14/H15 belonging to the same, separate spin system.

Correlation between H{11,11'} and C8 in the HMBC support the existence of an C8–C10 bond. The high chemical shift of C10 for a tertiary carbon is also in line with the presence of a nitrogen atom in the direct neighbourhood of C10. This leads to 4F-PHP as a candidate. Assignments for proton and carbon resonances are summarized in Table 5.

<u>Powder 5</u>

Since powder 5 in acetonitrile-d_3 led to a set of overlapping of signals from different spin systems in the aromatic region (Figure 33 for the ^{1}H spectrum in ACN-d_3, COSY spectrum in ACN-d_3 not shown) and overall low intensity leading to missing signals for all spectra involving ^{13}C, another solvent was chosen. DMSO-d_6 was selected because of the risk of signal overlap for the 4.93 ppm signal with the expected 4.87 ppm signal of methanol.

No fluorine signal was detected in ^{19}F NMR.

3,4-methylenedioxy-N-benzylcathinone (BMDP) shares the same 1-(2*H*-1,3-benzodioxol-5-yl) core structure as dibutylone. The same pattern of four resonances corresponding to the ortho- para-trisubstituted phenyl and acetal moieties is therefore detected, with similar coupling constants for both compounds. Contrary to powder 2 where H8 and H8' differ lightly in chemical shifts, these two protons can be seen as almost isochronic for powder 5 in DMSO-d_6 (Figure 34 and Figure 35).

This leaves five additional aromatic protons, corresponding to four ^{13}C resonances in a $1:2:2:1^q$ intensity pattern at δ_C = 128.9, 128.4, 130.0 and 131.9 ppm. This points towards a mono-substituted phenyl ring, directly attached to an CH_2 moiety (correlation peak in HMBC between H{17, 21} and C15 as shown in red in Figure 36 and Figure 37). The chemical shift of C15 suggests that it is directly bonded to a heteroatom, in this case, a nitrogen atom (a CH_2 bonded to an oxygen atom would be expected at an even higher chemical shift). Correlation between H11 and H14 in the COSY spectrum corresponds to an –$CH(CH_3)$- moiety (Figure 38).

As for Dibutylone, the detection of correlation peaks between the benzodioxyol ring protons H2 and H6 and the carbonyl C10 is in line with a direct connection of the carbonyl moiety to the benzodioxyol core. Correlation between H14 and C10 (HMBC spectrum, Figure 39) allows to link the -$CH(CH_3)$- moiety to the carbonyl moiety. The chemical shift of C11 at δ_C = 56.9 ppm, suggests that this moiety is directly bonded to a heteroatom, in our case nitrogen, thus leading to BMDP. This connection is confirmed by the examination of powder 5's COSY spectrum: cross peaks are detected between the two labile NHs and both H{15,15'} and H11 (Figure 38). The presence of two resonances integrating for one proton each instead of only one expected labile proton suggests that Powder 5 contains BMDP in salt form, for example as hydrochloride (Figure 40). All proton and carbon assignments to BMDP are summarized in Table 6.

The proposed structure of BDMP was confirmed by recording another ^1H NMR spectrum in DMSO-d_6 to match the reference data published as an analytical report by Slovenia's National Forensic laboratory.

Powder 6

The labelling of the bag indicates only "MeO-PCP" (methoxy-phencyclidine). The nitrogen rule applied to powder 6 indicates that its molecular formula contains an uneven number of nitrogen atoms. The spectra of powder 6 was recorded in acetonitrile-d_3 (Figure 41 for the ^1H spectrum). The sum of proton integrals amounts to at least 24 (some signals obviously lie under the water resonance).

Examination of the aromatic region of the ^{1}H spectrum of powder 6 in acetonitrile-d_3 led to the exclusion of 4-MeO-PCP as the right candidate because it shows four signals in 1:1:1:1 intensity ratio, which is not compatible with a local C_{2v} symmetry along the {C13-C16} axis. Instead, this 1:1:1:1 motive is consistent with a meta di-substituted phenyl ring. This is in line with the detection of 6 aromatic carbons in the $^{13}C\{^{1}H\}$-NMR spectrum (Figure 42), two of which being quaternary (see comparison with DEPT135,Figure 43). A correlation peak between H20 and C15 in HMBC (Figure 45) confirms the assignment of the ^{13}C signal detected at δ_c = 161.2 ppm, whose chemical shift is consistent with the unscreening effect of a directly bonded oxygen atom. The assignment of {H16 - C16} and {H18-C18} CH pairs respectively to the 7.05 (115.3) and 7.18 (123.0) ppm ^{1}H doublets (^{13}C singlets) was supported by several considerations:

- An OMe moiety located in ortho position screens considerably more the ipso carbon than if located in para (-14.4 ppm, compared to -7.8 ppm) while aliphatic C-N and bulky substituents such as tert-butyl groups have much smaller effects (<± 4 ppm), similar in intensity for ortho and para positions.
- A correlation peak between H18 and C7 is detected in HMBC. The in the ^{1}H spectrum overlapping H14 signal which also correlates with C7 is however unfortunately located, so that this correlation peak is only detected as a shoulder of the latter (Figure 44).
- All ^{1}H NMR chemical shift simulations based on the structure of 3-MeO-PCP using MestreNova® and Chemdraw® chemical shift predictors locate H18 at higher field than H16.

C7 was identified as a quaternary carbon using DEPT (Figure 43). It is quite high chemical shift (δ_{C7} = 71.8 ppm) suggests that one of its substituents is an electronegative atom, probably a nitrogen (in the case of an oxygen, it would be expected at about 80 ppm). The remaining aliphatic carbons (apart from C20) are all CH_2 moieties, which hints to (hetero)cycles.
4 out of the 6 remaining carbons display higher intensity than two last, suggesting the presence of a symmetry axis within the ring(s). This is confirmed by the ^{1}H, ^{13}C edited gHSQC (Figure 43 and Figure 45), which allows to identify pairs of diastereotopic protons:

- The protons correlated to the carbons at δ_C = 48.2 and 31.2 ppm unambiguously integrate for 2+2 protons each, confirming that they correspond to 2 equivalent CH_2 moieties each.
- The proton correlated to the carbon at δ_C = 25.7 ppm unambiguously integrates for 1+1 protons.
- The presence of overlaps in the proton spectrum at δ_H = 1.71 and δ_C = 1.17 ppm combined with the resolution of our HSQC spectrum along the F1 dimension, which make it difficult to

distinguish the remaining three carbons, do not allow to pair the carbons with their respective proton pairs using only HSQC (Figure 45).

- The remaining proton signals integrate for 2:2:3:3 protons respectively, which hints towards three proton pairs of 2+2:2+2: 1+1 intensity respectively. The last one can easily be ascribed to C4 as the only remaining single CH_2 (the C4 signal intensity in $^{13}C\{^1H\}$ is comparable to the one of C10, and about the half of the two other remaining ones – Figure 46).

On the one hand, the chemical shift of C{2,6} is in line with a neighbouring nitrogen atom. On the other hand, the protons ascribed to H{8',12'} show a correlation to C13 in HMBC (Figure 44), including C7 in a 6-membered ring. Precise attribution within the rings could be achieved by analysing the COSY spectrum (Figure 47 and Figure 48). The proton and carbon assignments are summarized in Table 7.

The proposed structure of 3-methoxy-phencyclidine (3-MeO-PCP) was confirmed (Figure 49) by recording another ^1H NMR spectrum in DMSO-d_6 to match the structurally confirmed reference data published as a SWGDRUG monograph by the US Department of Justice – Drug Enforcement Administration's Special Testing and Research Laboratory.

Powder 7

From the content of bag 7 escaped an acrid stench, strongly reminding acetic acid. Acetic acid was indeed detected as main contaminant: in the proton spectrum (Figure 50 and Figure 51 - singlet at 1.96 ppm, presence of a labile H at 11.83 ppm, whose integral hints either to acetic acid or to the protonated form of alpha-pyrrolidinopropiophenone -α-PPP) as well as in $^{13}C\{^1H\}$ NMR (Figure 52) at 19.1 ppm and 170.8 ppm (detected only in HMBC, Figure 53).

The main compound resonances integrals in the proton spectrum of powder 7 in acetonitrile-d_3 (Figure 50) sum for 18 (17+1 protons because of an acid pH). 11 resonances ascribed to the main substance of Powder 7 are detected in the corresponding $^{13}C\{^1H\}$ NMR spectrum (Figure 52).

The aromatic region of the proton spectrum (Figure 51) shows the typical pattern of a mono-substituted benzene ring: three signals of relative intensities 2:2:1, thus mirroring the local axis {9 – 13} of symmetry. This is also the case in the carbon spectrum, where four resonances are detected in the aromatic region, three of which being tertiary carbons (CH), the fourth one being quaternary. Precise assignment could be achieved, first by taking into account the H13 smaller expected integral, then by analysis of the corresponding parts of the COSY and HSQC spectra (Figures 54 to 56). A cross peak corresponding to the three bond correlation between H{12, 14} and C9 is also detected in the HMBC spectrum (Figure 53).

An intense cross-peak between H{11, 15} and the ketonic C8 indicates a close through-bond proximity, which is in line with the phenyl moiety being directly connected to C8.

As for powder 1, the diastereotopy of H{3,3'} and H{4,4'} (as well as H{5, 5') and H{6,6'}) arises from the presence of the stereogenic carbon C2. C3, C4, C5 and C6 are all individually detected in $^{13}C\{^{1}H\}$ and DEPT135 as CH_2 moieties. Furthermore, the unscreened chemical shifts of C2, C3, C4 on one hand, H2, H{3,3'} and H{4,4'} on the other hand, indicate that C2, C3 and C4 are all directly connected to the nitrogen atom. This is supported by the fact that H2 seems to be three-bond connected to the unscreened C3 or C4 (cross peak at δ_C= 53. 7 ppm in HMBC).

All assignments are summarized in Table 8.

The proposed structure of α-PPP was confirmed by recording another ^{1}H NMR spectrum in D_2O to match the reference data published as a SWGDRUG monograph by the US Department of Justice – Drug Enforcement Administration's Special Testing and Research Laboratory.

Conclusion

The use of NRM spectroscopy is necessary to collect enough data to identify 7 unknown small bags of powders, involved in a complex toxicological fatal case. This method allowed to identify the powders unambiguously and to measure their purity. Only NMR spectroscopy allows to unambiguously identify the supposed compound, possible remains of chemical reagents, determine the purity when the reference standard is missing and establish with high degree of certainty the chemical structure.

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Tableau / Table

Powder	Identification by ^{1}H-NMR	Purity (%)
1	4-chloro-alpha-pyrrolidinopentiophenone	81.2
2	dibutylone	59.6
3	4-chloroethcathinone	82.0
4	4-alpha-pyrrolidinohexuiphenone	87.7
5	3,4-methylenedioxy-N-benzylcathinone	50.8
6	3-methoxy-phencyclidine	72.9
7	alpha-pyrrolidinopropiophenone	89.5

Table 1: Identification of 7 powders, by 1H-NMR and purity

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm)
1, 3	8.13 ppm, m, 2H	130.8 ppm, s, 2*C*H
4, 6	7.66 ppm, m, 2H	131.8 ppm, s, 2*C*H
9	5.47 ppm, m, 1H	70.4 ppm, s, 1*C*H
13, 13', 16, 16'	3.43 ppm, broad, 1H 3.71 ppm, broad, 2H 3.13 ppm, broad, 1H	52.6 ppm, s, 1CH2 55.6 ppm, s, 1CH2
14, 14', 15, 15'	2.25 ppm – 2.18 ppm, m, broad, 4H	23.4 ppm, broad s, 2CH_2
11, 11'	2.07 ppm, m, 2H	31.6 ppm, s, 1CH_2
17, 17'	1.31 ppm, m, 1H 1.21 ppm, m, 1H	17.7 ppm, s, 1CH_2
18	0.90 ppm, t, J=7.3 Hz, 3H	14.1 ppm, s, 1CH_3
2	-	133.3 ppm, s, 1 C_q
5	-	142.1 ppm, s, 1 C_q
8	-	196.4, s, 1 C_q=O

Table 2 - Attribution of 1H and 13C signals to 4-Cl-α-PVP for powder 1 in MeOD-d4.

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm)
8, 8'	6.05 ppm, s, 2 H	100.0 ppm, s, 1 C
15, 16	2.87 ppm, broad s, 6 H	Detected as very broad signal ~41 ppm, 2 CH_3
11	5.04 ppm, dd, J_{HH} = 3.96 Hz, J_{HH}=6.30 Hz, 1 H	67.3 ppm, s, 1 *C*H
12, 12'	2.07 ppm, m, 2 H	19.4 ppm, s, 1 CH_2
13	0.74 ppm, t, J_{HH} = 7.55 Hz, 3 H	4.4 ppm, s, 1 CH_3
10	-	192.0 ppm, s, 1 C_q=O
2	-	125.5 ppm, s, 1 C_q-C
4	-	145.8 ppm, s, 1 C_q-O
5	-	151.2 ppm, s, 1 C_q-O
1	7.63 ppm, dd, J_{HH}=1.87 Hz, J_{HH}=8.37 Hz, 1 H	124.1 ppm, s, 1 *C*H
3	7.41 ppm, d, J_{HH}=1.86 Hz, 1 H	105.1 ppm, s, 1 *C*H and 105.9 ppm, s, 1 *C*H[1]
6	6.96 ppm, d, J_{HH}=8.37 Hz, 1 H	

Table 3 – Attribution of 1H and 13C signals to dibutylone for powder 2 in D2O

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm)
1,3	8.00 ppm, m, 2 H	129.9 ppm, s, 2 *C*H
4,6	7.58 ppm, m, 2 H	128.7 ppm, s, 2 *C*H
9	4.97 ppm, q, J_{HH} = 7.1 Hz, 1 H	56.9 ppm, s, 1 *C*H
11	1.59 ppm, d, J_{HH} = 7.1 Hz, 3 H	14.7 ppm, s, 1 CH_3
{12, 12'}	3.10 ppm, m, 1 H 2.99 ppm, m, 1 H	40.5 ppm, s, 1 CH_2
14	1.39 ppm, t, J_{HH} = 7.2 Hz, 3 H	10.0 ppm, s, 1 CH_3
5	–	1 C_q, not detected
2	–	1 C_q, not detected
8	–	195.9 ppm, s, 1 C_q=O

Table 4 - Attribution of the 1H and 13C NMR chemical shifts to 4-CEC for powder 3 in Acetonitrile-d3.

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm), multiplicity	δ_F (ppm)
Labile H/NH+	**11.34 ppm, broad s, 1H**	**–**	**–**
4,6	8.11 ppm,	132.9 ppm, d, JCF = 9.9 Hz, 2 CH	–
3,7	**7.31 ppm,**	**117.2 ppm, JCF = 22.6 Hz, 2 CH**	**–**
10	5.18 ppm,	68.4 ppm, s, 1 CH	–
13, 13' or 16, 16'	**3.76 ppm, broad m, 1H** **3.16 ppm, broad m, 1H**	**53.5 ppm, s, 1 CH2-N**	**–**
16, 16' or 13, 13'	3.63 ppm, broad m, 1H 3.09 ppm, broad m, 1H	54.5 ppm, s, 1 CH2-N	–
14,14' or 15, 15'	**2.06 ppm, m, 2H**	**24.3 ppm, s, 1CH2**	**–**
15, 15' or 14, 14'	2.13 ppm, m, 2H	24.5 ppm, s, 1CH2	–
11, 11'	**2.05 ppm, m, 2H**	**30.5 ppm, s, 1 CH2**	**–**
17, 17'	1.39 ppm, m, 1H 1.14 ppm, m, 1H	27.6 ppm, s, 1 CH2	–
18, 18'	**1.22 ppm, m, 2H**	**23.1 ppm, s, 1 CH2**	
19	0.77 ppm, t, JHH = 7.1 Hz, 3H	13.8 ppm, s, 1 CH3	–
2	**–**	**167.5 ppm, d, JCF = 257.8 Hz, 1 CqF (HMBC)**	**–**
5	–	132.8 ppm, d, JCF = 2.3 Hz, 1Cq	–
8	**–**	**195.8 ppm, d, JCF = 1.4 Hz, 1 Cq=O**	**–**
1	–	–	-104.5 ppm

Table 5 - Attribution of the 1H and 13C NMR chemical shifts of 4F-PHP in powder 4 in Acetonitrile-d3

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm), multiplicity
Labile H (NH/NH2+)	**10.24 ppm, broad s, 1H**	-
Labile H (NH/NH2+)	9.41 ppm, broad s, 1H	-
19	**7.40 ppm, m, 1H**	**128.9 ppm, s, 1CH**
6	7.53 ppm, d, JHH=1.8 Hz, 1H	107.6 ppm, s, 1CH
3	**7.13 ppm, d, JHH=8.2 Hz, 1H**	**108.3 ppm, s, 1CH**
2	7.69 ppm, dd, JHH=1.8 Hz and JHH=8.3 Hz, 1H	125.5 ppm, s, 1CH
{18,20}	**7.41 ppm, m, 2H**	**128.4 ppm, s, 2CH**
{17,21}	7.57 ppm, m, 2H	130.0 ppm, s, 2CH
11	**5.19 ppm, m, 1H**	**56.9 ppm, s, CH**
15, 15'	4.20 ppm, d, JHH = 13.1 Hz, 1H 4.05 ppm, d, JHH = 13.3 Hz, 1H	48.7 ppm, s, CH2
14	**1.52 ppm, d, JHH = 7.1 Hz, 3H**	**16.2 ppm, s, CH3**
8, 8'	6.20 ppm, m, 2H	102.4 ppm, s, CH2
16	**–**	**131.9 ppm, s, Cq**
1	–	127.4 ppm, s, Cq
5	**–**	**148.2 ppm, s, Cq-O**
4	–	152.7 ppm, s, Cq-O
10	**–**	**193.9 ppm, s, Cq=O**

Table 6 - Attribution of 1H and 13C NMR signals of BMDP for powder 5 in acetonitrile-d3

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm), multiplicity
2 or 6	3.62 ppm, d, 2H ~2.26 ppm, m, 2H (underlying signal)	48.2 ppm, s, $2CH_2$
3 or 5	~2.28 ppm, m, 2H (underlying signal) 1.71 ppm, m, 2H (overlapping signal)	23.7 ppm, s, 2 CH_2
4	1.71 ppm, m, 1H (overlapping signal) 1.16 ppm, m, 1H (overlapping signal)	23.0 ppm, s, 1 CH_2
7	–	71.8 ppm, s, $1C_q$
8 or 12	2.88 ppm, d, 2H 2.41 ppm, td, 2H	31.2 ppm, s, 2 CH_2
9 or 11	1.80 ppm, d, 2H	23.7 ppm, s, 2 CH_2
10	1.55 ppm, m, 1H 1.28 ppm, m, 1H	25.7 ppm, s, 1 CH_2
13	–	133.7 ppm, s, 1 $=C_q$
14	7.16 ppm, s, 1H	116.9 ppm, s, 1 CH
15	–	161.2 ppm, s, 1 $=C_q$–O
16	7.05 ppm, dd, J_{HH} = 8.2 and 2.5 Hz, 1H	115.3 ppm, s, 1 CH
17	7.45 ppm, t, J_{HH} = 8.0 Hz, 1H	130.8 ppm, s, 1 CH
18	7.18 ppm, d, J_{HH} = 8.40 Hz (HBMC), 1H	123.0 ppm, s, 1 CH
20	3.86 ppm, s, 3H	56.1 ppm, s, 1 CH

Tableau 7 - Chemical shifts assignments to 3-MeO-PCP for powder 6 in acetonitrile-d3

Atom numbering	δ_H (ppm), multiplicity	δ_C (ppm), multiplicity
Acid H of acetic acid / NH^+	11.83 ppm, broad s, 1H	-
11, 15	7.99 ppm, m, 2H	129.8 ppm, s, 2*C*H
13	7.71 ppm, m, 1H	135.6 ppm, s, 1*C*H
12, 14	7.57 ppm, m, 2H	130.2 ppm, s, 2*C*H
2	5.15 ppm, p, J_{HH}= 7.1 Hz, 1H	65.0 ppm, s, 1*C*H
3, 3' or 4, 4'	3.77 ppm, m, 1H 3.14 ppm, m, 1H	53.2 ppm, s, 1CH_2
4, 4' or 3, 3'	3.68 ppm, m, 1H 3.07 ppm, m, 1H	53.7 ppm, s 1CH_2
5, 5' and 6, 6'	2.06 ppm, m, 4H	24.6 ppm, s, 1CH_2 24.5 ppm, s, 1CH_2
7	1.64 ppm , d, J_{HH}= 7.2 Hz, 3H	16.3 ppm, s, 1CH_3
9	-	134.7 ppm, s, $1C_q$
8	-	196.7 ppm, s, 1C_q=O
CH_3- of acetic acid	1.96 ppm, s, 3H	19.1 ppm, s, 1CH_3
C=O of acetic acid	-	170.8 ppm, s, 1(C_q=O)O

Table 8 - Chemical shifts assignments to α-PPP for powder 7 in acetonitrile-d3

Figure 1

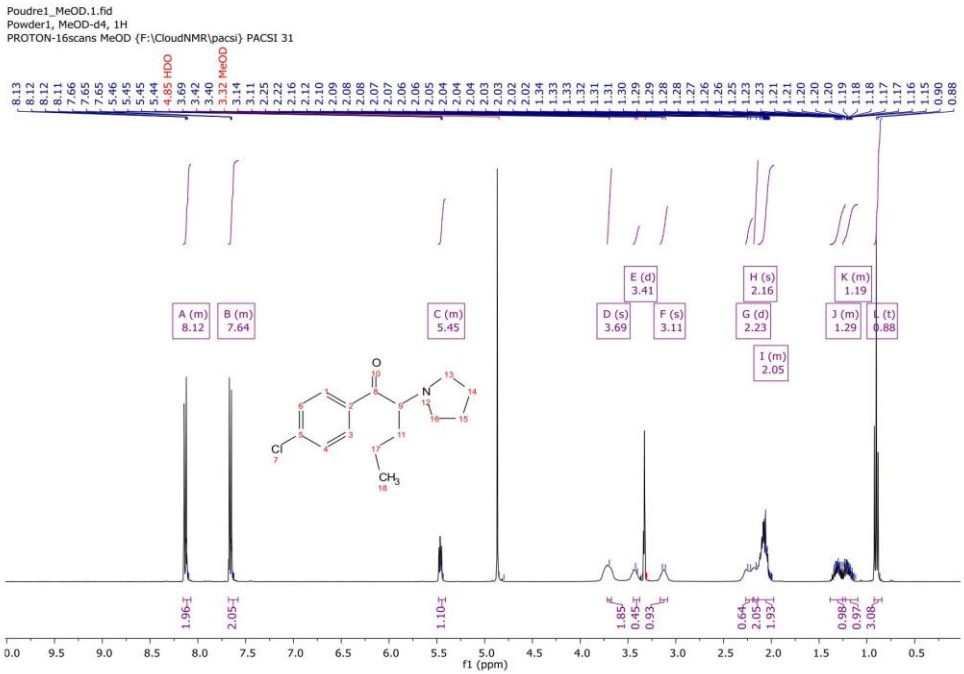

Figure 1- ^{1}H NMR spectrum of powder 1 in MeOD-d_4

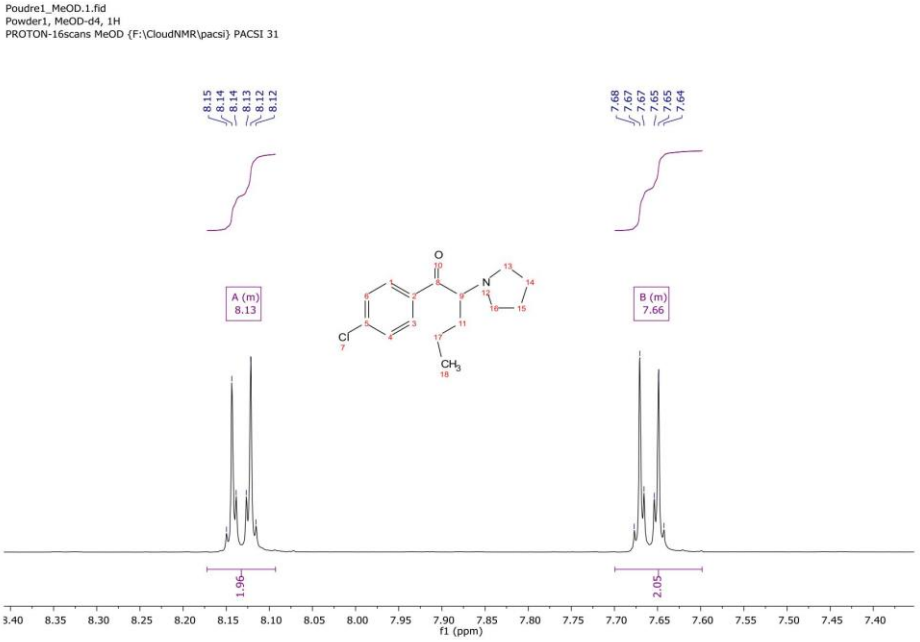

Figure 2 - Zoom of the aromatic region of the ^{1}H NMR spectrum of powder 1 in MeOD-d_4.

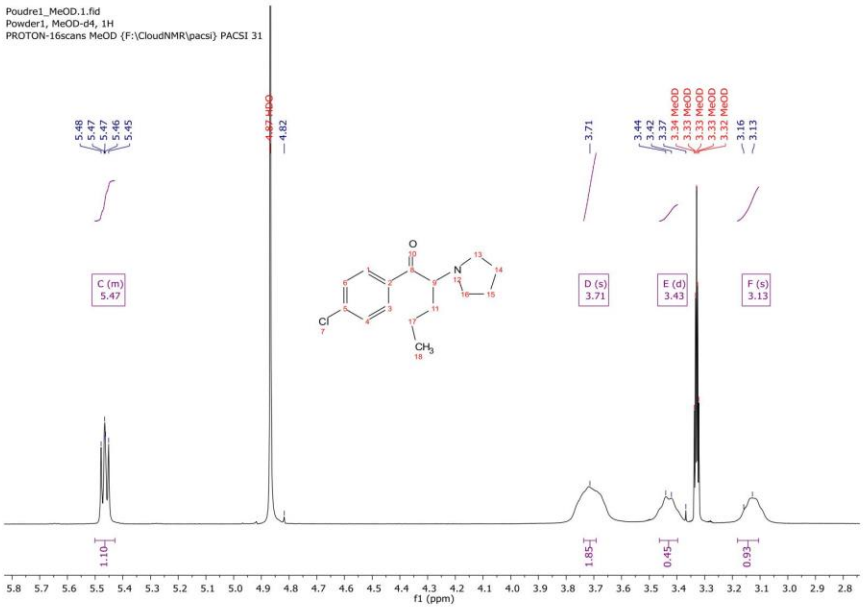

Figure 3 - Zoom of the higher frequency part of the aliphatic region of the ^{1}H spectrum of powder 1 in MeOD-d_4

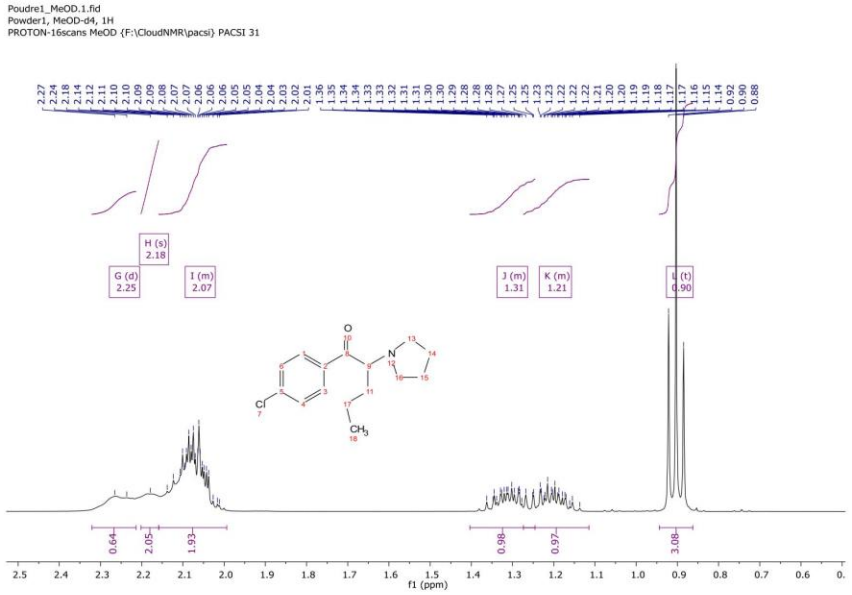

Figure 4 -Zoom of the lower frequency part of the aliphatic region of the ^{1}H spectrum of powder 1 in MeOD-d_4

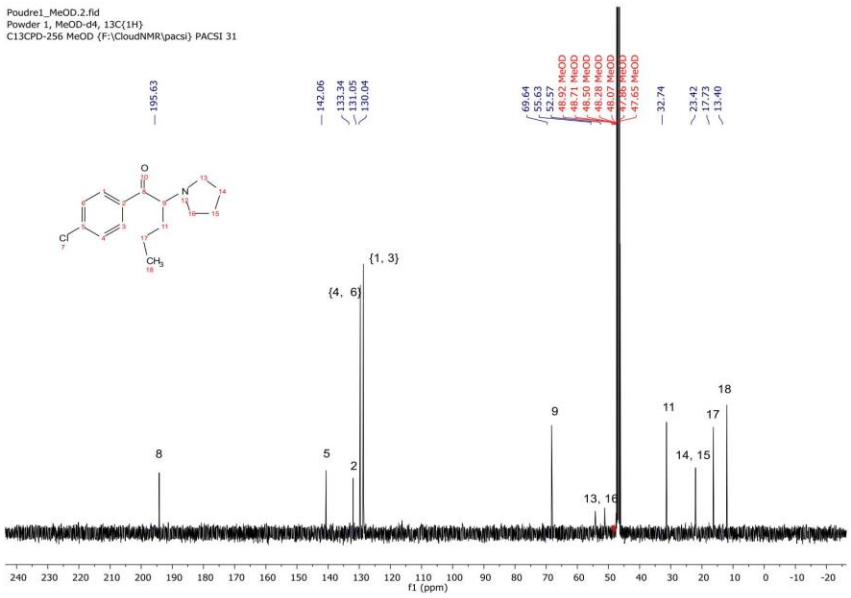

Figure 5 - $^{13}C\{^{1}H\}$-NMR spectrum of powder 1 in MeOD-d_4

Figure 2

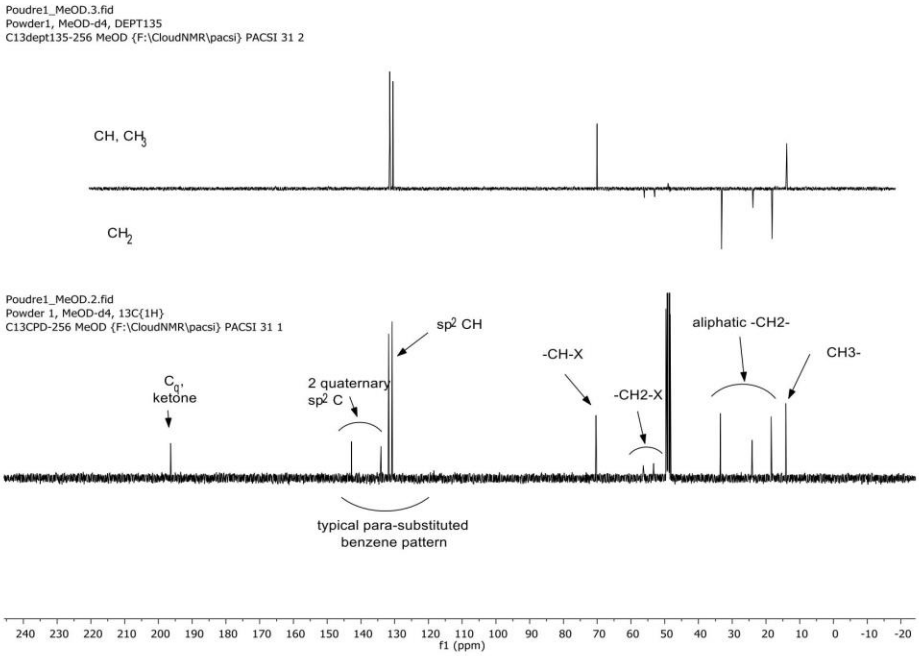

Figure 6 - Superimposed annotated $^{13}C\{^1H\}$ and DEPT135 spectra of powder 1 in MeOD-d_4. X stands for electronegative atom. Please note that quaternary C are not visible in DEPT135.

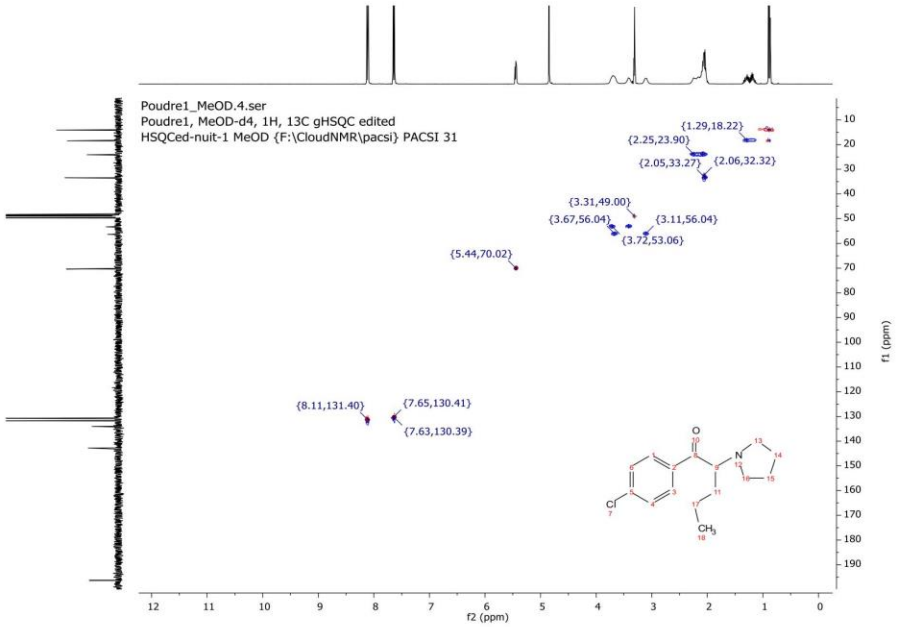

Figure 7 - 1H, 13C edited gHSQC of powder 1 in MeOD-d4. Red dots correspond to CH and CH3 moieties while CH2 cross peaks are depicted in blue.

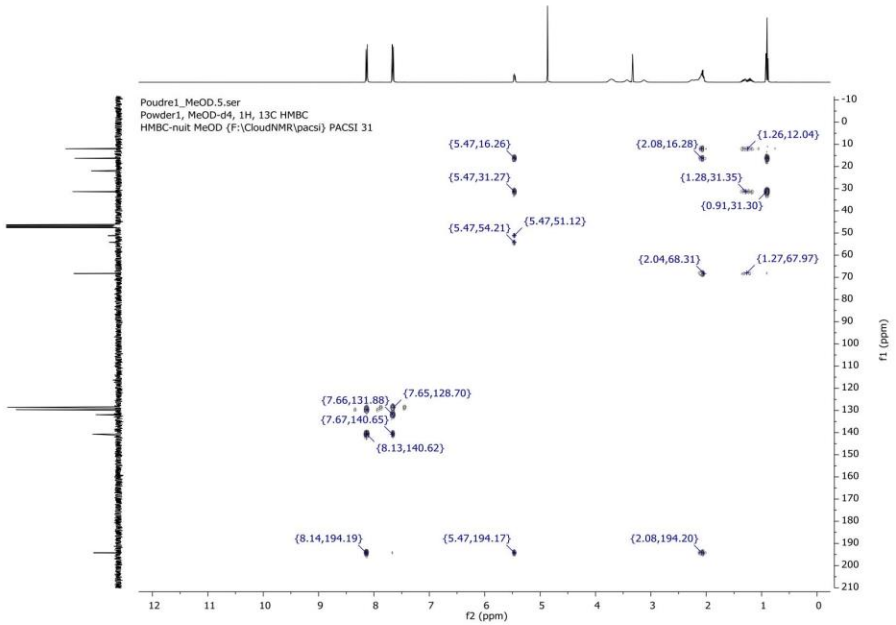

Figure 8 - ^{1}H, ^{13}C gHMBC of powder 1 in MeOD-d_4

Figure 3

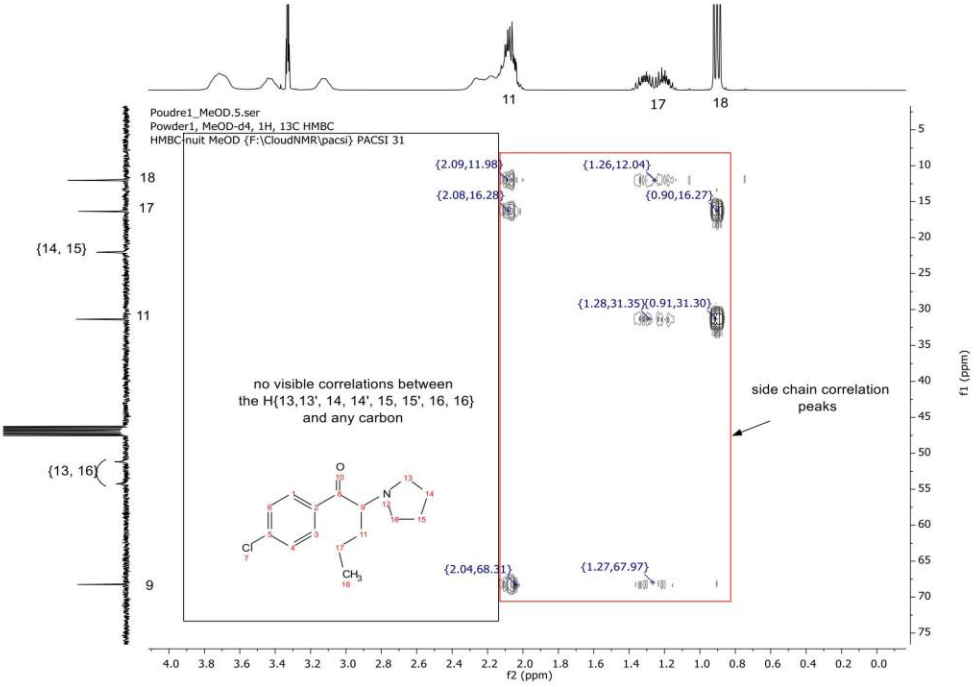

Figure 9 - Zoom of the ^{1}H, ^{13}C gHMBC spectrum of powder 1 in MeOD-d_4.

Figure 4

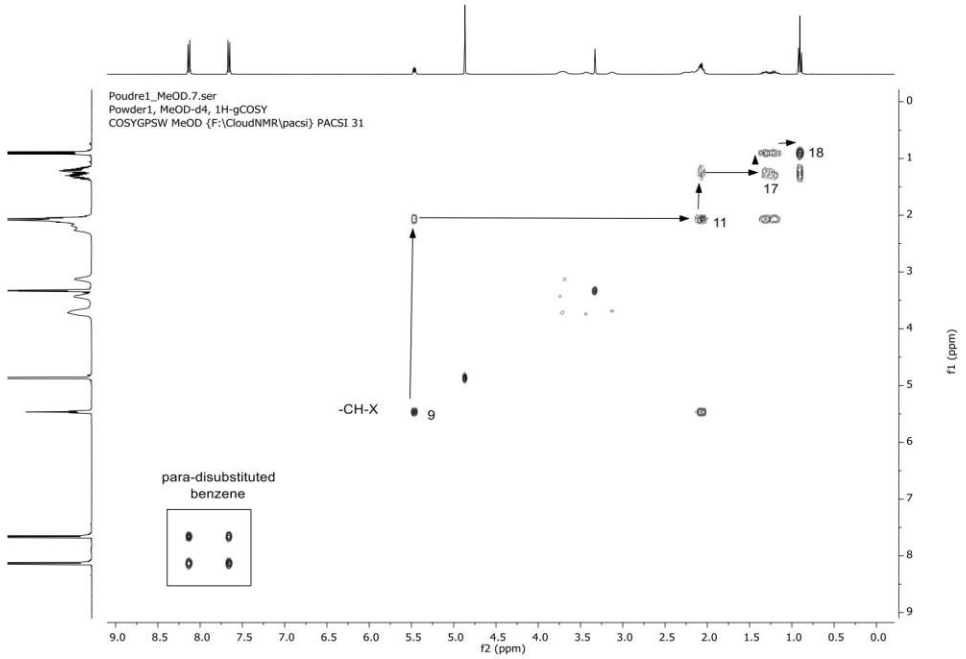

Figure 10 - ^{1}H gCOSY spectrum of powder 1 in MeOD-d_4.

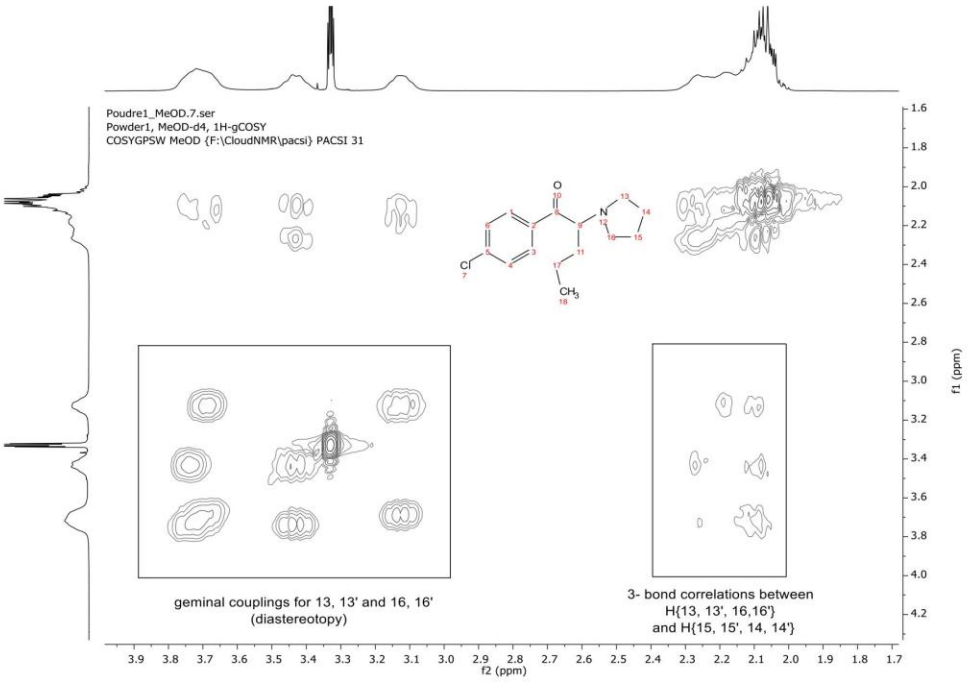

Figure 11 - Zoom of the ^{1}H gCOSY spectrum of powder 1 in MeOD-d_4.

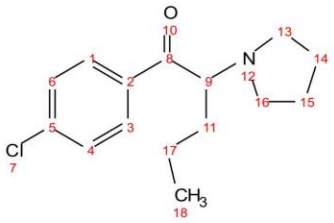

Figure 12 - Structure of 4-Cl-α-PVP

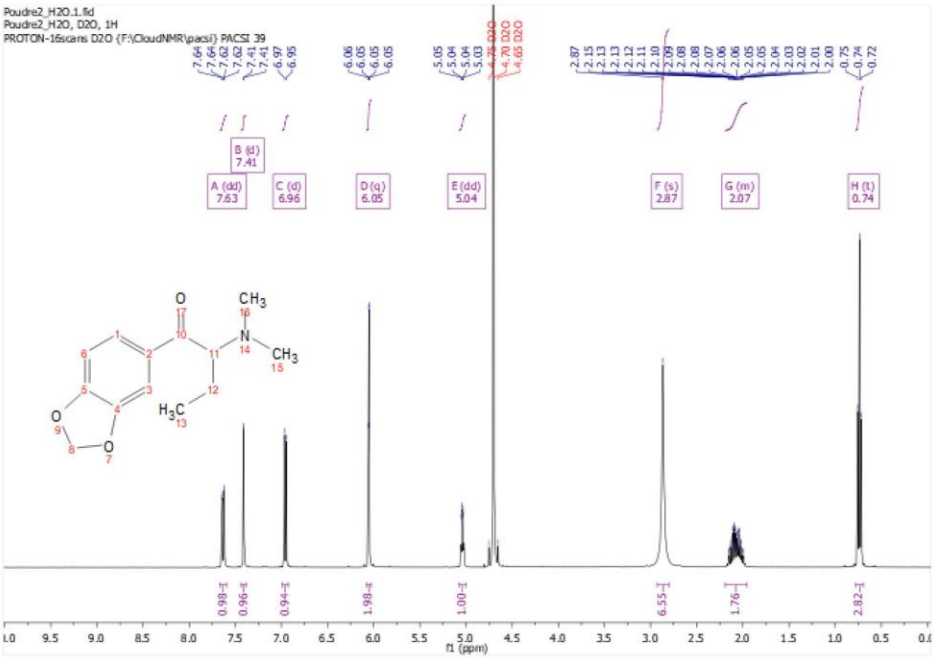

Figure 13 - ^{1}H NMR spectrum of powder 2 in D_2O.

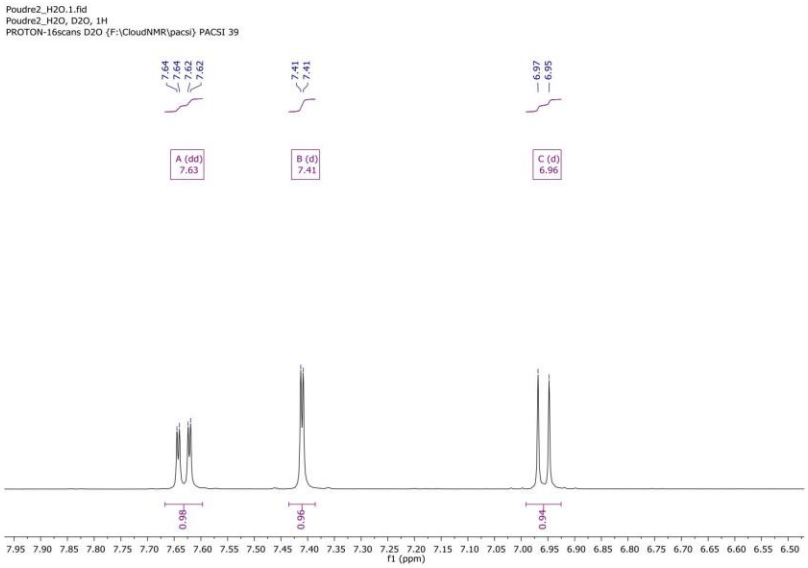

Figure 14 - Zoom on the aromatic region of the ^{1}H NMR spectrum of powder 2 in D_2O.

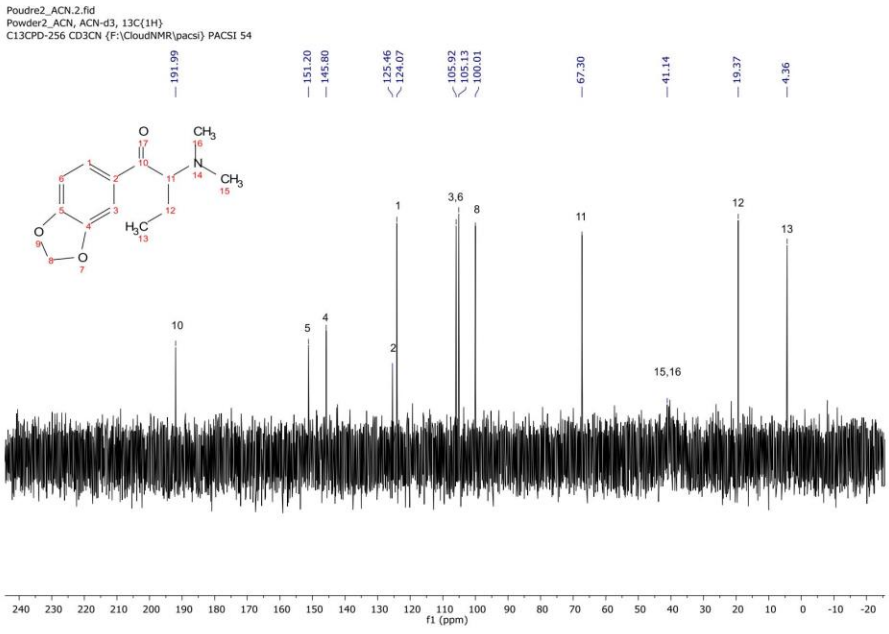

Figure 15 - $^{13}C\{^{1}H\}$ NMR spectrum of powder 2 in D_2O.

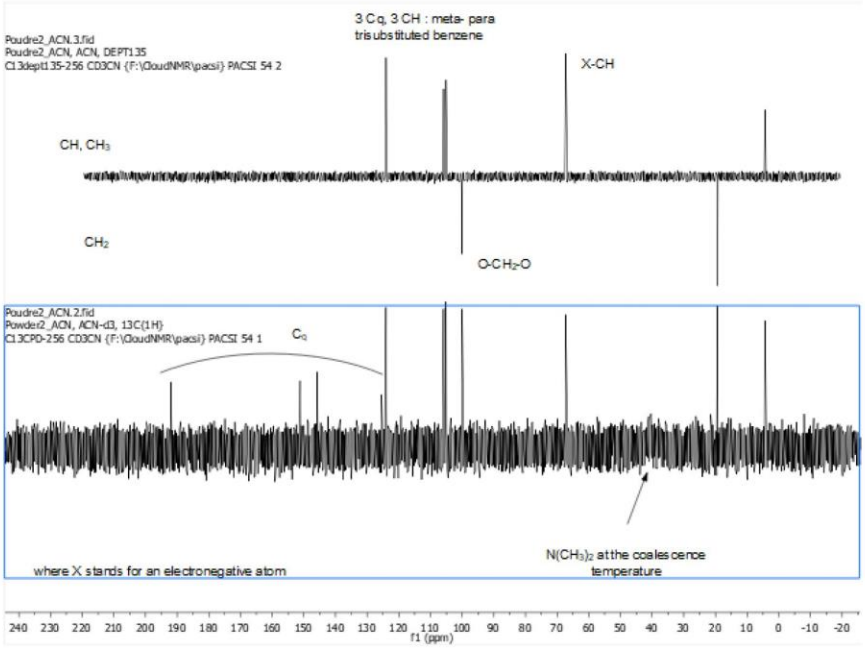

Figure 16- Comparison between the DEPT135 and $^{13}C\{^1H\}$ NMR spectra of powder 2 in D_2O.

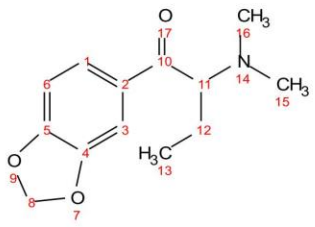

Figure 17 - Structure of dibutylone

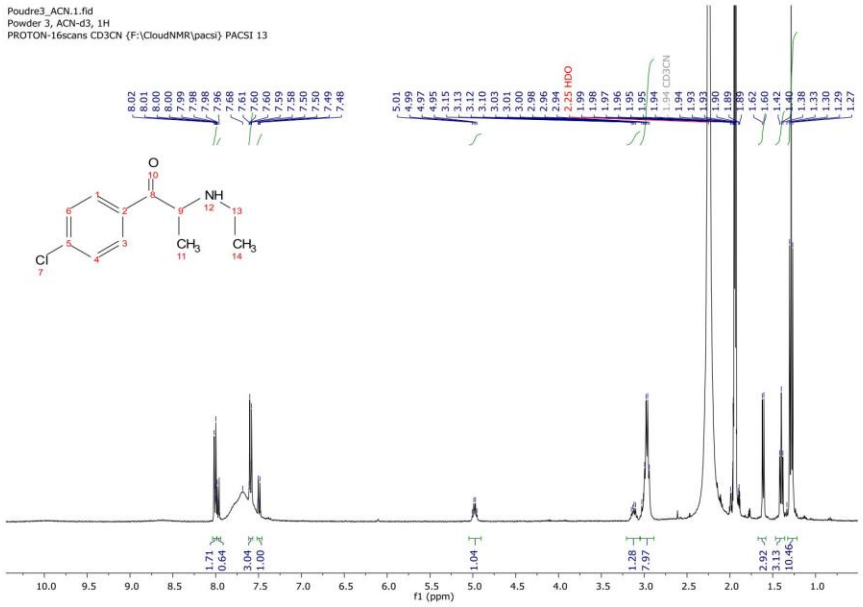

Figure 18 - ^{1}H NMR spectrum of powder 3 in Acetonitrile-d_3

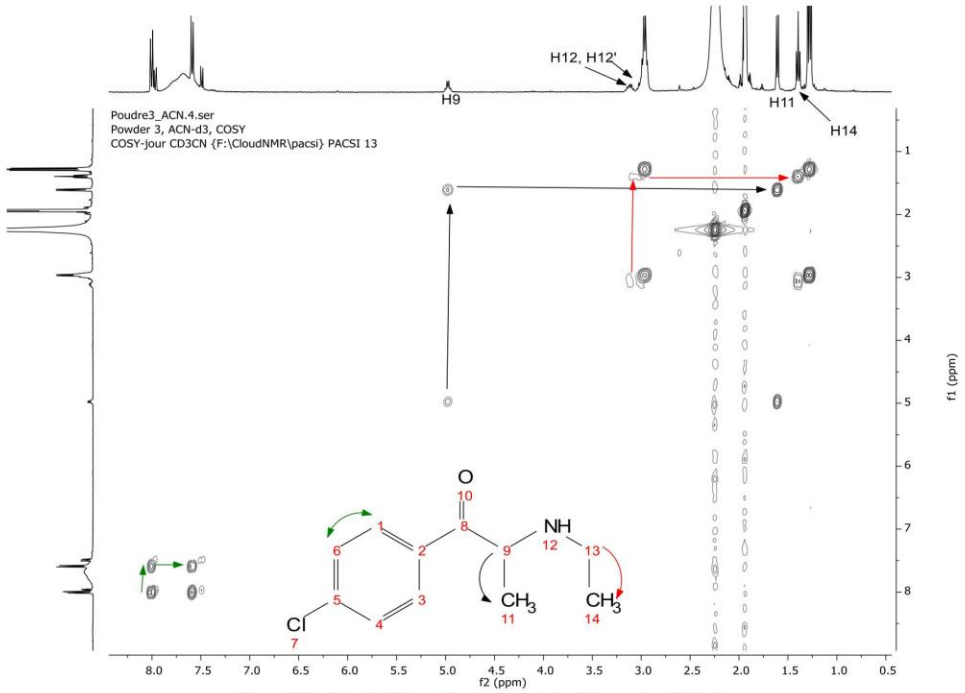

Figure 19 - ^{1}H gCOSY spectrum of powder 3 in acetonitrile-d_3

Figure 5

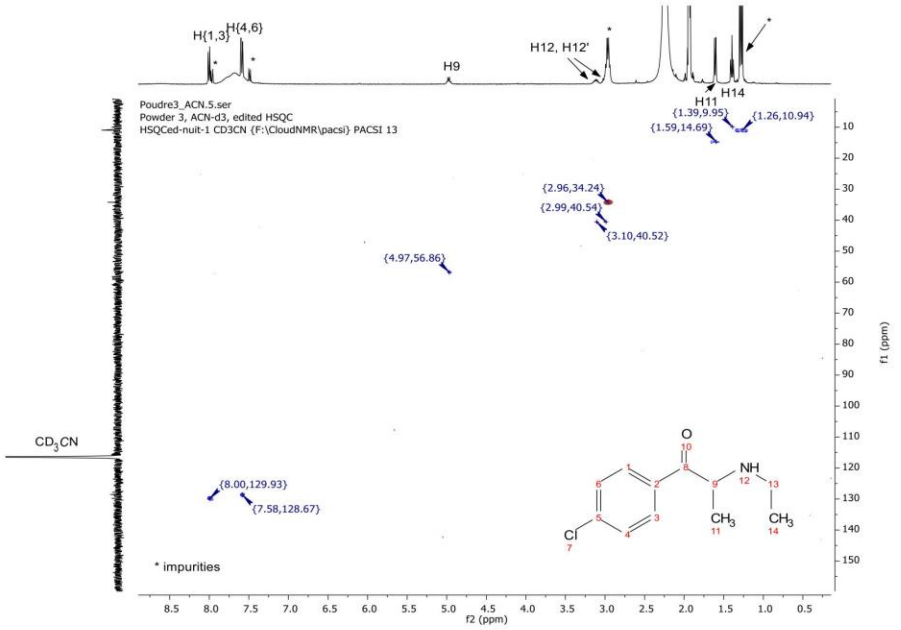

Figure 20 - ^{1}H, ^{13}C edited gHSQC of powder 3 in acetonitrile-d_3.

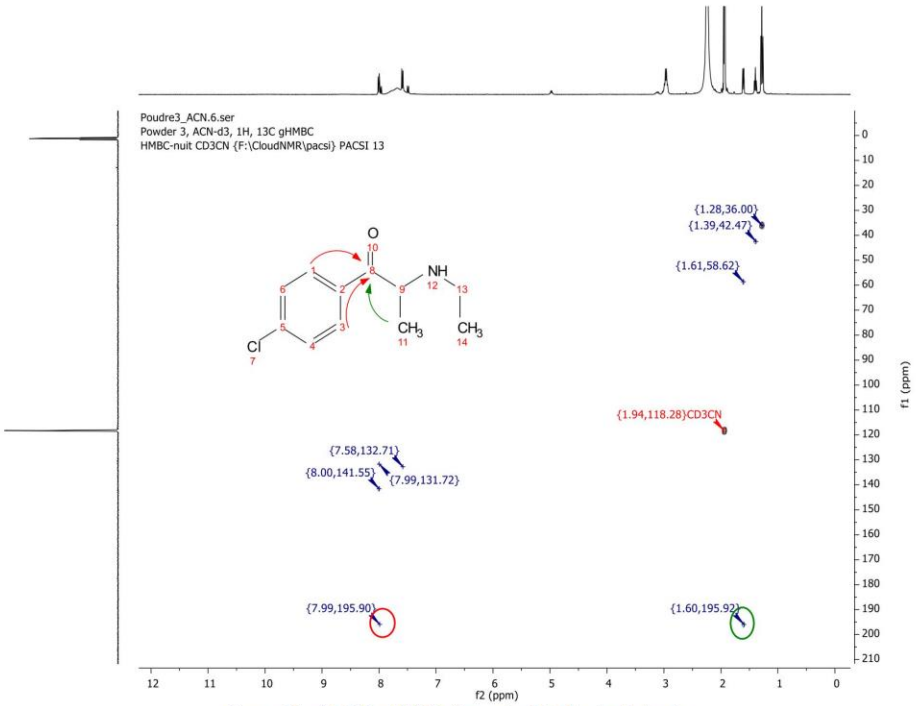

Figure 21 - ^{1}H, ^{13}C gHMBC of powder 3 in Acetonitrile-d_3.

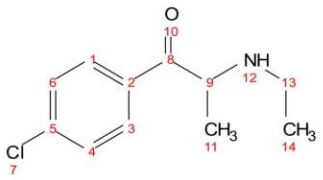

Figure 22 - Structure of 4-CEC

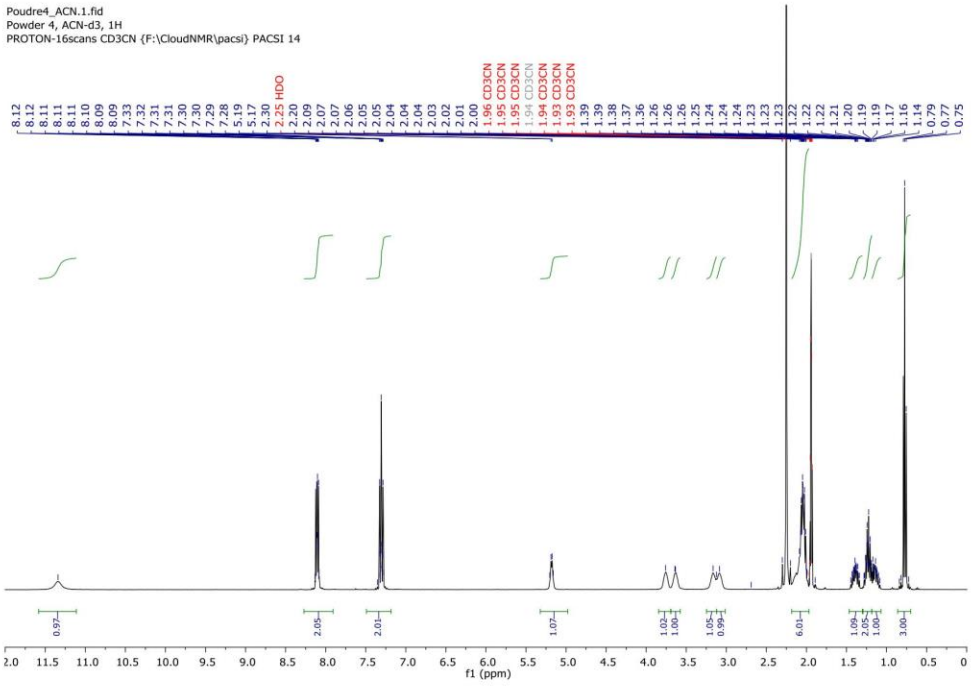

Figure 23 - ^{1}H NMR spectrum of powder 4 in acetonitrile-d_3.

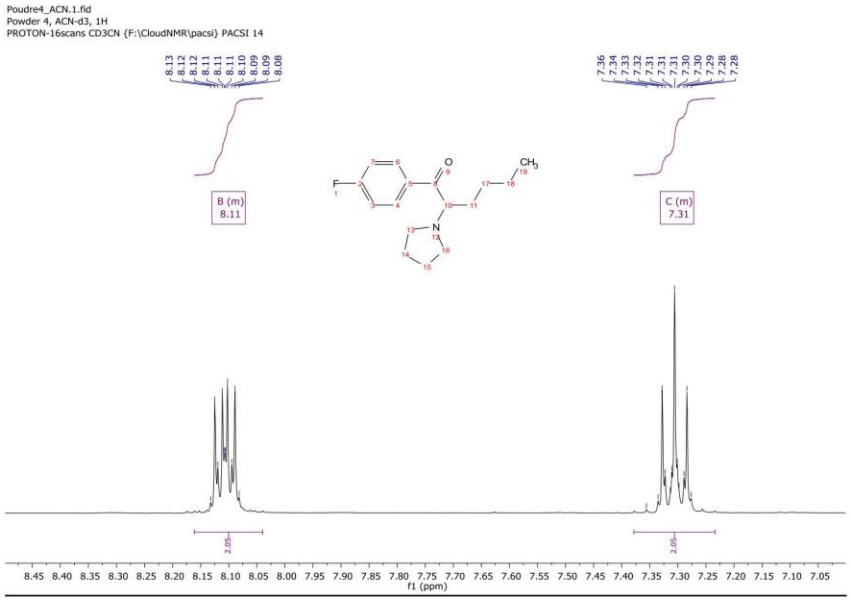

Figure 24 - Zoom on the 8.5–7.0 ppm region of the ^{1}H NMR spectrum of powder 4 in acetonitrile-d_3.

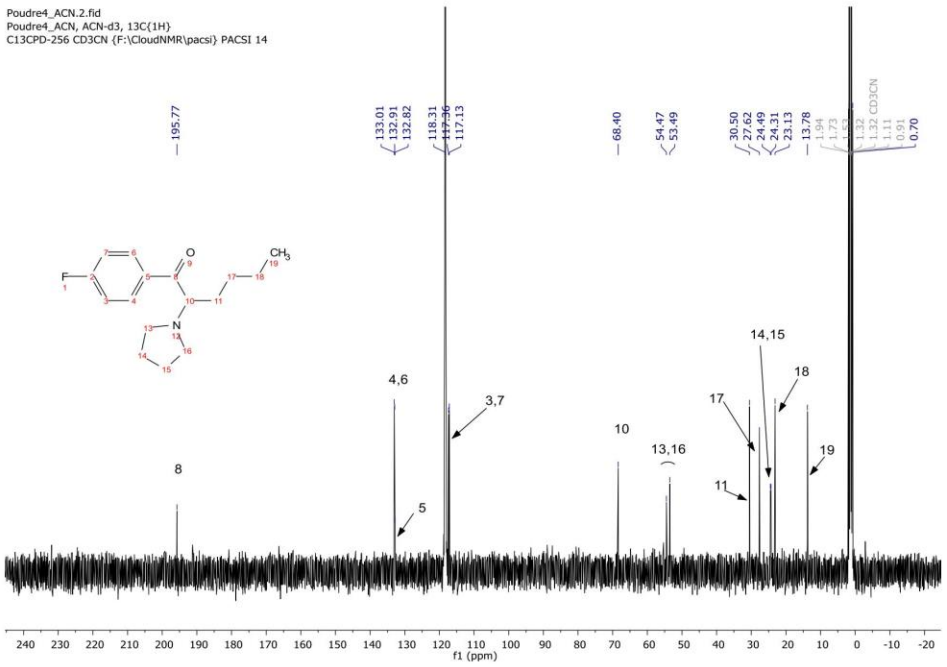

Figure 25 - $^{13}C\{^{1}H\}$ NMR spectrum of powder 4 in acetonitrile-d_3

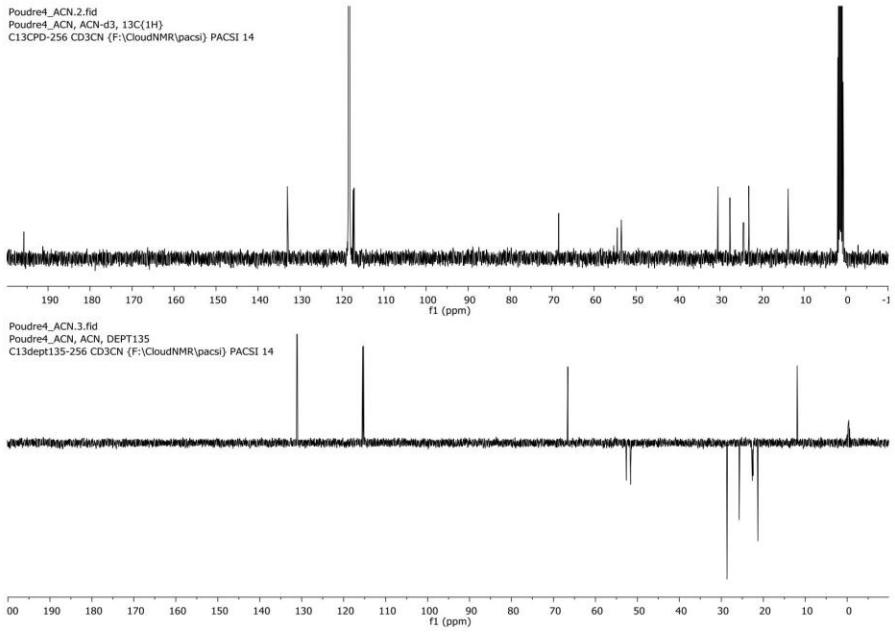

Figure 26 - Comparison between the DEPT135 and $^{13}C\{^{1}H\}$ NMR spectra of powder 4 in acetonitrile-d_3.

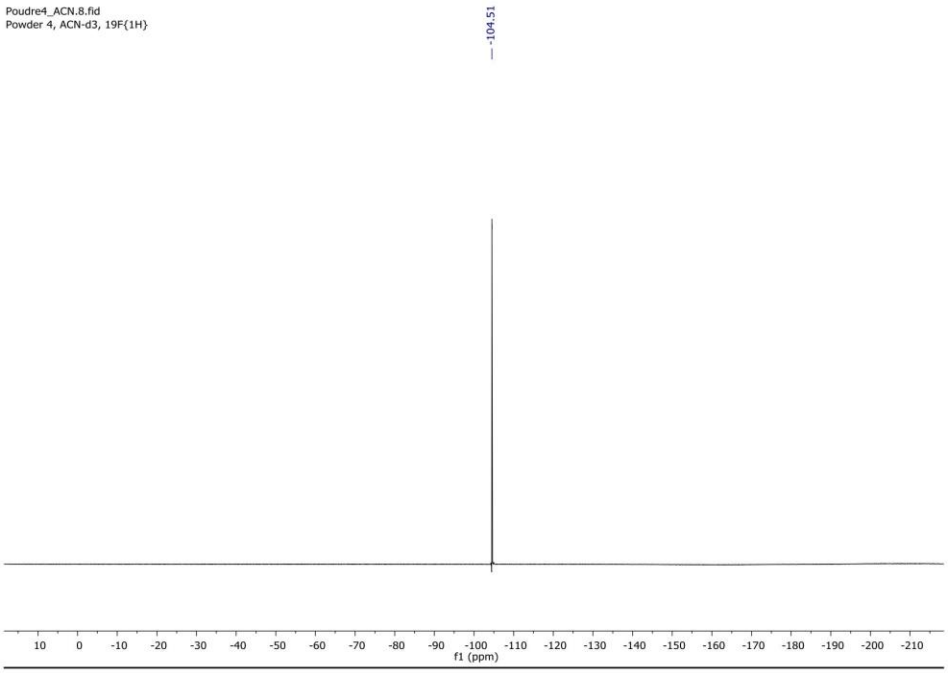

Figure 27 - $^{19}F\{^{1}H\}$ NMR spectrum of powder 4 in acetonitrile-d_3

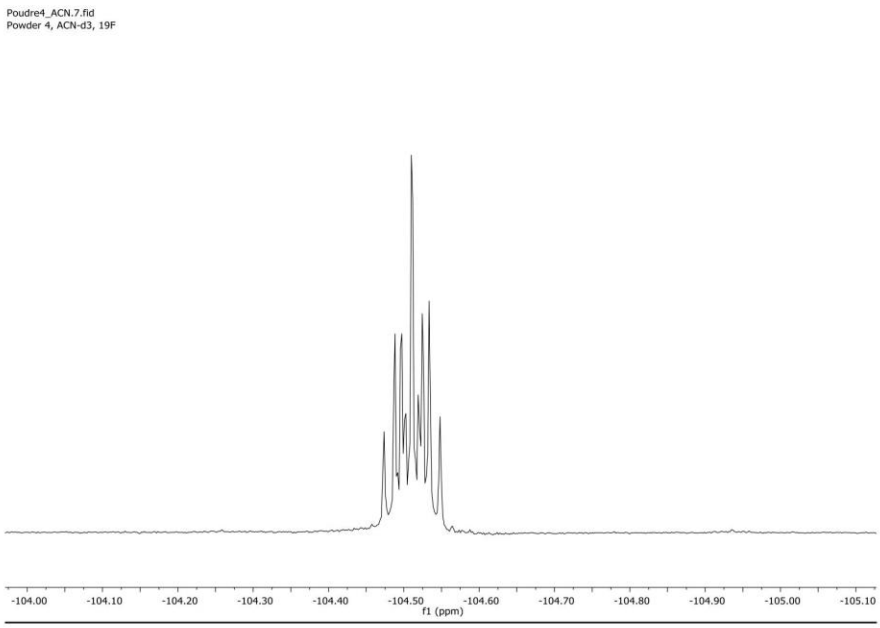

Figure 28 - Zoom of the ^{19}F NMR spectrum of powder 4 in acetonitrile-d_3

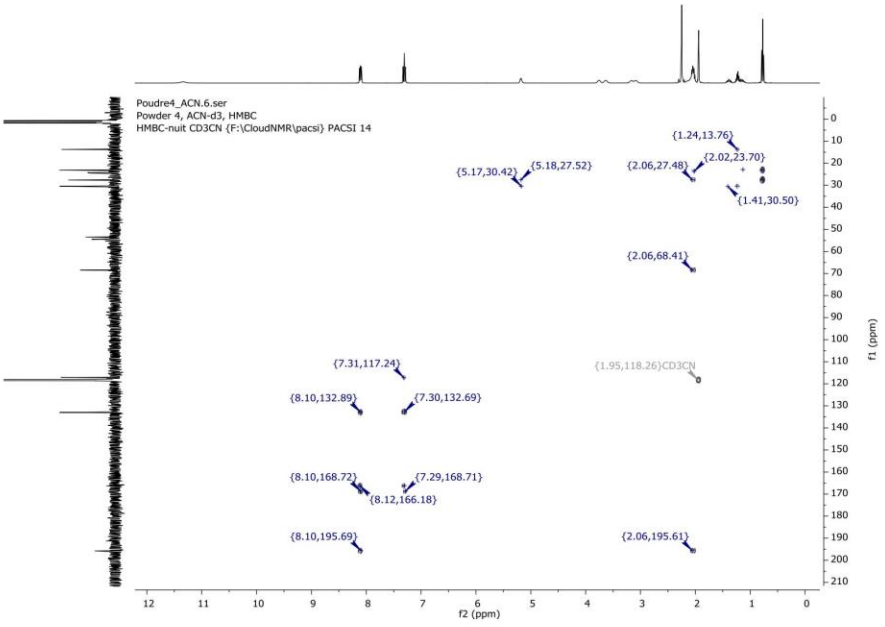

Figure 29 - ^{1}H, ^{13}C gHMBC of powder 4 in acetonitrile-d_3.

Figure 6

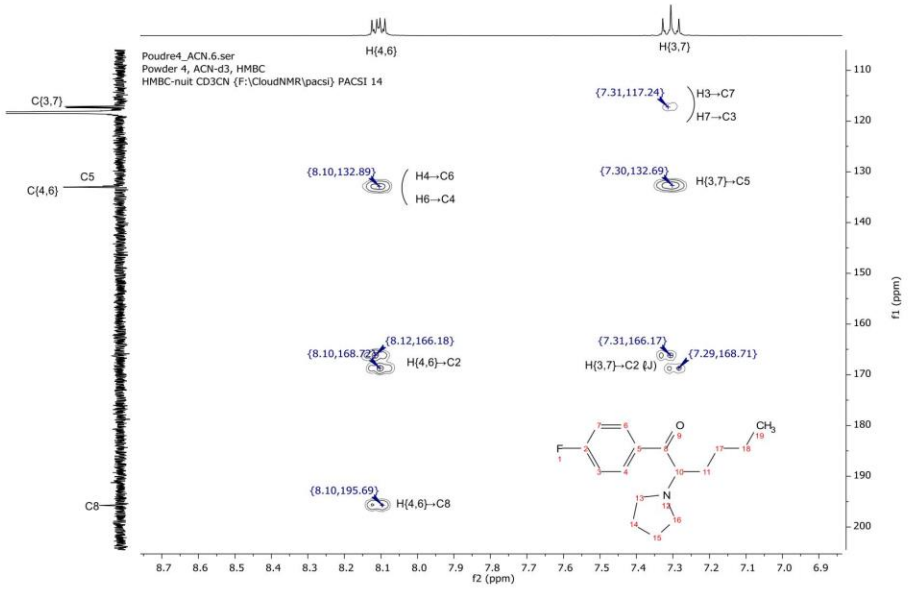

Figure 30 - Zoom on the aromatic region of the ^{1}H, ^{13}C gHMBC spectrum of powder 4 in acetonitrile-d_3.

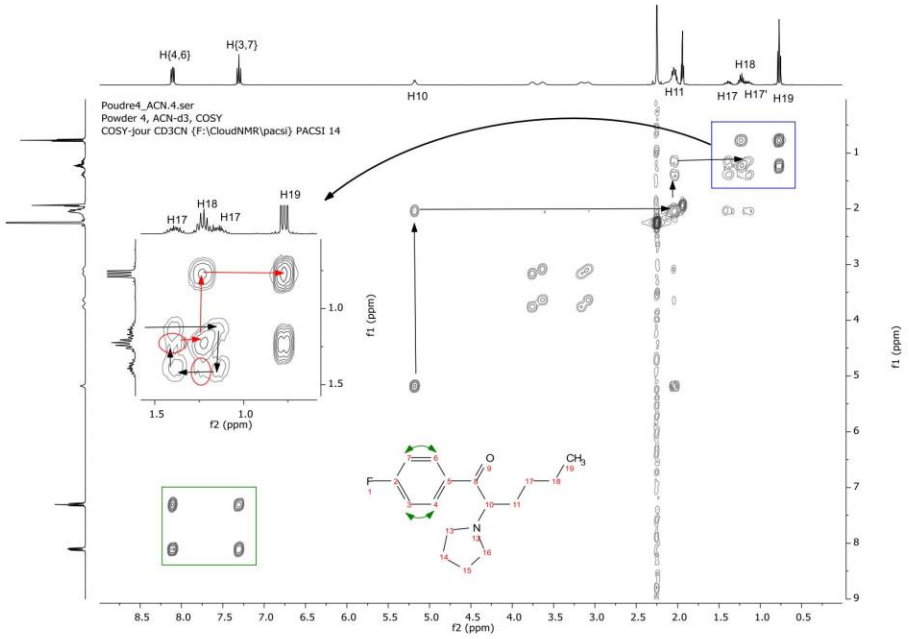

Figure 31 - ^{1}H gCOSY NMR spectrum of powder 4 in acetonitrile-d_3.

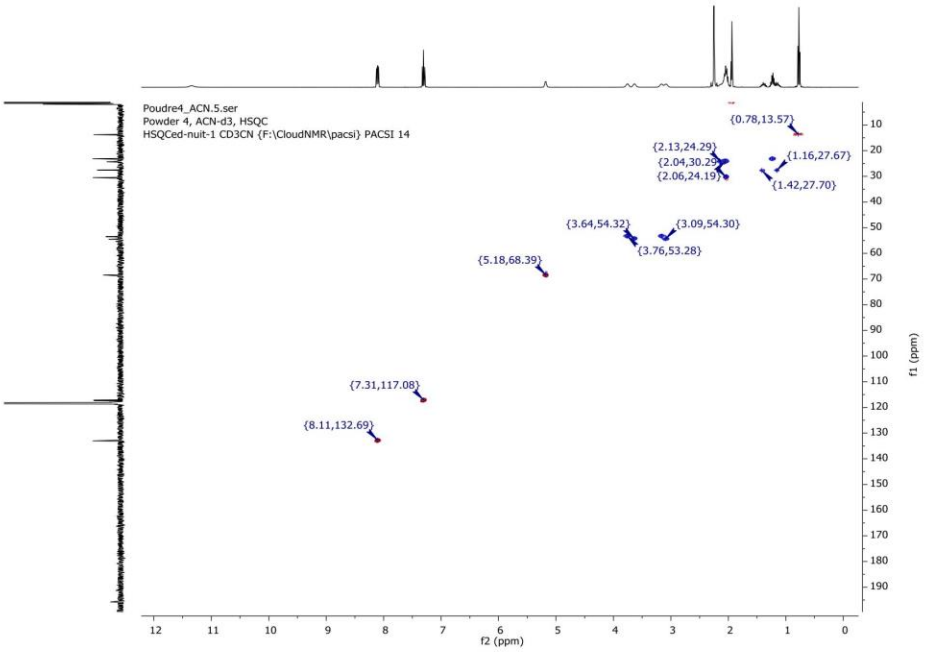

Figure 32 - ^{1}H, ^{13}C edited gHSQC spectrum of powder 4 in acetonitrile-d_3.

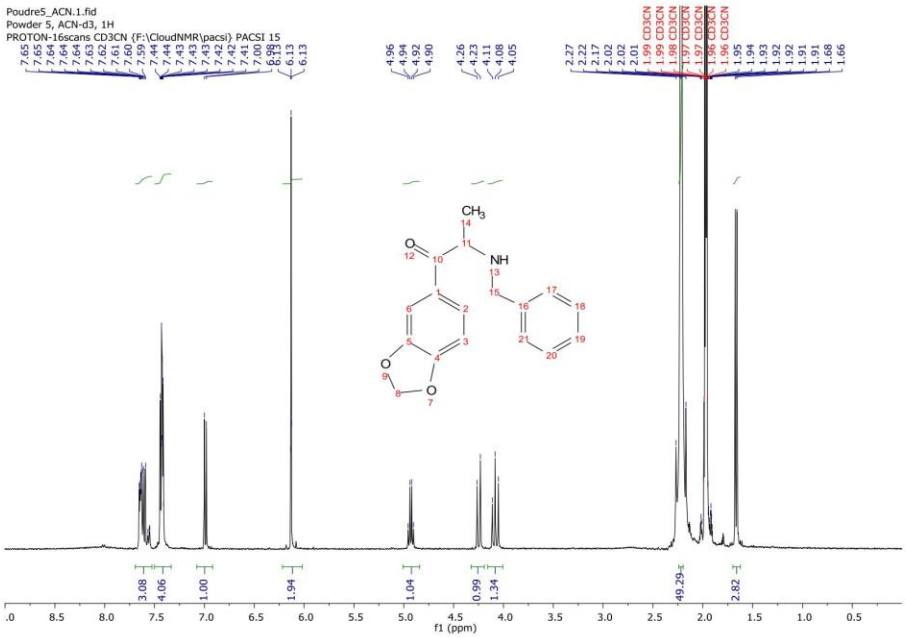

Figure 33 - ^{1}H NMR spectrum of powder 5 in acetonitrile-d_3.

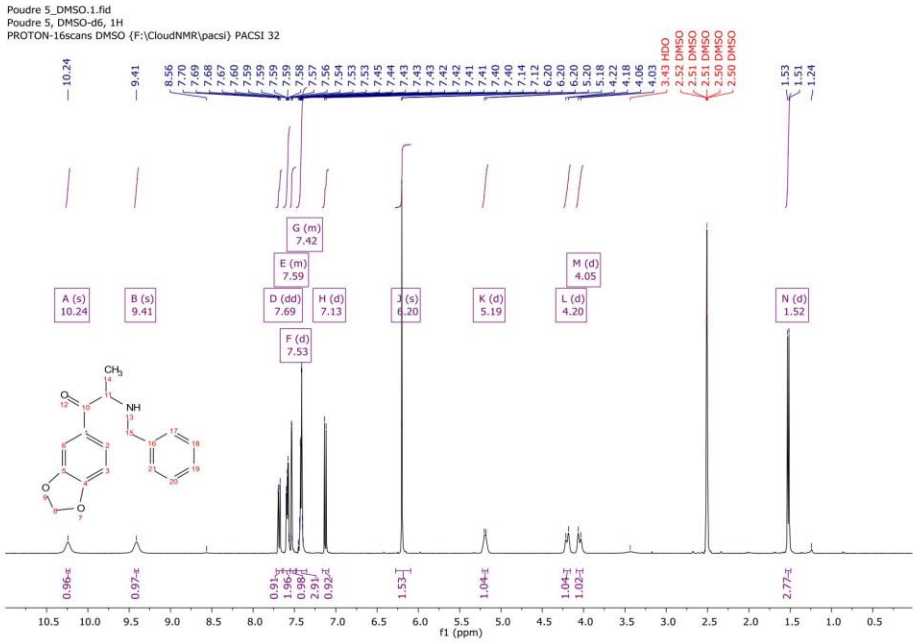

Figure 34 - ^{1}H NMR spectrum of powder 5 in DMSO-d_6.

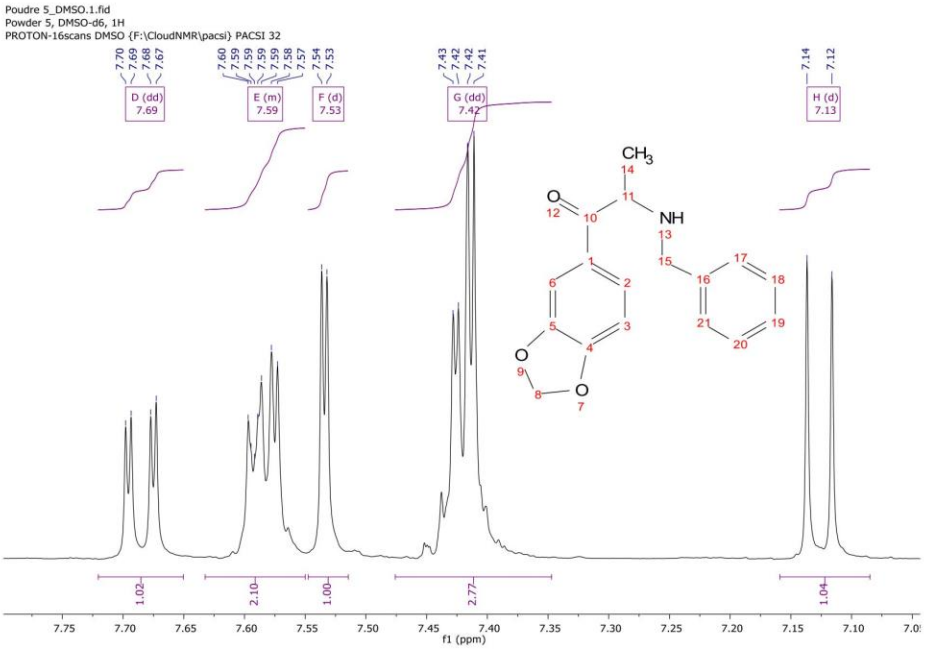

Figure 35 - Zoom of the 8.0 – 7.0 ppm region of the ^{1}H NMR spectrum of powder 5 in DMSO-d_6.

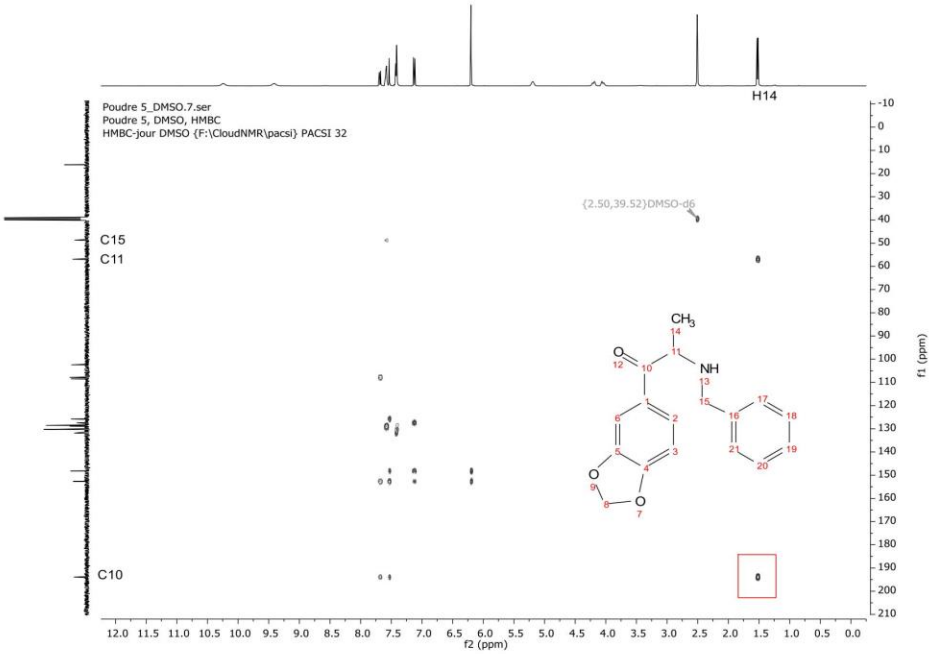

Figure 36 - ^{1}H, ^{13}C gHMBC of powder 5 in DMSO-d_6.

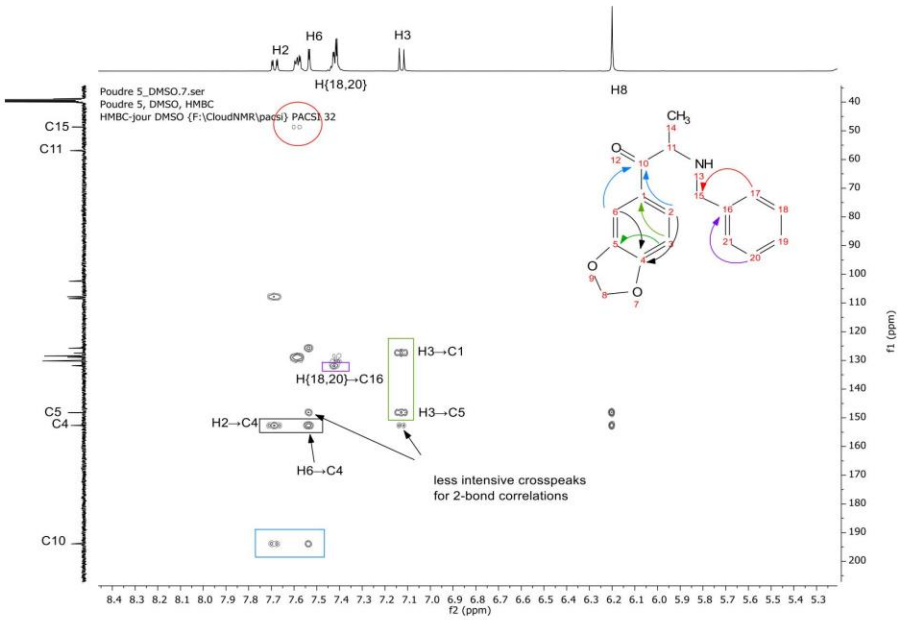

Figure 37 - Zoom on the ^{1}H, ^{13}C gHMBC of powder 5 in DMSO-d_6.

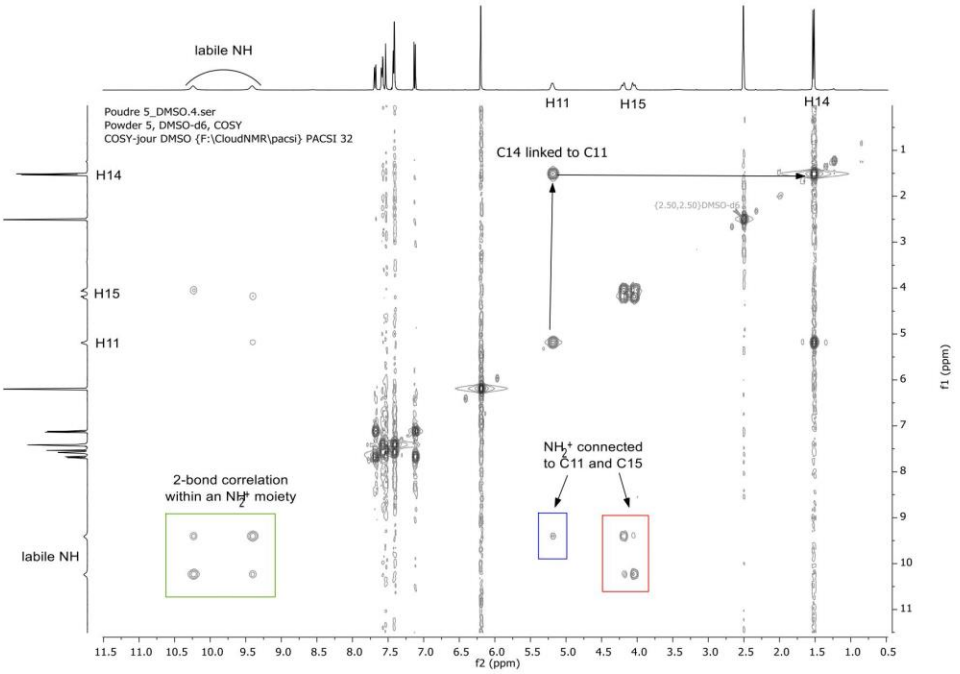

Figure 38 - ^{1}H gCOSY of powder 5 in DMSO-d_6.

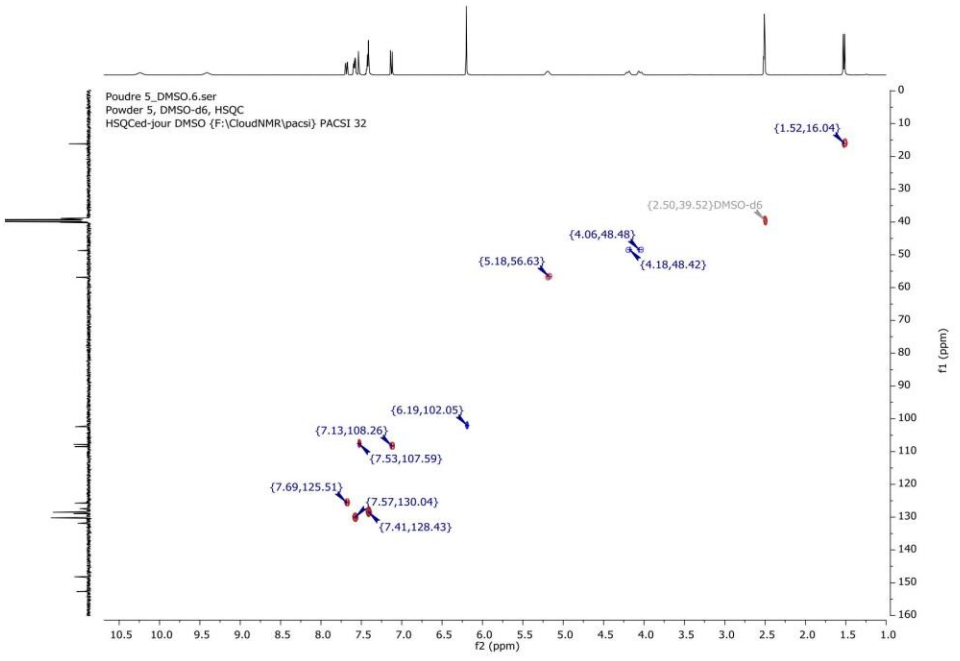

Figure 39 - ^{1}H, ^{13}C edited gHSQC of powder 5 in DMSO-d_6.

Figure 7

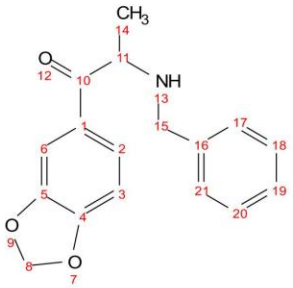

Figure 40 - Structure of BMDP

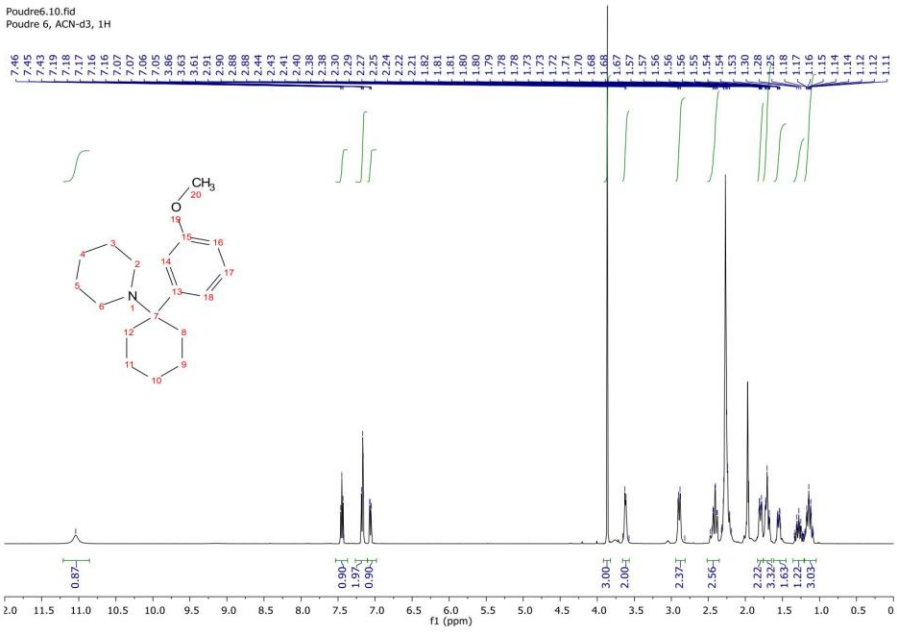

Figure 41 - ^{1}H NMR spectrum of powder 6 in acetonitrile-d_3

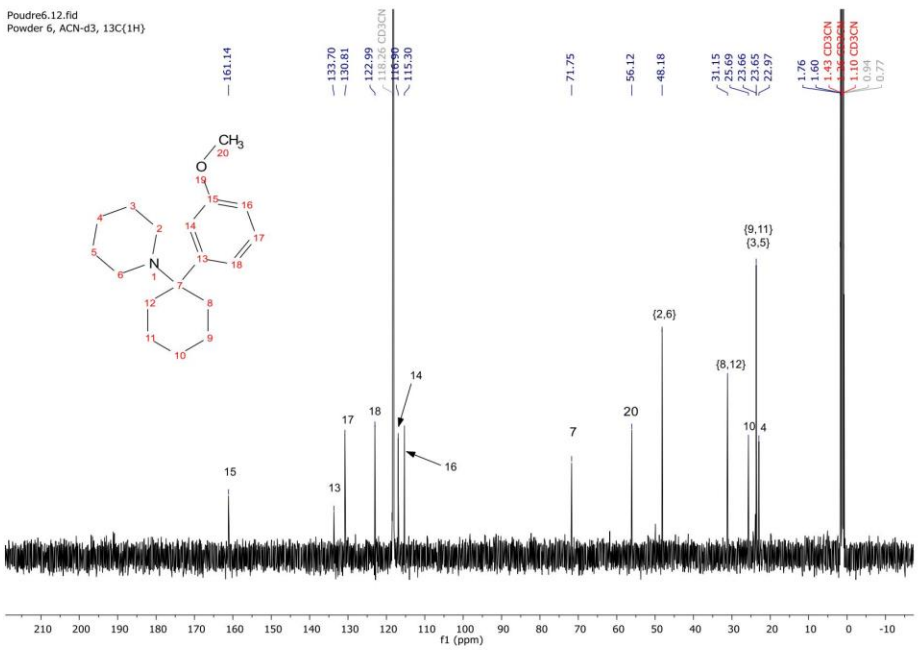

Figure 42 - $^{13}C\{^{1}H\}$ NMR spectrum of powder 6 in acetonitrile-d_3.

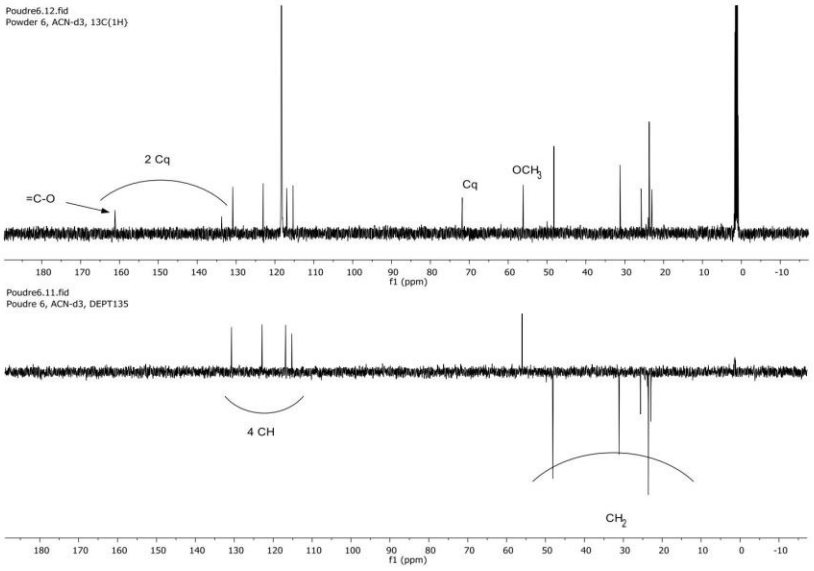

Figure 43 - Comparison of DEPT135 and $^{13}C\{^{1}H\}$ NMR spectra of powder 6 in acetonitrile-d_3.

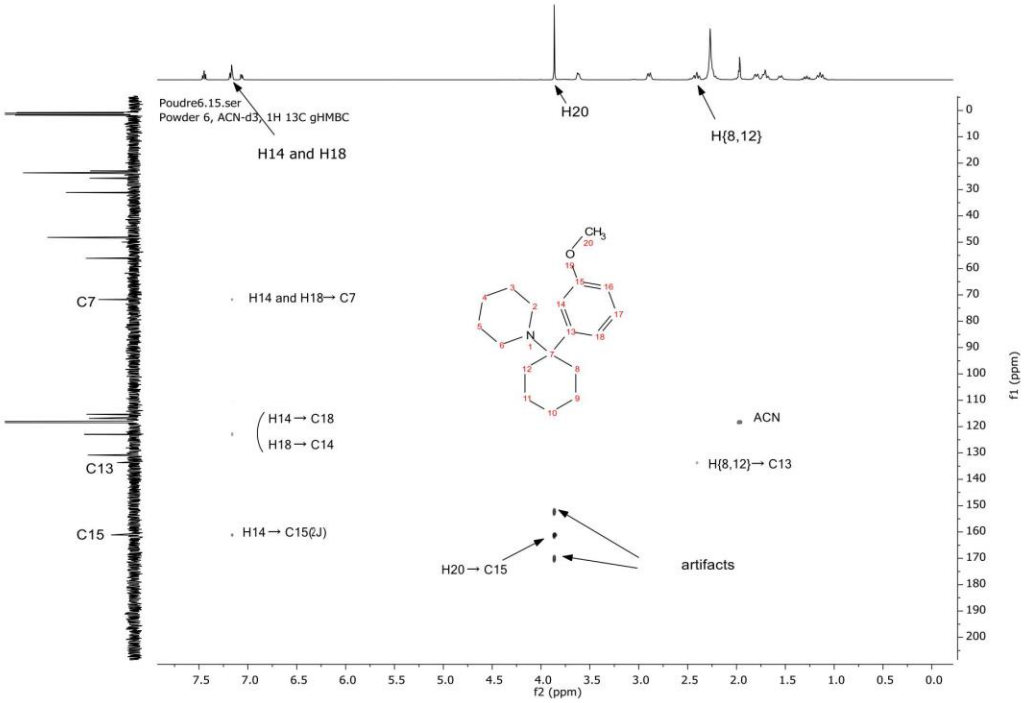

Figure 44 - ^{1}H, ^{13}C gHMBC of powder 6 in acetonitrile-d_3.

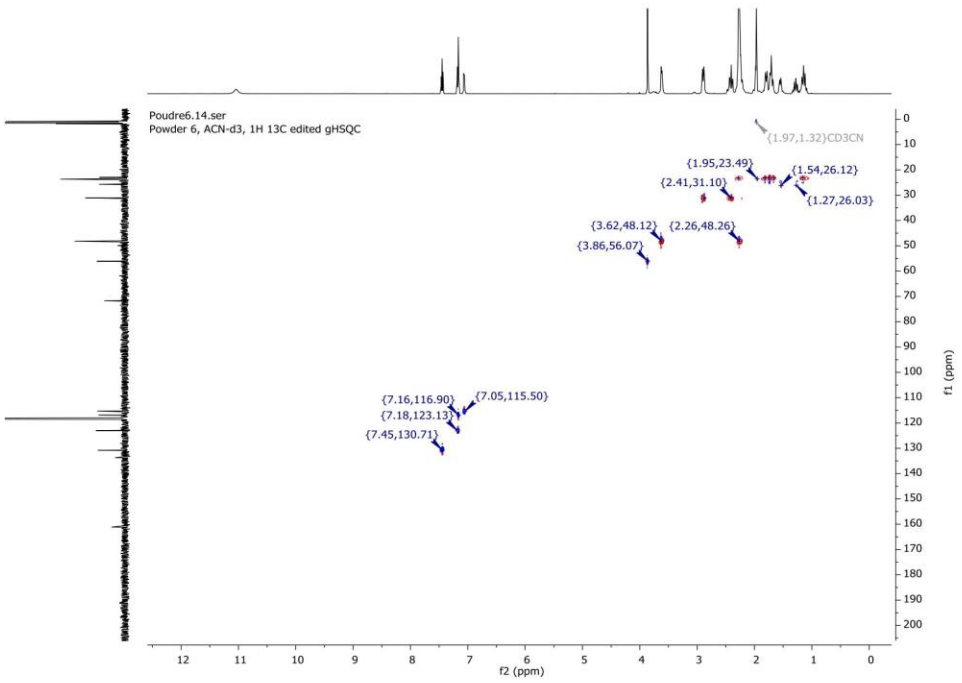

Figure 45 - ^{1}H, ^{13}C edited gHSQC spectrum of powder 6 in acetonitrile-d_3

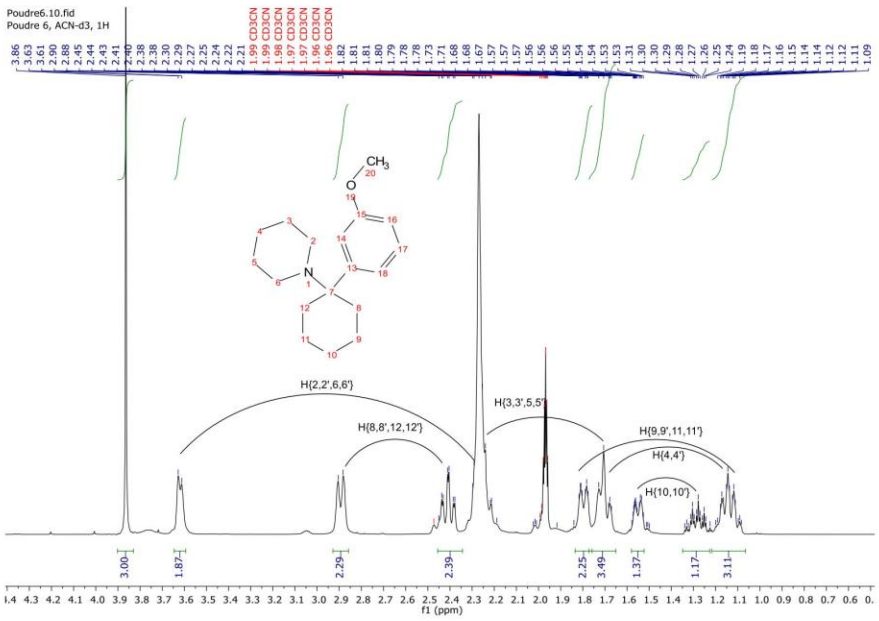

Figure 46 - Zoom on the aliphatic region of the ^{1}H NMR spectrum of powder 6 in acetonitrile-d_3.

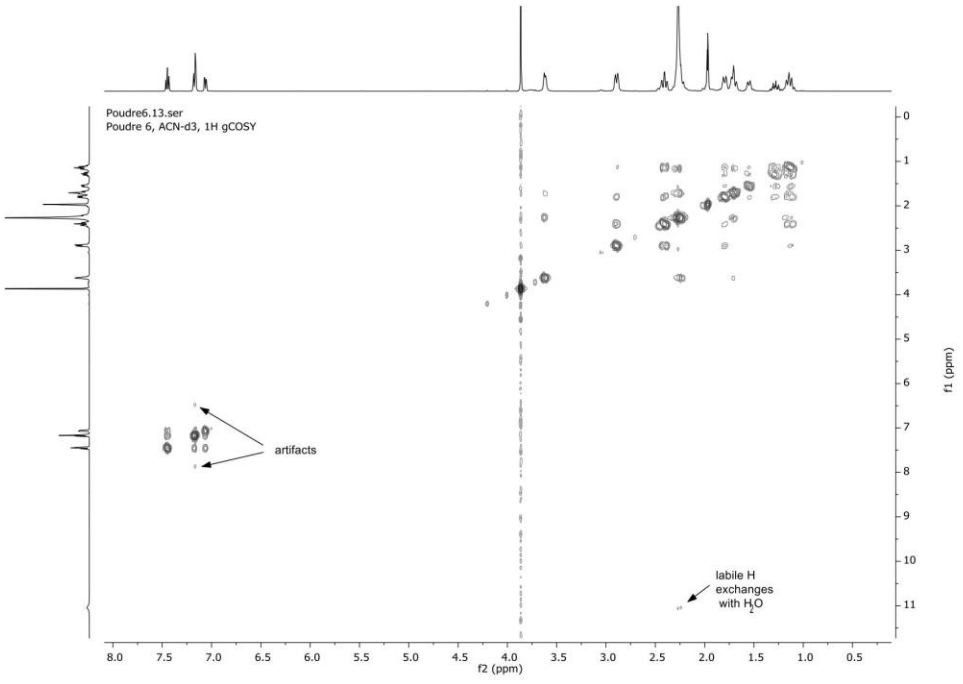

Figure 47 - ^{1}H gCOSY spectrum of powder 6 in acetonitrile-d_3.

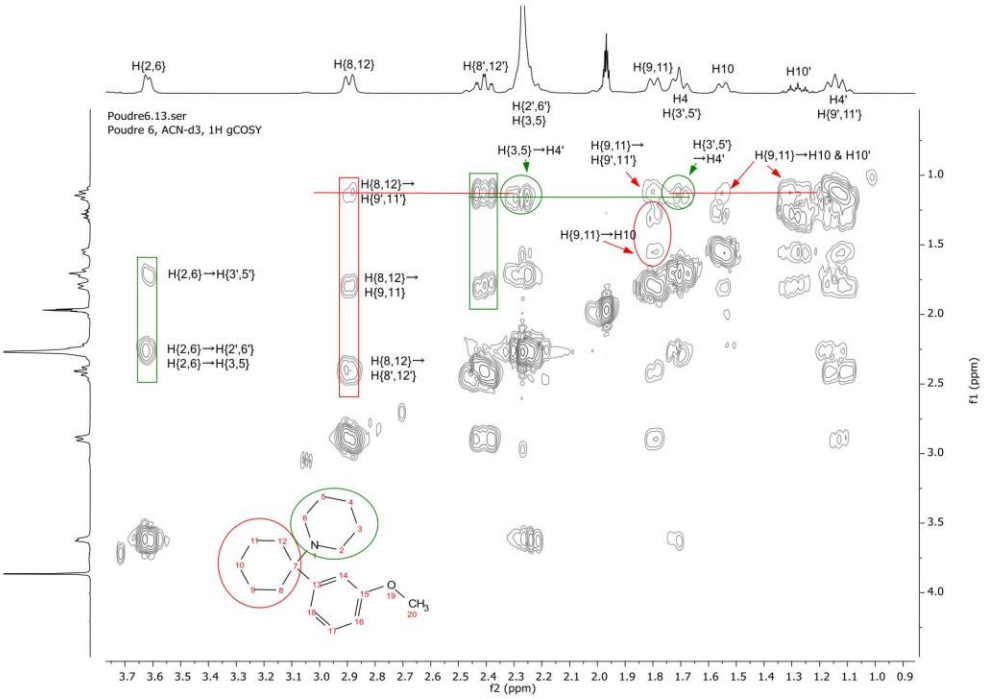

Figure 48 - Zoom of the aliphatic region of the ^{1}H gCOSY of powder 6 in acetonitrile-d_3.

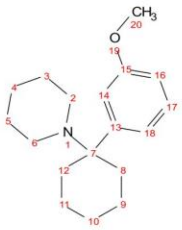

Figure 49 - Structure of 3-MeO-PCP

Figure 8

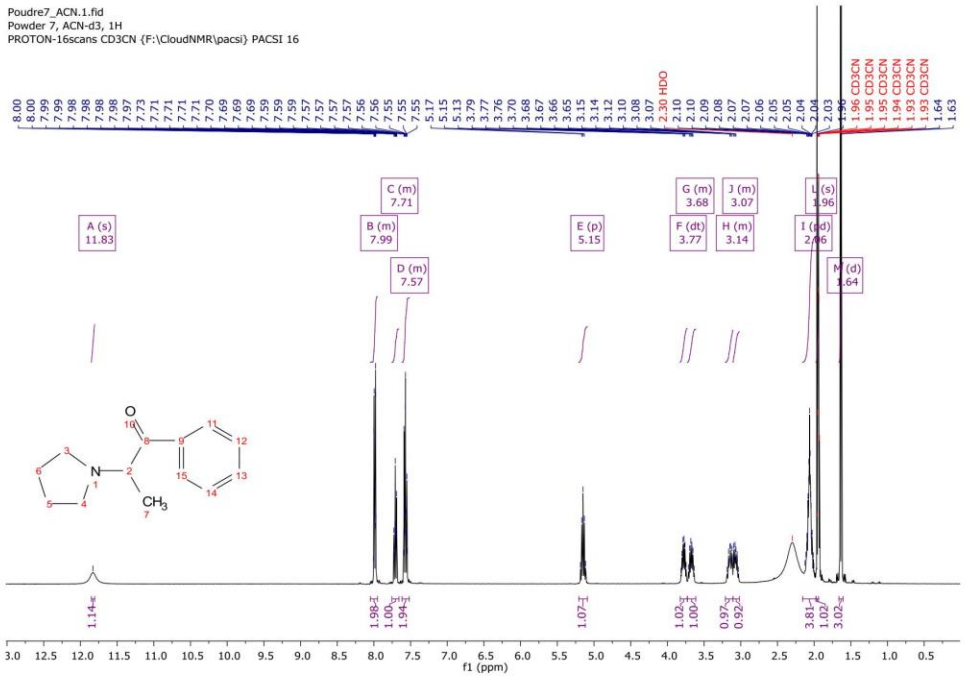

Figure 50 - ^{1}H NMR spectrum of powder 7 in acetonitrile-d_3.

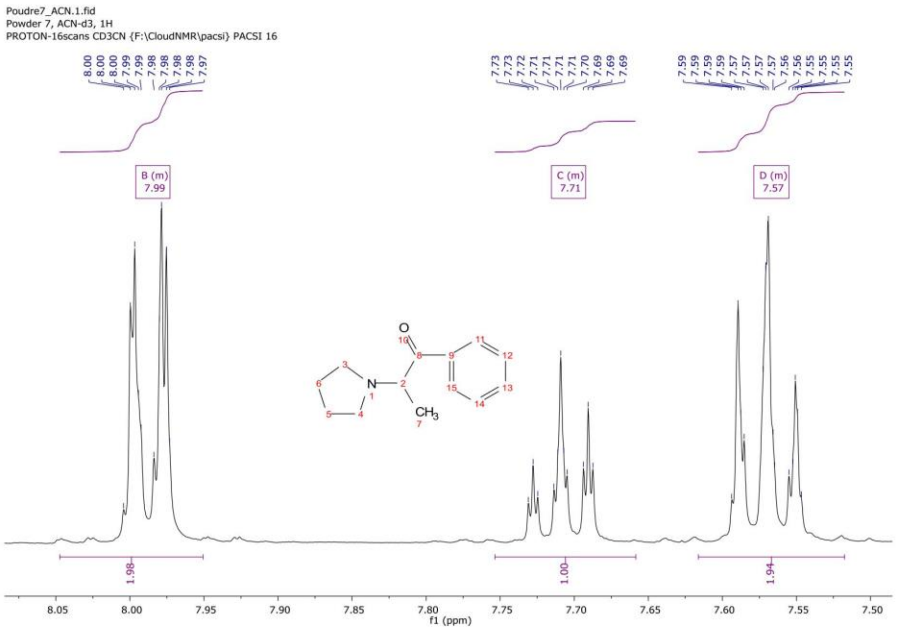

Figure 51 - Zoom on the aromatic region of the ^{1}H NMR spectrum of powder 7 in acetonitrile-d_3.

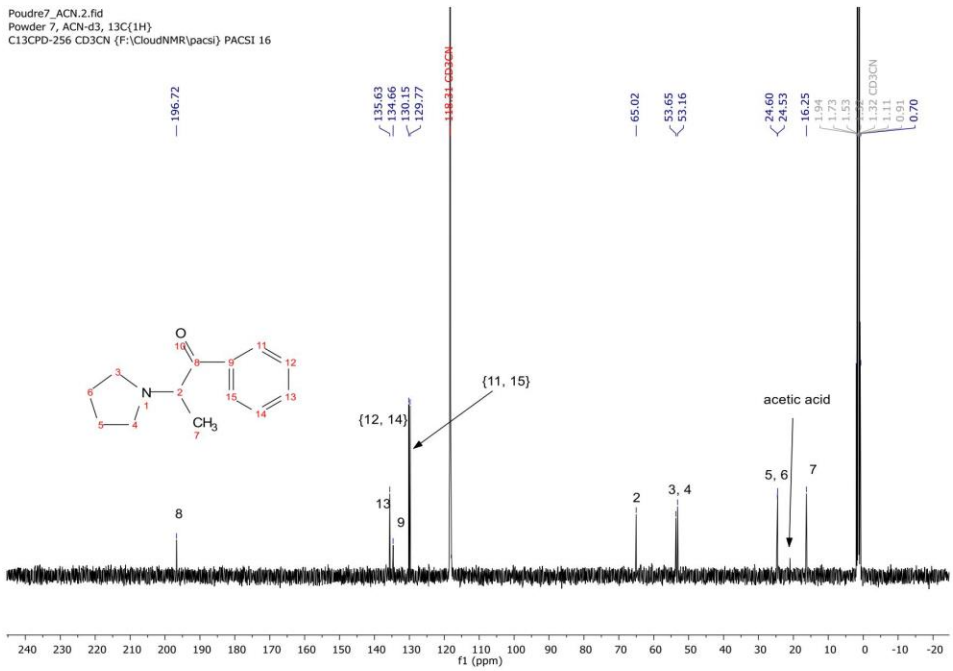

Figure 52 - $^{13}C\{^{1}H\}$ NMR spectrum of powder 7 in acetonitrile-d_3.

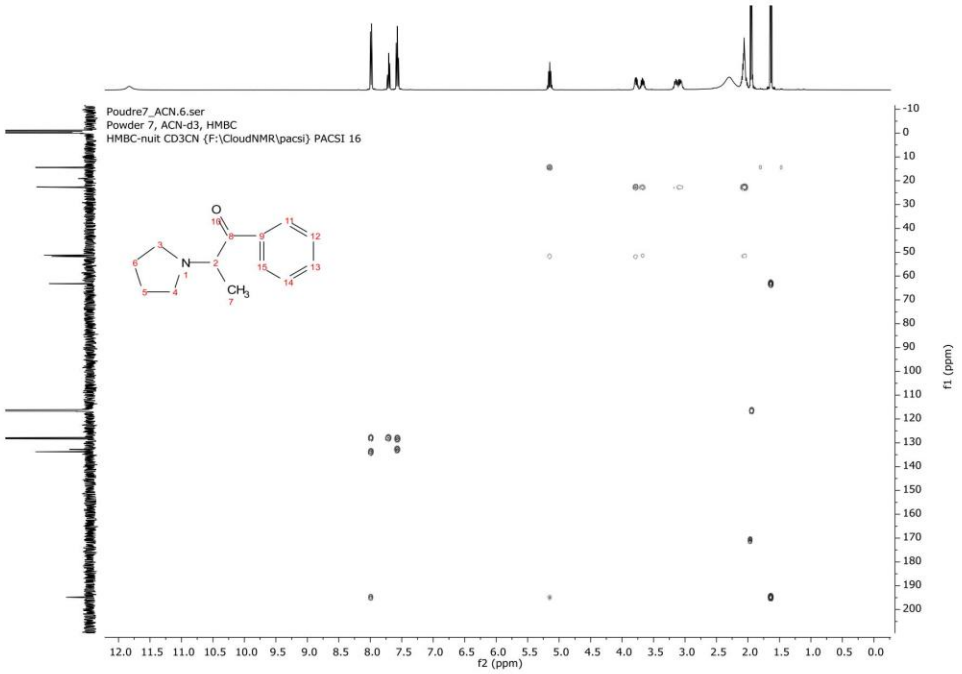

Figure 53 - ^{1}H, ^{13}C gHMBC of powder 7 in acetonitrile-d_3.

Figure 9

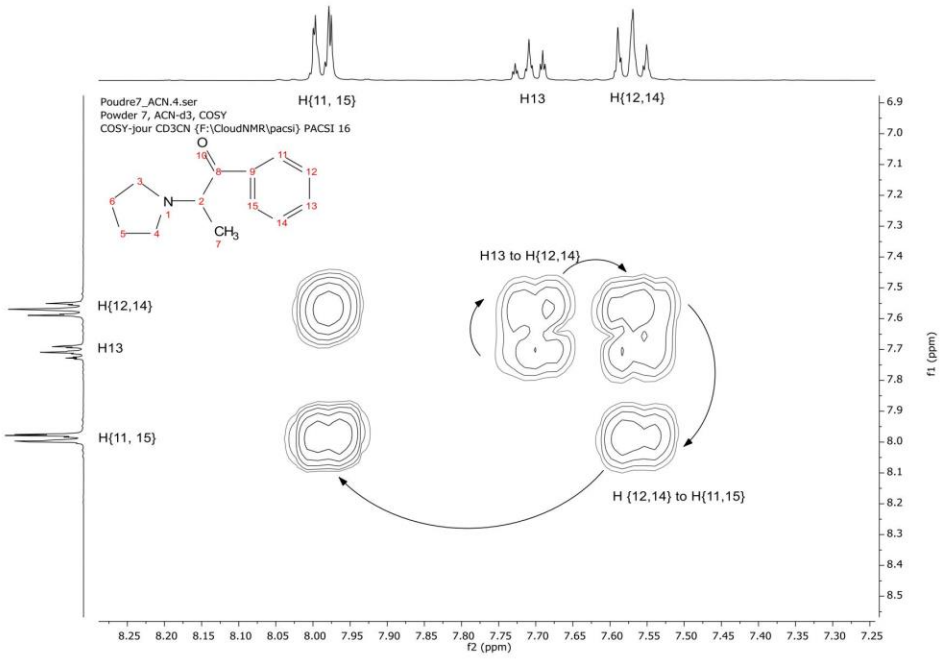

Figure 54 - Zoom of the aromatic region of the COSY spectrum of powder 7 in acetonitrile-d_3

Figure 10

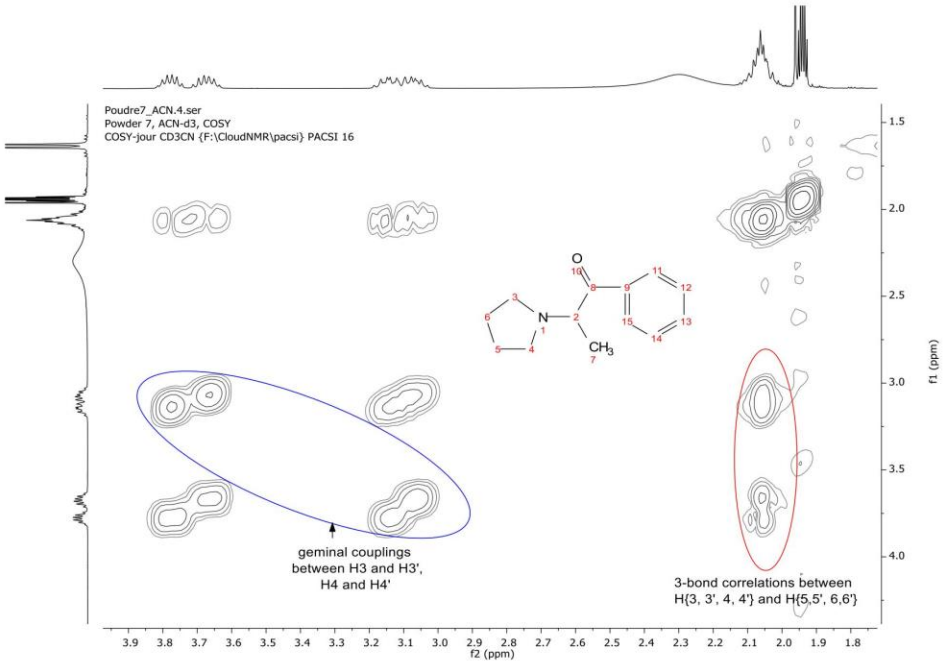

Figure 55 - Zoom on the 4.0 –1.7 ppm region of the COSY spectrum of powder 7 in acetonitrile-d_3.

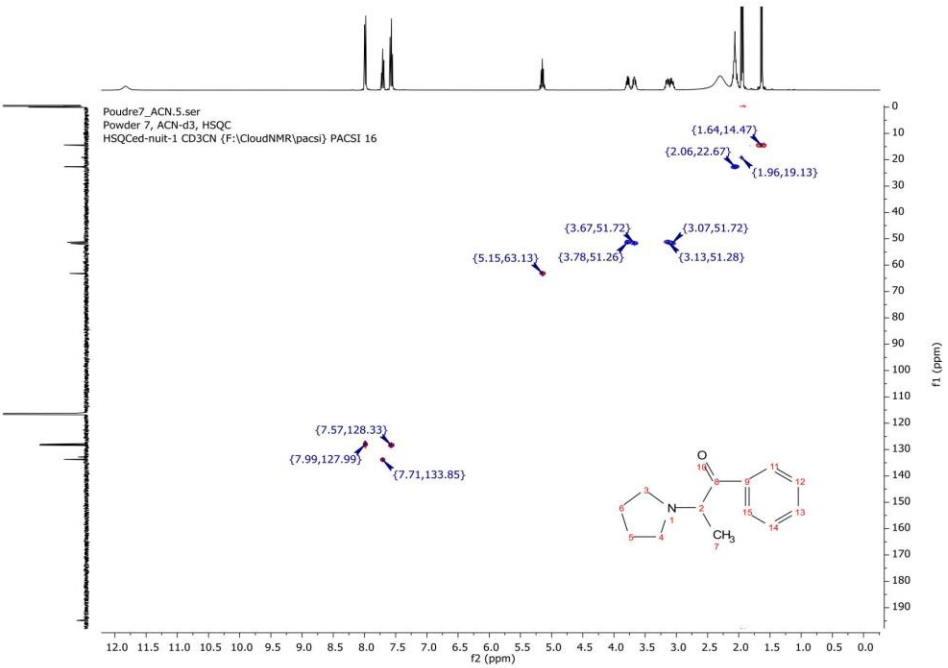

Figure 56 - edited ^{1}H, ^{13}C gHSQC of powder 7 in acetonitrile-d_3.

Discussion finale et conclusion

Alors … est-il trop tard ?
La lutte contre les NPS a-t-elle des limites, la guerre chimique est-elle perdue ?

Depuis une dizaine d'années, la mise sur le marché de NPS n'a cessé d'augmenter. Face à ce fléau dévastateur pour la santé publique, le développement d'une méthode analytique et d'une stratégie d'identification de ces substances sont apparus comme un sujet évident pour mon projet de thèse.

L'objectif principal était de mettre au point une méthode analytique robuste, exhaustive et sensible, permettant d'identifier un maximum de NPS. En effet, en 2016 le laboratoire de toxicologie de l'IML de Strasbourg ne disposait pas de méthode dédiée aux NPS, alors même que la littérature médicale et les présentations lors de congrès indiquaient une émergence particulièrement préoccupante. Le manque d'information concernant ces nouvelles substances, le peu de données épidémiologiques disponibles et les difficultés analytiques (la multitude de NPS, la modification à l'infini des structures chimiques, l'absence de référencement dans les bibliothèques de spectrométrie de masse commerciales, les difficultés rencontrées pour acquérir les standards de référence, les doses actives très faibles et par conséquent les concentrations sanguines et urinaires également faibles) sont les principales complications liées aux NPS et qui ont impactées notre démarche. La méthode développée est actuellement utilisée en routine au laboratoire et la bibliothèque de spectre MS comporte 114 NPS, bien au-delà des recommandations de la SFTA.

Très rapidement, après les premiers résultats, la communauté hospitalière alsacienne s'est intéressée à la puissance de l'outil analytique afin de documenter des observations difficiles. Régulièrement, le laboratoire est interrogé sur la possibilité d'investigations complémentaires, permettant au mieux d'expliquer une symptomatologie compliquée.

En parallèle, les forces de l'ordre, en particulier la brigade des stupéfiants, ont utilisé les services de l'IML. Il apparait, au décours des échanges avec les différents partenaires, que la notion même de NPS n'est pas encore correctement définie. En effet, la multiplication des molécules actives rend difficile une identification formelle, en particulier dans un contexte de restriction budgétaire. A titre d'exemple, le centre Ithaque de Strasbourg s'intéresse de près aux possibilités analytiques de laboratoire et souhaiterait une collaboration très étroite dans ce domaine.

La versatilité des prélèvements (échantillons solides et liquides, prélèvements biologiques dont les cheveux, taches de vomissements, *etc.*) associée à l'importance du parc analytique disponible (chromatographie gazeuse et chromatographie liquide couplée à la spectrométrie de masse dont de type haute résolution, résonnance magnétique nucléaire) a permis de répondre de façon satisfaisante et dans des délais raisonnables aux différents prescripteurs.

Plusieurs cas d'intoxication et de décès ont ainsi pu être mis en évidence à l'IML de Strasbourg, mais également en collaboration avec plusieurs laboratoires français. Ces cas ont donné lieu à la rédaction de 18 publications nationales et internationales. La pérennité des collaborations mises en place et l'enrichissement régulier des bibliothèques de spectres MS vont continuer au-delà de cette thèse afin de se rapprocher au mieux de la liste des substances comptabilisées par le système d'alerte précoce de l'UNODC et de lutter contre ce phénomène.

Les différentes publications et communications à des congrès nationaux et à l'étranger ont montré le nombre important de molécules différentes, retrouvées dans le cadre de l'activité hospitalo-universitaire du laboratoire. La diversité des structures chimiques et des familles pharmacologiques a rendu obligatoire l'utilisation de techniques séparatives sélectives et ultra sensibles.

Rien n'est définitivement acquis et de nouvelles familles de NPS ont fait leur apparition sur le e-commerce. Récemment des anabolisants de synthèse ont été observés (tétrahydrogestrinone ou THG, methylstenbone, mentabolan, stenbolone, *etc.*). Ces substances ne sont pas uniquement utilisées par des athlètes de haut niveau, mais également par des amateurs qui s'approvisionnent directement dans leurs salles de fitness ou sur Internet, et qui ne mesurent pas la dangerosité de ces substances *[Gheddar, 2019]*.

Au terme de cette thèse, les perspectives futures sont les suivantes :

- alimenter en continu la bibliothèque de spectres MS
- appliquer la méthode de criblage des NPS en routine afin de documenter les demandes hospitalières du CHU de Strasbourg, mais aussi des hôpitaux périphériques, dans des situations d'altération de la vigilance ou de coma inexpliqué, en particulier lorsque le criblage toxicologique initial par immunochimie est négatif
- réaliser une recherche systématique de NPS dans toutes les expertises médico-judiciaires, bien au-delà de la demande de la note de la Chancellerie du 6 novembre 2017. En effet, il n'y a pas d'âge pour consommer des NPS, le premier cas observé à l'IML ayant concerné un homme de 69 ans.

- identifier la nature chimique de substances inconnues, ou peu connues, par résonnance magnétique nucléaire et l'étendre à tous les composés rencontrés dans la pratique quotidienne, tels que l'adrafinil, un psychostimulant observé récemment, ou encore le ligandrol (LDG-4033), un modulateur sélectif des récepteurs androgènes, objet d'une demande de confirmation dans un contexte de dopage.
- produire des métabolites à partir de microsomes hépatiques humains, afin de déterminer leurs caractéristiques chromatographiques (temps de rétention et transitions ioniques) pour les comparer avec les résultats obtenus dans des prélèvements biologiques, reçus dans le cadre hospitalier ou judiciaire. Cette approche pourrait permettre, par exemple, d'augmenter la fenêtre de détection dans les urines. Elle est également utile pour mettre en évidence des interactions médicamenteuses entre deux molécules, comme par exemple la rilpivirine et le budésonide.
- enfin, sensibiliser les forces de l'ordre, les magistrats et les médecins, permettant ainsi de rester vigilants et de développer des programmes d'éducation, de réduction des risques et de prise en charge

Trois années d'activité professionnelle dans le cadre de la préparation de cette thèse ont permis la rédaction de 18 publications, à la fois dans des journaux internationaux, mais aussi français, pour partager ma recherche avec la communauté scientifique. A l'heure du bilan, il s'avère que mon quotidien est allé au-delà du travail de paillasse. La participation à des réunions scientifiques, la mise en place de collaborations avec plusieurs structures publiques et privées, les très nombreux échanges avec des toxicologues partageant les mêmes problématiques, la possibilité offerte pour enseigner ce que j'ai acquis et la maitrise des outils analytiques disponibles au laboratoire m'ont ouvert naturellement des perspectives d'avenir dans le monde de la toxicologie. La reconnaissance par la SFTA de mes travaux (prix de la meilleure communication orale au congrès d'Avignon en 2017 et prix de la meilleure publication dans Toxicologie Analytique et Clinique en 2018) m'encourage de façon forte à poursuivre dans cette matière. Il apparait donc évident que je conçois de continuer mon implication en toxicologie médico-judiciaire dans cette structure qui m'a donnée le goût de la recherche, m'a appris la rigueur, l'opiniâtreté, la persévérance et l'aspect convivial du travail en équipe.

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Alice AMELINE

Aspects analytiques, cliniques et médico-judiciaires des nouvelles substances psychoactives

Résumé

En raison de la diffusion incontrôlée sur le e-commerce, la sécurité et l'alternative légale aux stupéfiants habituels, les nouvelles substances psychoactives (NPS), d'apparition récente (2008), sont au cœur des phénomènes récents d'addiction et de décès mal expliqués. Au-delà des différents défis dans nos sociétés (prévention, législation), la capacité d'identifier les NPS dans des échantillons biologiques pour caractériser leur utilisation, présente de nombreux challenges analytiques. L'objectif principal de cette thèse a été de collecter des échantillons biologiques (sang, urine, cheveux) provenant de cas d'exposition à des NPS et d'y caractériser les substances présentes à l'aide de méthodes analytiques originales, dans le but d'enrichir les librairies de spectres de masse et d'améliorer, en conséquence, la détection de la consommation de NPS. En particulier, il s'agissait d'augmenter la fenêtre de détection de la prise de NPS en se focalisant sur les métabolites qui sont, le plus souvent, les produits majeurs d'élimination. Le développement analytique, par chromatographie liquide ultra haute performance couplée à la spectrométrie de masse en tandem (UHPLC-MS/MS), a demandé plusieurs mois d'optimisation afin d'obtenir une méthode robuste, exhaustive et sensible. Actuellement, la librairie de spectres MS comporte 114 NPS et est mise à jour régulièrement. A la suite de ce développement, ma thèse a porté sur l'étude de cas d'intoxication vus au service des urgences du CHU de Strasbourg, mais aussi en médecine légale, avec des situations de décès et d'identification de produits inconnus provenant de saisies (poudres et cristaux). Il a également été nécessaire de développer des outils analytiques complémentaires, tels que la caractérisation de métabolite(s) par étude sur microsomes hépatiques humains (HLMs), et l'utilisation de la spectroscopie par résonance magnétique nucléaire (RMN) afin d'identifier avec certitude certains composés et de déterminer leur degré de pureté. Les outils analytiques développés et la stratégie mise en place ont permis la rédaction de 18 publications, ainsi que l'agencement de nombreuses collaborations.

Mots clés : Nouvelles substances psychoactives (NPS), chromatographie liquide ultra haute performance couplée à la spectrométrie de masse en tandem (UHPLC-MS/MS), métabolites, microsomes hépatiques humains (HLMs), résonance magnétique nucléaire (RMN), intoxication, décès

Abstract

Due to the uncontrolled spread on the Internet and their legal alternative to usual drugs, the new psychoactive substances (NPS), recently appeared (2008), are at the center of recent phenomena of addiction and badly explained deaths. Beyond different challenges in our societies (prevention, legislation), the ability to identify NPS in biological samples, in order to characterize their use, presents many analytical challenges. The main objective of this thesis was to collect biological samples (blood, urine, hair) from cases of exposure to NPS and to characterize the substances present using original analytical methods, in order to enlarge the libraries of mass spectra and improve, as a result, the detection of NPS consumption. In particular, it was intended to increase the detection sensitivity of NPS intake by focusing on the metabolites that are often the major products of elimination. This analytical development, by ultra-high liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), required several months of optimization in order to obtain a robust, exhaustive and sensitive method. At present, the mass spectra database has 114 NPS and is regularly updated. Thereafter, ma thesis focused on the study of cases of intoxication observed in the emergency department of Strasbourg, but also in legal medicine with situations of deaths and identification of unknown products collected from seizures (powders and crystals). It has also been necessary to implement complementary analytical tools, such as the characterization of metabolites by human liver microsomes (HLMs), and the use of nuclear magnetic resonance (NMR) spectroscopy to accurately identify the compounds and establish their purity degrees. The analytical tools developed, and the strategy adopted, allowed the writing of 18 publications, as well as the setting up of numerous collaborations.

Keywords: New psychoactive substances (NPS), ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), metabolites, human liver microsomes (HLMs), nuclear magnetic resonance (NMR), intoxication, death