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Potential of high-resolution mass spectrometry for the detection of drugs and metabolites in hair: methoxetamine in a real forensic case.

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ABSTRACT

The analysis of drugs of abuse in hair and other biological matrices of forensic interest requires great selectivity and sensitivity. This is done traditionally through target analysis, with one or more analytical methods, or with different and specific preanalytical phases, and complex procedures performed by the toxicological laboratories, and there is no exception with ketamine-like compounds, such as methoxetamine, a new psychoactive substance (NPS) whose use has increased in the last decades, and continues to grow quickly year by year. More validated methods of analysis are needed to detect these substances in low concentrations selectively. Reanalyzing the samples of a former case of a polydrug consumer accused of a crime against public health in Spain, five metabolites of methoxetamine (normethoxetamine, O-desmethylmethoxetamine, dehydromethoxetamine, dihydronormethoxetamine and hydroxynormethoxetamine) were tentatively detected using a high-resolution technique that is liquid chromatography coupled to high-resolution mass spectrometry (LC-HR-MS/MS). The most selective analytical LC-HR-MS/MS method together a universal and simpler pretreatment stages has demonstrated to allow faster analysis and more sensitivity than the one

performed traditionally at the INTCF laboratories, which was gas chromatography coupled to mass spectrometry (GC-MS).

KEYWORDS

Methoxetamine, new psychoactive substances (NPS), metabolites, hair, high-resolution mass spectrometry, liquid chromatography coupled to high-resolution tandem mass spectrometry (LC-HR-MS/MS), gas chromatography coupled to mass spectrometry (GC-MS)

INTRODUCTION

Polydrug consumption is becoming a popular practice among people. This habit of consumption is related to higher mortality rates, as well as physical and psychological comorbidity [1], and prolongation of medical treatments [1, 2]. In our time, new molecules with psychoactive properties are sold in the Internet and in the black market. These compounds are produced to mimic the effects of already controlled psychoactive drugs. They are called novel psychoactive substances or NPS. Currently, there is a big number of different types of NPS, *i.e.*, arylcyclohexylamines, synthetic cannabinoids, cathinones, piperazines, phenylethylamines, fentanyls and other substances. Nevertheless, this list keeps growing year by year [3, 4].

Many of these new drugs are eliminated quickly from the body; therefore, the different matrices analyzed in forensic toxicology such as blood, humor vitrous or urine may not be useful without any other additional analysis. Hair analysis allows the investigation with a longer detection window (months to years), because the compounds and their metabolites may remain in the

hair matrix for a longer time without significant loss or degradation [5, 6]. This way, hair samples allow us to investigate consumption patterns retrospectively and to known the poly-consumption and prevalence of drugs and NPS consumed. In addition, hair sampling is not invasive, and the samples can be stored easily [5]. Hair samples have been employed to study drug consumptions retrospectively in daily analyses at the National Institute of Toxicology and Forensic Sciences (INTCF) for the last 25 years.

There are limited screening procedures for NPS analysis in hair and, in fact, we will quote some of them in scientific literature [5, 7-9]. The sample pretreatment procedures employed traditionally in target methods for hair analysis usually take much time and effort [10].

Particularly, the detection and identification of arylcyclohexylamines in the hair matrix have been slightly investigated for the last decades. Ketamine is the most consumed out of all arylcyclohexylamines so far. There are around a hundred publications where its determination can be found. To achieve such purpose, studies usually employ chromatographic separation techniques coupled to mass spectrometry [11-14], and scarcely electrochemical techniques [15]. It is also of forensic interest to analyze the metabolites of drugs in hair, because their presence confirms the actual consumption of that substance, while ruling out the possibility of external contamination.

Many of the studies carried out so far do not contemplate ketamine analogues, such as methoxetamine. Methoxetamine is a dissociative drug classified as an arylcyclohexylamine-type drug. It is produced as a "ketamine-like drug" in the illicit market. However, methoxetamine presents some chemical differences with ketamine: the chlorine group on the phenyl ring is replaced by a methoxy group (R2), and the N-methyl group by a N-ethyl group (R1) [16]. In Figure 1, the structural differences between ketamine and

methoxetamine are represented.

Though methoxetamine is considered a fashionable drug that mimics the psychoactive effects of ketamine in humans, in fact, its long-term effects still remain unknown, as the studies have only been carried out in animals [17-20]. This NPS is potentially dangerous and may remain undetected, as it is sold on the Internet as a "research chemical" or "legal high" accompanied by the indication that they are "not for human consumption" [21].

The toxic effects of methoxetamine, consist in hallucinations, derealization, sensory deprivation and dissociation between mind and body [22]. Other desired effects are euphoria, feeling of peace and increased empathy and social interaction [23].

New psychoactive substances (NPS), alongside their metabolites, should be detected with a technique that is sensitive and selective enough. Traditional combined techniques sometimes do not fulfill this necessity in the forensic analysis of hair. Less is known about the metabolism of arylcyclohexylamines in the human body and the possibility to report the identification of the different metabolites in hair. Figure 2 shows the different metabolic pathways of interest followed for methoxetamine. The enzymatic reactions taking place are N-dealkylation, O-demethylation, hydroxylation, reduction and dehydrogenation [16].

This work aims to report the analytical approach followed in the National Institute of Toxicology and Forensic Sciences (INTCF) for the identification of methoxetamine and five of its main metabolites in hair related to a criminal case occurred in Spain involving a drug consumer. To achieve this, the specific objetives pursued in this work were: (i) evaluate the simplification of the traditional GC-MS method with a simpler LC-HR-MS/MS method for drug

detection in hair to confirm the poly-consumption that was previously observed; and (ii) add the identification of methoxetamine and its main metabolites in the hair sample of the revised case.

1.1. Case history

A drug dealer whose age was unknown, probably young, was charged with a crime against public health in Spain. The judicial requirement was to establish and corroborate whether the seized drug was for own consumption or not. This substance was a white powder and was provided due to the fact that the seized drug was sent to another laboratory different to INTCF.

The medical examiner of the Ministry of Justice referred two locks of black scalp hair (4 cm each) from the defendant. The entire length was collected and prepared for toxicological analysis. In Spain, this type of analysis is frequently asked for reduction of penalty alleging consumption, since this fact is contemplated as an attenuator.

2. EXPERIMENTAL

2.1. Standards and reagents

Classical drug standards of amphetamines (amphetamine, 3,4 methylendioxy-N-methyl-amphetamine (MDMA), 3,4 methylendioxy-N-ethyl-amphetamine (MDEA), 3,4 methylendioxyamphetamine (MDA); as well as arylcyclohexylamines or ketamine analogues (ketamine, norketamine and methoxetamine) were provided by LGC Promochem Cerilliant (Teddington, Middlesex, UK) and Lipomed (Arlesheim, Switzerland) as pure solutions in methanol or acetonitrile at 1.0 mg/ mL. As internal standards (IS), cocaine-

d3, BE-d3, morphine-d3, 6-AM-d3, codeine-d3, methadone-d9, ketamine-d4, amphetamine-d5 and MDMA-d5 were purchased from LGC Promochem Cerilliant, at 1.0 mg/ mL solutions in methanol. Stock solutions of 1.0 mg/ mL methanol were stored at 4 ºC.

The solutions used during sample treatment were of analytical grade (LC-MS grade) and, as derivatizing agents pentafluoropropionic anhydride (PFPA) and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) were used, obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Hair samples

Two locks of hair samples were used: (i) one lock of blank hair was used to make standard solutions for calibration, collected from drug-free volunteers; (ii) and one real sample from the drug dealer case, kept in custody by the Madrid Department of the INTCF, to carry out the high-resolution identification analysis.

3. METHODS

The sampling of the hair for both samples was carried out following the guidelines addressed by the Society of Hair Testing [24]. The Madrid Department of the INTCF assessed a method developed and used for the study of hair using a simple preanalytical phase to prepare the sample for a screening analysis, followed by a high-resolution identification technique, which is liquid chromatography coupled to high-resolution mass spectrometry (LC-HR-MS/MS) for chemical characterization. This method, contrary to the targeted gas chromatography coupled to mass spectrometry (GC-MS) traditionally used, was developed for the screening of untargeted substances and reporting their identification. The results were compared against the ones obtained using GC-MS used to solve the case in the past, in order to observe

whether there would be actual advantages in drug and metabolite characterization or not.

The methods for sample pretreatment are described and compared in full detail in section 3.1. When the case was under investigation, the methodology employed for drug analysis was that applied for amphetamines and ketamine analysis previously reported by Matey et al. [25] (named method-1). On the contrary, method-2 referred to the LC-HR-MS/MS method.

3.1. Preanalytical hair sample treatment

All the hair samples were subjected to a pretreatment before the analytical determination of drugs. Two steps were necessary: decontamination (to remove possible external drugs and interferents) and extraction. Others were optional and depended on the method of analysis and its limitations (purification, derivatization, etc). Table 1 shows both the steps followed for the pre-treatment of the hair samples and the experimental conditions used for both of the analytical methods used. After decontamination, the total amount of hair analyzed in both methods was previously dried with nitrogen flow for 10 minutes. Trituration was performed for sample homogenization, using a Precellys Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) and spiked with the internal standards of AMP-d5, MDMA-d5, KT-d4 for method-1 (extraction by basic digestion); and cocaine-d3, 6-MAM-d3, morphine-d3, methadone-d9, codeine-d3, benzoylecgonine-d3, AMP-d5, MDMA-d5 and KT-d4 for method-2 (extraction by methanolic incubation). Purification by (LLE) and (SPE) was made using OASIS MCX cartridges (3 cm^3, 60 mg of sorbent and 30 μm particle size, Waters Corporation, Milford, Massachusetts, US), and derivatization only necessary in method 1 (GC-MS).

3.2. Instrumental methods

The hair routine GC-MS methodology implemented in the INTCF laboratory was used to characterize drugs and metabolites in the suspect's hair and solve the case. As briefly indicated in Table 1, 1 μL aliquot was injected into the Agilent gas chromatograph (6890A or 7890A) with an Agilent MS/EI Detector 5973 or 5975C (Agilent Technologies, Palo Alto, CA, USA) for selected-ion monitoring (SIM) mode. The ions monitored, with the retention time and their respective ion target and qualifiers, are listed in Matey et al [25]. Helium gas was set at constant pressure and was used as carrier. The injector and quadrupole temperatures were 230 ºC and 150 ºC, respectively. The column temperature was set to 60 ºC for 1 min, then 30ºC/ min up to 116 ºC, then 5 ºC/ min up to 150 ºC, and finally 20 ºC/ min up to 300 ºC for 1 min [26].

For the LC-HR-MS/MS methodology (method-2 in table 1) a 1 μL aliquot was injected into a Vanquish Flex Binary UHPLC system, coupled with an Orbitrap Q Exactive Focus system (Thermo Fisher, Hemel Hempsted, UK) for full-scan ion monitoring (FS), with data-dependent acquisition (DDA) for fragmentation in MS/MS spectrometry. An initial isocratic elution, followed by a gradient elution, increasingly adding the organic B eluent, performed at a flow rate set to 0.5 mL/ min, The gradient was programmed as follows: 0-1.0 min 1% B, 1-10 min to 99% B, 10-11.5 min to 1 % B and 11.5 -13.5 min hold 1% B. An electrospray ionization (H-ESI II) set in the positive ionization mode was performed. The settings for full scan ion monitoring were the same reported in the study performed by Hans H. Maurer in their papers [27, 28]. Briefly, it consisted in positive/negative switching ionization mode with FS and a subsequent DDA mode, modifying the list of fragmentation acquisition (inclusion list) for different NPS and their metabolites. This was a screening method, which was validated in terms of selectivity (given by the hyphenation of the separation technique with an exact mass spectrometer)

and limits of detection (LODs) [29-31]. In this LC-HR-MS/MS method (method-2), all the resulting chromatograms and MS spectra were compared to the ones available at the INTCF library, which contains more than 1,550 standards stored up in the database, with a particular spectrum of MS/MS database included.

3.3. Data treatment: LOD calculations

The limits of detection (LODs) for GC-MS were obtained from the validation data of the method-1 calculated experimentally obtaining different readings with half of the amount of the first calibration point, prepared similarly as the calibration curves, described in more detail by Matey et al [25]. For method-2 the LODs for LC-HR-MS/MS Orbitrap were determined using decreasing concentrations of drug-fortified blank hair samples with ketamine and methoxetamine: 25, 20, 15, 10, 5, 2 and 1 pg/mg. The limits of detection were defined as the lowest concentrations exhibing a signal of 3-times the background height (background noise) in the chromatogram profiles [29].

4. RESULTS AND DISCUSSION

LC-HR-MS/MS Orbitrap is a type of high-resolution mass spectrometry technique that was recently acquired in the Madrid Department of the INTCF. The potential of this technique is assessed in this report by reanalyzing a previous solved forensic case, pursuing: (1) to evaluate the simplification of the routine methodology for hair sample analysis in the INTCF laboratory by GC-MS when a developed LC-HR-MS/MS method was applied to the same real case involving poly-consumption; (2) and, then, to identify methoxetamine and

its metabolites in the hair sample of the studied , where the ketamine detection suggested the potential presence of other arylcyclohexylamines,

4.1. Substances determined in the hair sample of the real forensic case.

Table 2 shows the substances detected by GC-MS [25] and LC-HR-MS/MS and their corresponding experimental LODs for arylcyclohexylamines. As expected by LC-HR-MS/MS, in addition to ketamine and its main metabolite norketamine, the presence of methoxetamine was confirmed. In addition, the identification was also positive, by GC-MS analysis, to amphetamine, MDMA, MDA, most likely as a metabolite of MDMA. The LODs for these substances were not calculated.

Obviously, the calculated LODs for the HR-LC-MS/MS methodology were lower than those values obtained for the conventional GC-MS methodology. Lower LOD values imply better capability to detect smaller concentrations of analyte in the sample. In fact, ketamine had LOD values about 2 orders of magnitude lower with LC-HR-MS/MS Orbitrap than with GC-MS. However, the interpretation of results from high-resolution techniques needs to be done by high-qualified personnel. In addition, the higher detection capacity of the high-resolution technique may facilitate the detection of occasional or single consumption, as for example in Drug-Facilitated Crimes (DFC). Besides, other substance was only detected using LC-HR-MS/MS, which is a big advantage because there is a greater number of identifications using a single method of analysis using the half amount of sample (20 mg) than using the routine and laborious GC-MS method (40 mg).

Furthermore, other substances of toxicological interest were identified in this hair sample, such as cocaine (and its main metabolites benzoylecgonine and

ethylbenzoylecgonine, or cocaethylene, when combined with alcohol), sildenafil (phosphodiesterase-5 inhibitor), a pharmaceutical often involved recreationally for chemical sex or "chemsex", [32, 33] and some usual adulterants of cocaine. These identified adulterants were levamisole, lidocaine and phenacetin. They are compounds commonly used as cutting agents of cocaine [34, 35]. In this case, in addition to conventional drugs of abuse (cocaine, amphetamine, MDMA, ketamine), a polydrug pattern consumption profile is observed and confirmed positive in the forensic case studied, detecting ketamine, methoxetamine and their metabolites, just as previously reported in similar cases [26, 36].

4.2. Identification of methoxetamine and metabolites by LC-HR-MS/MS.

In this work, the LC-HR-MS/MS method was used to study methoxetamine and its metabolites. Figure 3 and Table 3 are complementary, showing systematically all the results obtained from the chromatographic separation and the LC-HR-MS/MS analysis of the ion fragments. In Figure 3, the chromatographic results and the MS/MS spectra obtained by LC-HR-MS/MS are reported for arylcyclohexylamines (ketamine and methoxetamine) and some of their main metabolites. The formulae, the peak chromatogram and the precursor area [M-H+], and HR-MS/MS spectra fragmentation are also collected, as well as the ion fragments corresponding to each signal. The CRM, as well as the library of MS/MS spectra, were available for ketamine, norketamine and methoxetamine. There was no information available for methoxetamine metabolites. For this reason, metabolites were tentatively identified according to the literature [16, 37] and spectral information. Those metabolites positively identified were: normethoxetamine, O-desmethylmethoxetamine, dehydromethoxetamine, dihydronormethoxetamine

and hydroxynormethoxetamine. The ion mass and ion MS/MS products or fragments detected for methoxetamine and its metabolites are given in Table 3. All fragments mass/charge (m/z) were sorted by number of ions detected. Table 3 also organizes comprehensively the data obtained by HR-MS/MS for methoxetamine compared with its metabolites. Just like with norketamine, normethoxetamine shows the same main ion fragments in MS/MS spectra as the parent drug, because the N-ethyl group of methoxetamine is not present in this fragment in both compounds. Consequently, the structural fragmentation is the same for ketamine and norketamine (see figure 3). With the other metabolites, the principal ion of methoxetamine (1* and 3*, figure 3) is present in the rest of metabolites detected with a major or minor abundance and with some structural variations, due to the metabolization process of methoxetamine. Ion fragment 2* is present in all metabolites with the exception of hydroxynormethoxetamine, which is not detected, probably because the precursor ion is detected with minor abundance. The same thing occurs with ion fragments 4* and 5*, which are not detected with these metabolites, not even with dehydromethoxetamine, probably because of the metabolite chemical structure.

5. CONCLUSIONS

This case report has demonstrated that LC-HR-MS/MS is a valid method for determining methoxetamine, and at least five of its metabolites in hair samples of one past forensic case. This is very relevant since the detection of the metabolites of methoxetamine allowed discriminating between active consumption and external contamination. The LC-HR-MS/MS method has also proved that it is possible to use a simpler pre-analytical procedure where laboratory efficiency is improved to be faster, with a universal extraction step,

such as methanolic incubation, that will lead to methods with greater capability for the study of hair matrices in the screening mode. This LC-HR-MS/MS screening method optimizes time use, resources and efficiency of the experiments. As another important advantage, only half of the seized sample previously studied was analyzed leading, additionally to obtain lower LODs in comparison with the targeted GC-MS method used routinely in the INTCF.

Methoxetamine and five of its metabolites (normethoxetamine, O-desmethylmethoxetamine, dehydromethoxetamine, dehydronormethoxetamine and hydroxynormethoxetamine) were detected in addition to conventional drugs of abuse (amphetamine, MDMA and ketamine) in the hair sample. GC-MS also gave a positive result for the same drugs, except for methoxetamine, which was not tested in routine method (GC-MS). The combination of these results confirm a polydrug consumption case, with at least six drugs found in the hair sample. Due to the 4 cm of hair length that were under study, the evaluated period corresponded to 4-5 months, assuming that it was cut from the root and that the growth is 1 cm per month. Furthermore, polydrug consumption is revealed as a common practice amongst drug users and, in this case, a drug poly-consumption involving the NPS methoxetamine was observed.

The high-resolution methodology provides higher detection capacity and makes it possible the screening of drugs and NPS substances in occasional or single consumptions, as for example in Drug-Facilitated Crimes (DFC). Herein lies the importance to incorporate untargeted LC-HR-MS/MS methods into routine analysis for drug detection and its identification.

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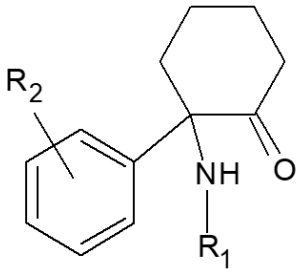


Arylcyclohexylamines	R_1	R_2
Ketamine	$-CH_3$	-Cl
Methoxetamine	$-CH_2-CH_3$	$-O-CH_3$

Figure 1. Structural differences between ketamine and its analog, methoxetamine.

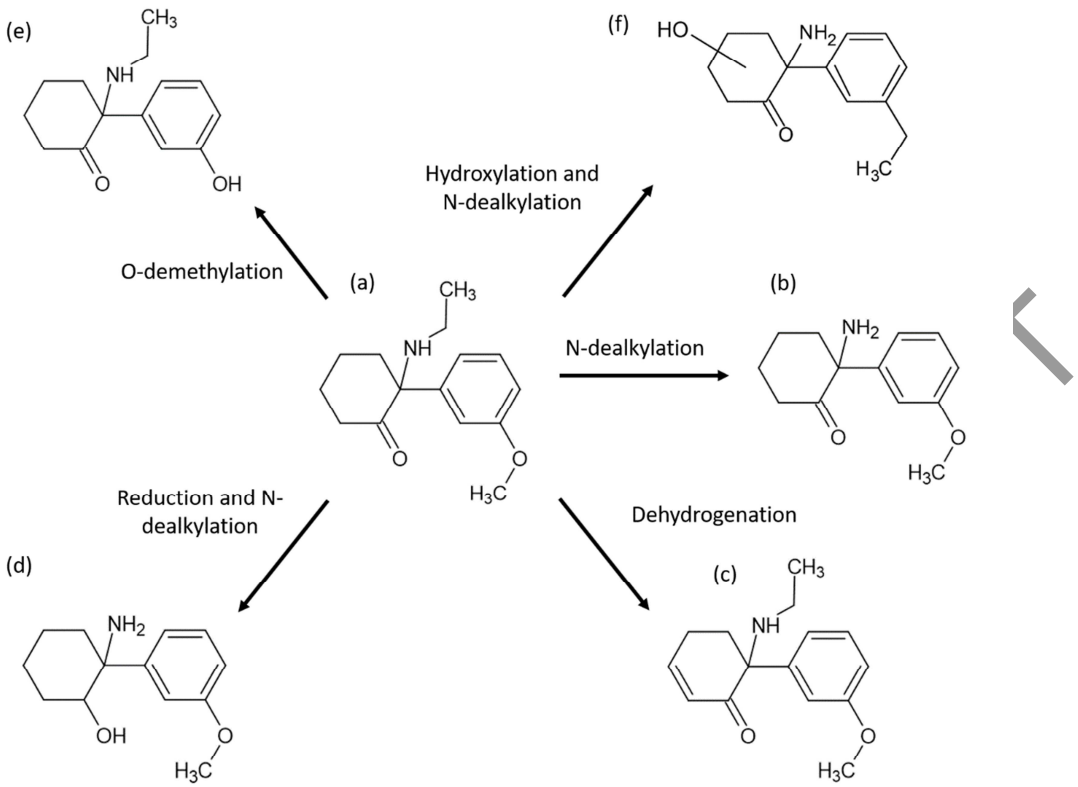
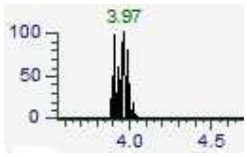
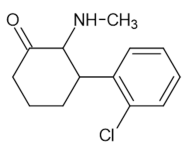


Figure 2. Metabolic pathways of (a) methoxetamine into five of its metabolites: (b) normethoxetamine, (c) dehydromethoxetamine, (d) dihydronormethoxetamine, (e) O-desmethylmethoxetamine and (f) hydroxynormethoxetamine [16].

KETAMINE





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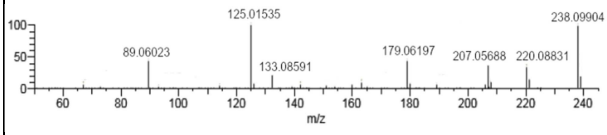
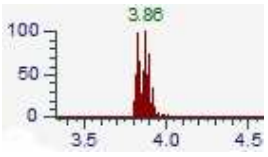
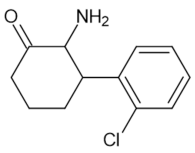


NORKETAMINE





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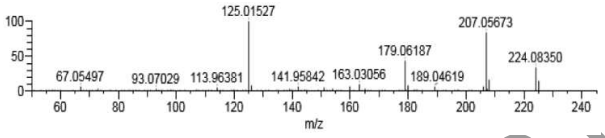
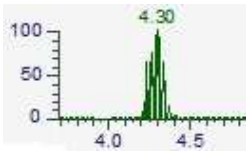
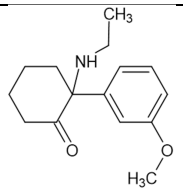


METHOXETAMINE





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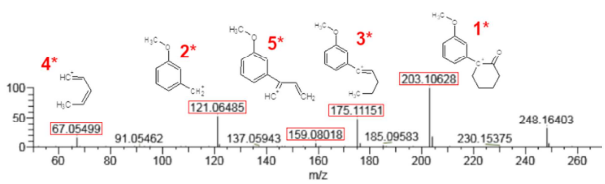
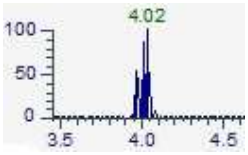
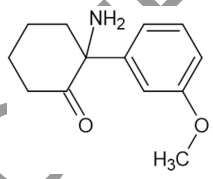


NORMETHOXETAMINE





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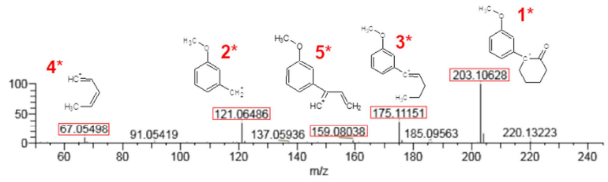
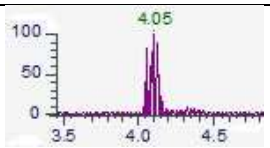
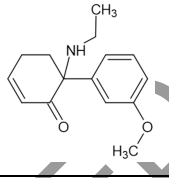


DEHYDROMETHOXETAMINE





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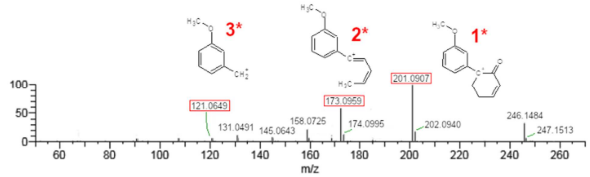
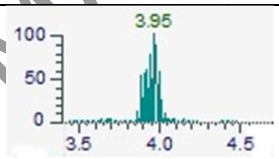
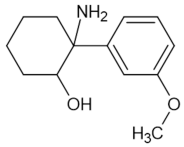


DIHYDRONORMETHOXETAMINE





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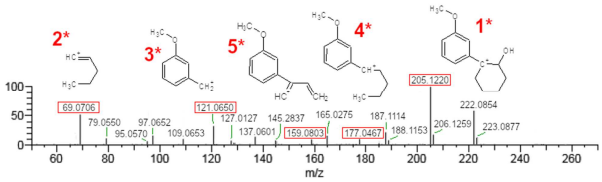
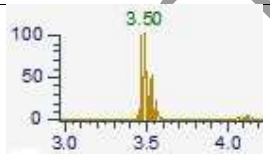
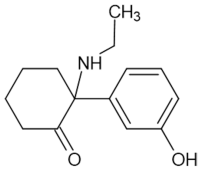


O-DESMETHYLMETHOXETAMINE





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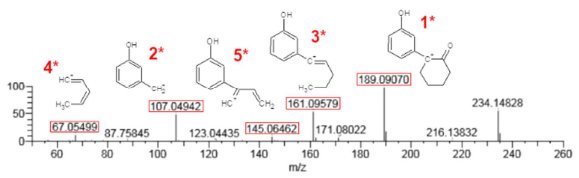
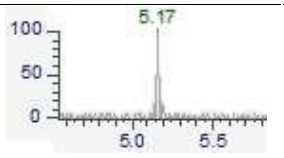
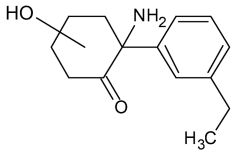


HYDROXYNORMETHOXETAMINE





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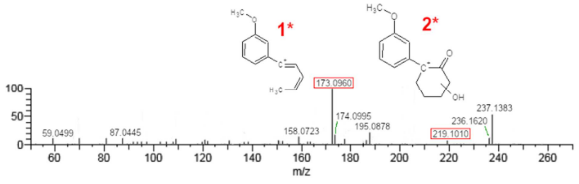


Figure 3. Chromatograms of the arylcyclohexylamines (ketamine and methoxetamine) and their metabolites detected and identified. Chemical formulae, peak chromatogram and precursor [M-H$^+$] area, and of HR-MS/MS Orbitrap fragmentation spectra

Table 1. Methodology followed to prepare the samples in both preanalytical and analytical phases for the two methods used.

Method	Hair amount (mg)	Preanalytical phase					Analytical phase
		(i) Decontamination	(ii) Extraction	(iii) Purification		(iv) Derivatization	Instrumentation
				LLE	SPE		
1	40	Double washing: 5 mL of dichloromethane	Basic digestion with NaOH 1 M, 1 mL, T= 95 ºC, t= 10 min	Simple extraction with n-hexane/ethyl acetate (9:1, v/v) after acidification with HCl 0.1M, 1 mL	Washing with water/acetic acid (98:2, v/v) and methanol/water/acetic acid (18:80:2, v/v/v). Elution with dichloromethane/ isopropanol/ammonium hydroxide (83:15:2, v/v/v)	PFPA and ethyl acetate (10:6, v/v); T= 90 ºC, t= 30 min	**GC-MS** Injection: 1 μL extract Column: 5% phenylmethylsiloxane (30 m and 0.25 μm id 0.25mm) Elution: gradient T= 60-300 ºC Detection: Agilent MS/EI Detector 5973 for SIM
2	20		Trituration of hair and incubation in methanol, t= 18 h	Not necessary			**LC-HR-MS/MS*** Column: phenylhexyl (100 mm x 2.1 mm x 2.6 μm) Elution: gradient MP_A: ammonium formate 2 mM with 0.1% formic acid (pH 3) and MP_B: acetonitrile/methanol (50:50, v/v) with 1% of MP_A Flow rate: 0.5 mL/ min Detection: Orbitrap Q Exactive Focus system

*High-resolution technique

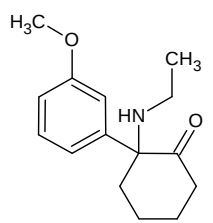
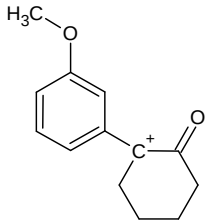
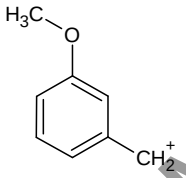
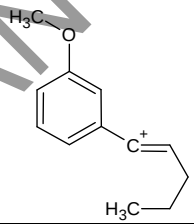
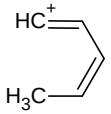
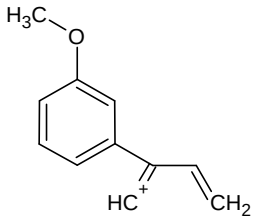
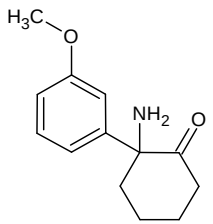
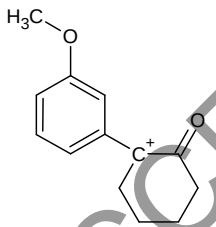
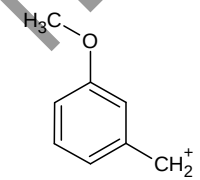
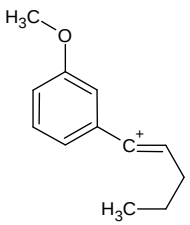
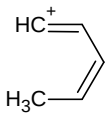
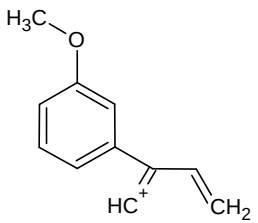
Table 2. Limits of detection (LOD) of the arylcyclohexylamines under analysis, using GC-MS and LC-HR-MS/MS techniques, with available CRM in the hair forensic case sample.

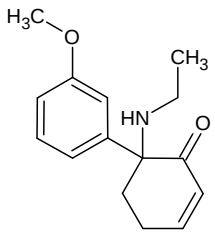
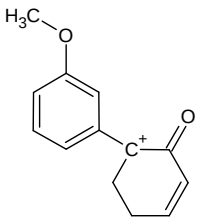
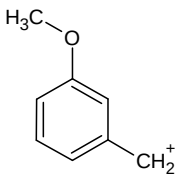
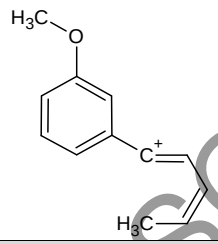
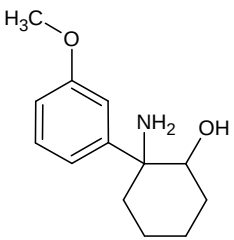
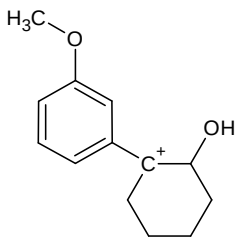
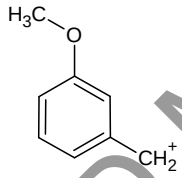
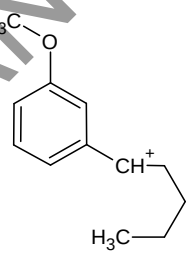
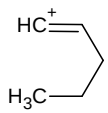
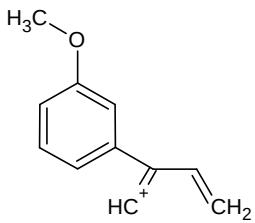
Drug group detected	**Drugs or metabolites**	**LOD_{GC-MS*} (pg/mg)**	**$LOD_{LC-MS/MS}$ (pg/mg)**
Arylcyclohexylamines	Ketamine	250	2
	Norketamine	50	2
	Methoxetamine	nt	5

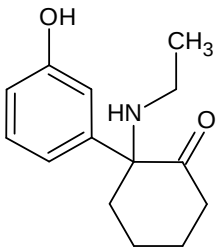
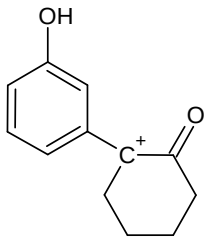
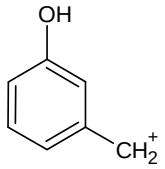
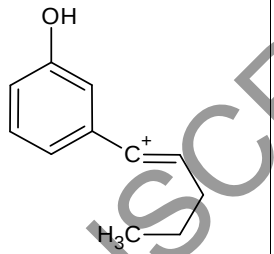
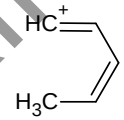
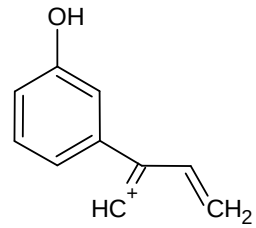
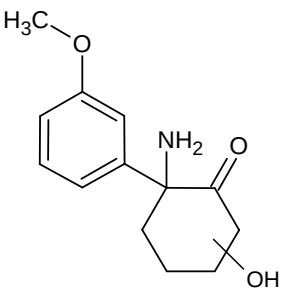
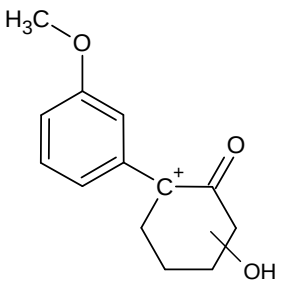
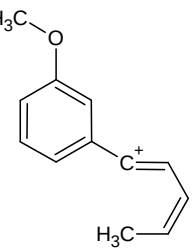
nt = not tested

* Calculated from the validation data of the GC-MS method (Matey et al.) [25].

Table 3. Ion precursors of methoxetamine and metabolites and calculated error mass measure (ppm) are comprehensively described. On the other hand, a comparison of the main HR-MS/MS fragments of methoxetamine (5 ion fragments) and the ion fragments detected in metabolite spectra as a mode of tentative are represented.

(a) METHOXETAMINE (MXE)					
PRECURSOR MS[1] Formula / Measure/ Error (ppm) / Structure	**FRAGMENT-1* (MS/MS)** Formula / Measure/ Error (ppm) / Structure proposed	**Fragment-2* (MS/MS)** Formula / Measure/ Error (ppm) / Structure proposed	**Fragment-3* (MS/MS)** Formula / Measure/ Error (ppm) / Structure proposed	**Fragment-4* (MS/MS)** Formula / Measure/ Error (ppm) / Structure proposed	**Fragment-5* (MS/MS)** Formula / Measure/ Error (ppm) / Structure proposed
$C_{15}H_{21}NO_2^+$ = 248.1639 (-2.32) 	$C_{13}H_{15}O_2^+$ = 203.1063 (-1.48) 	$C_8H_9O^+$= 121.0649 (1.65) 	$C_{12}H_{15}O^+$ = 175.1116 (-0.57) 	$C_5H_7^+$= 67.0550 (12.63) 	$C_{11}H_{11}O^+$ / 159.0803 (-0.63) 
(b) NORMETHOXETAMINE (Nor-MXE) (N-dealkylation process)					
$C_{13}H_{17}NO_2^+$ = 220.1328 (-1.82) 	$C_{13}H_{15}O_2^+$ = 203.1063 (-1.48) 	$C_8H_9O^+$= 121.0649 (1.65) 	$C_{12}H_{15}O^+$ = 175.1116 (-0.57) 	$C_5H_7^+$= 67.0550 (12.63) 	$C_{11}H_{11}O^+$ / 159.0803 (-0.63) 
(c) DEHYDROMETHOXETAMINE (Dehydro-MXE) (Dehydrogenation process)					

$C_{15}H_{19}NO_2^+$ = 246.1486 (-1.22) 	$C_{13}H_{13}O_2^+$ = 201.0907 (-0.99) 	$C_8H_9O^+$ = 121.0649 (1.65) 	$C_{12}H_{13}O^+$ = 173.0959 (-0.58) 	Not detected	Not detected
(d) DIHYDRONORMETHOXETAMINE (Dihydro-nor-MXE) (Reduction and N-dealkylation processes)					
$C_{13}H_{19}NO_2^+$= 222.1484 (-2.25) 	$C_{13}H_{17}O_2^+$ = 205.1220 (-0.98) 	$C_8H_9O^+$ =121.0650 (2.48) 	$C_{12}H_{17}O^+$=177.0467 (0.00) 	$C_5H_9^+$= 69.0706 (11.98) 	$C_{11}H_{11}O^+$ = 159.0803 (-0.63) 

(e) O-DESMETHYLMETHOXETAMINE (O-desmethyl-MXE) (O-demethylation process)					
$C_{14}H_{19}NO_2^+$ = 234.1487 (-0.85) 	$C_{12}H_{13}O_2^+$ =189.0907 (2.64) 	$C_7H_7O^+$ =107.0494 (1.65) 	$C_{1a}H_{13}O^+$= 161.0961 (-1.86) 	$C_5H_7^+$= 67.0557 (23.57) 	$C_{10}H_9O^+$= 145.0648 (-1.38) 
(f) HYDROXYNORMETHOXETAMINE (Hydroxy-nor-MXE) (Hydroxylation and N-dealkylation processes)					
$C_{13}H_{17}NO_3^+$ = 236.1281 (0.00) 	$C_{13}H_{15}O_3^+$ =219.1010 (1.83) 	Not detected	$C_{12}H_{13}O^+$ = 173.0960 (0.00) 	Not detected	Not detected

Author's Response to Decision Letter for (JAT-19-3046.R1)

Potential of high-resolution Orbitrap technology for the detection of drugs and metabolites in hair. Special focus in ketamine and methoxetamine in a real forensic case.

Simon Elliott, Ph.D.
Director, Elliott Forensic Consulting
Visiting Professor, King's College London
TIAFT Special Issue Editor, Journal of Analytical Toxicology

Dear Editor, Bruce A. Goldberger, Ph.D.,
Editor-in-Chief, Journal of Analytical Toxicology,
Professor, University of Florida

Dear Editors,
First of all, we thank the decision and advice to revise our manuscript. We greatly thank reviewers for their time to revise our study and the careful evaluation made to our work, improvable through their pertinent comments. We have consider them for preparing our revision (changes are highlighted in red in the corrected manuscript). As stated below, we have evaluated each comment and provided our responses:

Reviewers' comments:

Reviewer: 1:
Comments to the Author The manuscript present a case report about the possibility to monitor the presence of methoxetamine in hair by using Orbitrap. In my opinion the manuscript can be of limited interest for the readers and to be published it should be carefully revised and improve. - There are several typing error throughout the text and the abstract. In many part the text is obscure and not clear. For example, the method description is not clear (p. 6-7).

Response: We have checked this page and cleared it according to the reviewer´s suggestion, particularly in the comparison between the two methods, GC-MS (method-1) and LC-HR-MS/MS (method-2,) for the identification of methoxetamine and metabolites.

Moreover, to be suitable for publication a careful revision of the English should be performed. - Introduction: page 6 line 26. What does the authors mean with the term “nitrogen extraction”? Please explain it.

Response: We have checked this paragraph and corrected it according to the reviewer´s suggestion.
“After decontamination, the total amount of hair analyzed in both methods was previously dried with nitrogen flow for 10 minutes”.
The next steps were (i) trituration (with Precellys), (ii) addition of internal standards, (iii) extraction (which depended on the methodology used), and (iv) purification and derivatization (for the method-1, GC-MS analysis)

- Introduction: page 6 line 35-36. The author should explain why they use “at least” 18 hours of incubation and what they intend with this term. Do they performed some experiments to establish the difference in the extraction recovery by using 18 hours (which is quite a long time) or more than 18 hours? - Do the authors performed an incubation of hair with methanol using precellysis homogenizer for 18 hours? It is not clear from the text.

Response: The reviewer is right. We have checked this paragraph and corrected it according to the reviewer´s suggestion.
For this case, in the method-2, the extraction process took 18 hours for methanolic incubation (method-2). The trituration and homogenization with Precellys were performed before the extraction process.

- P. 11, lines 42-47. This sentences should be totally re-written in order to clarify what the authors intend. - My major concern about the paper is the fact that HRMS technology has extensively been applied in the determination of drugs in hair. It doesn’t seem from the data presented in this paper that Orbitrap technology can really be superior over other HRMS techniques or other publication. For example the LOD of the proposed approach for drug of abuse appears to be comparable or with those obtained with GC-MS from other lab (see for example K. Lachenmeier et al. / Forensic Science International 159 (2006) 189–199). Moreover, the author cited reference 11, by Favretto et al. This reference describe the determination of ketamine in hair, by using Orbitrap and only 2 mg of hair with a LOD of 20 pg/mg . An example of the determination of methoxetamine in real hair sample can be found in Laurent Imbert et al., Journal of Analytical Toxicology 2014 Sep;38(7):410-5, where using 50 mg of hair they could reach a limit of detection of 0.5 pg/mg for methoxetamine, so much more lower than those obtained from the authors.
In my opinion, the novelty of the present paper is only limited to the identification of the methoxetamine metabolite in real hair samples and the paper should be re-written to emphasized this aspect.

Response: We have checked and revised the manuscript following these criteria, resulting in:

First: we have omitted from the title and from other parts of the manuscript the word Orbitrap and we have left only high-resolution mass spectrometry.
Second: we have focused the manuscript in what is novel: both methoxetamine and metabolites detected in hair from a real forensic case.
Third: Also, we have performed a new experimental analysis for the LODs for LC-HR-MS/MS using decreasing concentrations of drug-fortified blank hair samples with ketamine and methoxetamine: 25, 20, 15, 10, 5, 2 and 1 pg/mg.
The results were ketamine and norketamine being 2 pg/mg and methoxetamine, 5 pg/mg.
It has to be taken into account that other references should have a lower detection limit, but it should be due to the amount of sample used (20mg) and the low pre-cleaning of the sample in our case.
In addition, Table 2 has been updated and modify to focus in the comparative analysis of LODS in the GC-Ms and LC-MS/MS techniques in the available CRM of drugs or metabolites in the hair case.