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A validated GC–MS method for ketamine and norketamine in hair and its use in authentic cases

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ABSTRACT

This work has shown that it is a valid method for determining ketamine, norketamine and amphetamines derivates in hair samples of forensic cases. This method was validated meeting the criteria of sensitivity and accuracy for detecting repeated consumption of ketamine in hair samples of forensic interest, according to the proposed cut-off for ketamine of 0.5 ng/mg. The detection of norketamine allowed discriminating between active uses and external contamination. The assessed method was applied for analyzing 1189 hair samples of judicial interest received in the INTCF along 15 months, obtaining 62 positive in ketamine consumption. This means a 5.2% of positivity. Ketamine consumers present a profile of young age (21–30 years old), polydrug use with consumption of synthetic substances preferably MDMA and, then, amphetamine. As consumer is collective, prone to consume new psychoactive substances, requires special attention due to they show a consumer profile with higher prevalence in MDMA than

amphetamine, indicating that ketamine consumers belong to a subgroup with a different profile within the INTCF casuistry.

The results of the exercises of the proficiency tests performed satisfactorily in all cases.

In conclusion, it is a suitable method also to evaluate the chronic consumption of ketamine, in addition to amphetamines in the same method of analysis.

KEYWORDS

Ketamine; Norketamine; Hair; Drugs; MDMA; Amphetamine

INTRODUCTION

The determination of toxics present in the hair matrix has been widely investigated in the recent decades. This matrix has been used to evaluate the presence of metals(arsenic, mercury, lead or thallium [1]); different drugs of abuse and/or their metabolites [2–4]; and biomarkers of drug consumption or stress, such as ethylglucuronide [1,5] or cortisol [6]. Hair is a very valuable matrix due to its particularities for fixation and incorporation of analytes. This makes it possible to detect consumptions during very long periods, which are impossible to be detected in other matrices.

Hair analysis is a common task in laboratories dealing with forensic analysis, such as detoxification in consumption or as a abstinence proof, ratification of chronic substance abuse, drug facilitated sexual assault (DFSA) and postmortem analysis. In addition, new analytical applications can be developed, although the framework of their applications would require legal regu- lations. For example, the Spanish legislation on hair analysis does not consider the drug testing issues in the workplace or driving testing following

substance abuse convictions [7]. For the latter, there is a single reference study for its implementation, such as the one conducted by Lendoiro et al. [8], where hair analysis and psychological tests were evaluated as tools for the detection of chronic alcohol and drug consumption.

The Spanish National Institute of Toxicology and Forensic Sciences (INTCF) has conducted judicial investigations with hair samples since 1993 [9]. This institute receives annually more than three thousand judicial cases collecting hair matrices. It is accredited for analysis of forensic hair samples since 2012 for ISO/IEC 17025. Among all drugs of abuse, cocaine and cannabis are the most prevalence [10]. However, as any other controlled substance, ketamine also needs to be determined in hair samples, more if we consider its high prevalence in Asia [11].

Ketamine, (also known as "Keta", "Special K" or only "k") is usually used illicitly, although, it has different clinical applications in veterinary and humans that cannot be ignored [12]. Nowadays, it is used as rapid-acting dissociative anesthetic in both animals and humans. However, it is abused by an increasing number of young people as a 'club drug' and is often distributed at 'raves' and parties. Teenagers and young people are the major abusers [13], probably because of its hallucinogenic and stimulant effects. Due to this illicit use, it has been classified in the list I of psychotropics and outlawed since 2010 [14].

Once in the body, ketamine (KT) is *N*-demethylated to norketamine (NK). As shown in Fig. 1, ketamine is metabolized in the liver by the P450 system, being the CYP3A4 the main enzyme responsible of *N*-demethylation into norketamine [15]. Therefore, in addition to the determination of the ketamine content, the determination of norketamine it is recommended to provide a positive result in hair samples.

In order to determine a habitual consumption of drugs that is repeated over time, i.e., a

chronic consumption, there are some guidelines that advise a threshold value of positivity or cut-off. There are some prestigious associations that agree these cut-off values such as: the Society of Hair Testing (SoHT) [16], the German Society of Toxicological and Forensic Chemistry (GTFCh) [17], the Substance Abuse and Mental Health Services Administration (SAMHSA) [18], and the European Workplace Drug Testing Society (EWDTS) [19]. However, the unique cut-off available for ketamine is that from EWDTS, which is only a proposal of 0.5 ng/mg for ketamine and 0.1 ng/mg for norketamine [20].

Different methods have already been reported for the determi- nation of ketamine in hair samples by GC–MS [13,21–26] and by LC– MS/MS [12,20,27–30]. In addition, there are different proficiency tests for both substances (ketamine and norketamine metabolite), where the INTCF laboratory has participated annually for different exercises of Arvecon (Walldorf, Germany). In 2016, ketamine was included together with amphetamines in the same in-house- validation analytical method in the INTCF.

Because of the increase in ketamine consumption among young people and its distribution in juvenile nightlife environ- ments, the aim of this work was to validate a CG–MS method for ketamine and norketamine in hair that satisfices the needs of the Spanish INTCF analysis. Finally, the validated method was applied to authentic forensic cases to detect the chronic consumption of ketamine.

MATERIALS AND METHODS

Standard and reagents

Ketamine, norketamine and amphetamines (amphetamine AMP, methamphetamine MAMP, methylendioxiamphetamine MDA, methylendioxymethamphetamine MDMA and methylen- dioxiethylamphetamine MDEA, ketamine and norketamine were provided by LGC Promochem Cerilliant (Teddington, Middlesex, UK) and Lipomed (Arlesheim, Switzerland) as pure solutions in methanol at 1.0 mg/mL. As internal standards (IS), AP-d5, MDMA- d5 and ketamine-d4 were purchased from LGC Promochem Cerilliant, at 1.0 mg/mL solution in methanol.

Pentafluoropropionic anhydride (PFPA) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). All chemicals and solvents were of analytical grade.

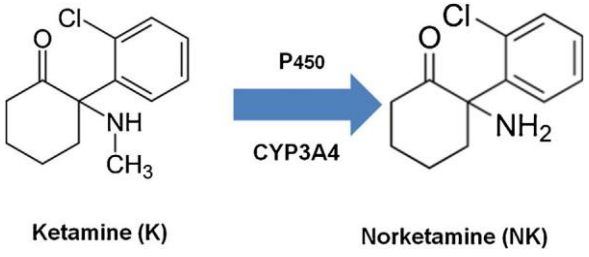

Fig. 1. Metabolism of ketamine to norketamine.

Hair samples

Three types of hair samples were used: (i) for the validation of the method and the internal quality control (QC), hair samples were collected from drug free volunteers (blank hair or negative hair); (ii) for the external quality control, the analysis of a Proficiency Tests from Arvecon (Walldorf, Germany) was per- formed; and (iii) real forensic samples from judicial cases, submitted to the laboratory of INTCF in the Madrid Department for a period of 15 months.

Preanalytical hair sample treatment

All hair samples needed a preanalytical treatment before the analysis of the drugs. The assessed method for ketamine and norketamine was based, with some modifications, in-house-validated analytical method of the INTCF laboratory for amphetamines [4].

Hair samples were treated as follows:

1 Decontamination by double washing, with 5 mL diclomethane for five minutes in each wash. All washes were individually retained for analysis, if necessary;

2 Extraction of 40 mg of the dried sample (with nitrogen) spiked with the internal standards of AMP-d5, MDMA-d5 and KT-d4 (200 ng, each), by a basic digestion (1 mL of 1 M NaOH, T = 95 °C, t = 10 min);

3 Purification of the resulted basic extract by Liquid–Liquid Extraction (LLE) and Solid-Phase Extraction (SPE). LLE was made after acidifying the basic extract (with 1 mL of 0.1 M HCl) and mixed it with 2 mL of an organic phase composed by *n*-hexane/ethyl acetate (9:1, v/v), subjected to subsequent centrifugation (4400 g for 5 min). This extraction was performed twice. For the SPE, the third remaining aqueous fractions were mixed in a test tube, where the pH was adjusted to 5.5 with

3.33 M H_3PO_4. Ketamine, norketamine and amphetamines were extracted from this solution using a SPE with OASIS MCX cartridges (3 cm^3, 60 mg of sorbent and 30 mm particle size, Waters Corporation). The cartridges were previously condi- tioned with methanol and deionized water. The samples were followed by sequential washes with water/acetic acid (98:2 v/v) and methanol/water/acetic acid (18:80:2 v/v/v). The final elution was performed with dichloromethane/isopropanol/ NH_4OH (85:15:2, v/v/v).

4 Derivatization with PFPA and ethyl acetate (10:6 v/v) were optimized for ketamine derivatization according to Refs. [22,26]. Briefly, it was performed adding the reagents to the obtained extract at 70 °C for 30 min. The obtained solution was evaporated to dryness and reconstituted in 100 mL of *n*-hexane

[4] prior to the GC–MS analysis.

GC–MS conditions

1 mL of each of the derivatized extracts was injected in splitless mode onto two similar instruments, specifically into Agilent gas chromatographs 7890A model, each of them equipped with a capillary column (30 m, 0.25 mm i.d., 0.25 mm thick film of 5% phenylmethylsiloxane) and coupled with an Agilent MS/EI Detector 5975C for selected-ion monitoring. Helium gas at constant pressure (with retention time locking) was used as carrier and the injector and the quadrupole temperatures were 230 °C and 150 °C, respectively. The column temperature program was 60 °C for 1 min, 30 °C/ min up to 116 °C, 5 °C/min up to 150 °C, 20 °C/min up to 300 °C for 1 min. The ions monitored, with the retention time and their respective ion target and qualifiers, are listed in Table 1.

Table 1. Detection characteristics of ketamine (including it IS) and norketamine PFPA derivatives by their GC–MS analysis. Ketamine-PFP, ketamine-d4-PFP and norketamine-PFP are given with their respective retention times (t_R), ions target (T) and qualifier (q).

Compound	t_R (min)	Ions (m/z)		
		Target (T)	Qualifier (q1)	Qualifier (q2)
Ketamine-PFP	14.68	320	312	–
Ketamine(d4)-PFP	14.67	324	316	–
Norketamine-PFP	13.58	334	306	264

Identification criteria

The identification criteria included retention time (t_R) within (2%) and 20% in relative ratios of the ions (qualifier/target) in the sample [31], which were compared with of average calibrator, or quality control (QC).

Validation

Method validation was performed following SWGTOX guide- lines [32]. Other literature references were also consulted [33]. Calibrators and quality controls (QCs) were prepared by fortifying blank hair samples. Validation parameters included linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy and precision, interferences, carryover and uncertainty.

Linearity was evaluated by least squares regression (r^2) with 6 non-zero calibrators on five different days. Acceptable linearity was achieved when $r^2 \geq 0.99$ and calibrators quantified within ±20%. LOD and LOQ were determined by decreasing concentrations of

drug-fortified blank hair samples. LOD was the lowest concentration with acceptable chromatography, signal/noise ratio ≥ 3. It was calculated higher than 3 times the signal to noise (S/N) from each peak height and noise by software data acquisition and allowing the identification of the targeted analytes according to previously described criteria (Section 2.4). LOQ was studied for the lowest concentration of the concentration levels prepared in the INTCF laboratory for the optimization of the method. It meets the criteria of the signal/noise ratio 10; the accuracy and precision were within ±20%.

The accuracy and precision were evaluated by measuring with spiked at three levels of concentration (low, medium, and high) of norketamine and ketamine in hair negative samples. They were prepared on different days (n = 12) for each of them. These three levels of control were studied to cover the entire calibration range, where the low level coincides with the LOQ and the high level studied coincides with the ULOQ. The LOQ values were the same than the values chosen and proposed by cut-off concentration to settle a repeated or habitual consumption [19]. Accuracy was evaluated for each concentration as 100 group mean observed concentration/ known concentration. Acceptable accuracy was within ±20% that is from 80% to 120% [31–33]. And the precision was evaluated with relative standard inter-day RSD (%). The acceptable precision was <20 RSD (%).

The study of interferences or selectivity of the method was conducted by the study of matrix interferences and exogenous interferences (other analytes that could be presents). For matrix interferences or endogenous interferences were reanalyzed 10 negative cases, and for the exogenous interferences, samples were fortifying with 100 mL of 1 mg/L of different types of substances: cocaine (cocaine, benzoylecgonine, cocaethylene, ecgonine methyl esther, norcocaine), opioids (6-monoacetylmorphine, codeine, morphine, methadone, EDDP) and amphetamines (amphetamines, methamphetamine, MDA, MDMA,

MDEA), directly, derivatized and reconstituted according to the method of analysis. The interferents were considered insignificant if the analytes of interest were <LOD.

Carryover was not detected by injecting the highest level of calibration, and in different real cases above of ULOQ, the next sample analyzed was not detected, or the analytes of interest were <LOD. The estimated uncertainty u (bias) has been calculated by the Eq. (1) [34].

$$u(bias) = \sqrt{(bias)^2 + \left(\frac{S_{bias}}{\sqrt{n}}\right)^2 + u(Cref)^2}$$

where bias is the accuracy, the second term depends on reproducibility of method, and the last term depends on the certified reference material used. The acceptable values of uncertainty are depending on the requirement of the laboratory and in turn on the accuracy and precision of the method. When the uncertainty of the certificated material reference is greater than 20% of the uncertainty of total, probably it is not good material references for us method.

In addition to the reported validation, a study of the assessment of precision, accuracy and uncertainty of our method through the analysis of proficiency test, which is a material reference certificated by the organizer, were evaluated (n = 9). In this case, the uncertainty provided of this reference material was calculated by the organizer through the standard deviation (Sd) for each substance, target value and number of laboratories tested (n for analyte), which allowed to obtain an uncertainty value of these certified material u(Cref) (%).

The criterion to evaluate the acceptability of the uncertainty of our method Uexp (%), either by the results obtained of the validation with fortified samples or those obtained of the analysis of different materials in proficiency tests, has been compared with the adopted

criterion given by the Proficiency Test analyzed (PT) Arvecon [35], given by the Eqs. (2) and (3):

$$Uexp\ (\%) \leq T\ 2 * (RSD_{HORWITZ}) \quad (2)$$

$$RSD_{HORWITZ}\ (\%) = 2^{(1—0.5\log C)} \quad (3)$$

where $RSD_{HORWITZ}$ is a theoretical algorithm applied to the analyses ofketamine and norketamine traces, and C isthe concentrationof the analyte expressed in g/mL, or expressed in g/g for solids.

RESULTS AND DISCUSSIONS

For the determination of ketamine and norketamine in hair samples, an established GC–MS method for amphetamine derivatives in hair samples was used, where only a new independent window acquisition has been added to the method. This method was accredited by ISO/IEC 17025 and it has been successfully adapted to the analysis of different INTCF laboratory recent needs, such as the determination of ketamine and norket- amine substances in hair samples presented in this paper. An evaluation of the analytical performance of the method, as well as different quality controls including the Prociency Test, were achieved prior its application to real forensic samples.

Method validation

Method was applied to fortified blank hairsamples to validate the method in term of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision, for the determination of ketamine and norketamine. The resultsare summarized in Table 2.

Linearity

Different amounts of ketamine and norketamine in methanol were added to 40 mg segments blanks hair samples. The linearity was verified in the concentration range comprise from 0.5–50 ng/mg

Table 2. CG–MS method validation for the determination of ketamine and norketamine. LOD, LOQ, cut-off or limit of decision recommended by Ref. [19], relative standard inter-day $RSD_{(T)}$ (%), accuracy error (%) and uncertainty, Uexp (%). Concentration levels (QC): low (LOQ) = 0.1 ng/mg (norketamine), 0.5 ng/mg (ketamine); medium = 1 ng/mg (norketamine), 5 ng/mg (ketamine); and high (ULOQ) 10 ng/mg (norketamine), 50 ng/mg (ketamine).

Analyte	Linearity range (ng/mg)	LOD (ng/mg)	LOQ (ng/mg)	QC levels	$RSD_{(T)}$ (%) (n = 12)	Accuracy (%) (n = 12)	Uexp. (%) (n = 12)
Ketamine	0.5–50	0.25	0.5	Low	8.4	6.10	17.4
				Medium	9.5	−8.9	19.7
				High	9.1	0.10	19.0
Norketamine	0.1–10	0.05	0.1	Low	10.0	2.0	21.0
				Medium	16.6	5.9	34.6
				High	12.2	−6.9	25.5

for ketamine, and 0.1–10 ng/mg for norketamine, in six concentra- tion levels by duplicate (Table 2). The result is calculated by a linear regression analysis of the ratio of the peak area of ketamine and norketamine to the peak area of the internal standard against drug concentration spiked (ng/mg). The calibration model was superior to $R^2 > 0.99$ and the study of residuals was less than 15% at each interpolated point of the different levels of the calibration.

That new calibration curve was constructed when it was necessary, evaluated with quality controls and calibrated, at least, every six months.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ was calculated using Reference Materials prepared in the same way as the calibrator or QC (see Section 2.6) [32]. The LOD concentration determined was half of the first point of quantification: 0.25 ng/mg for ketamine, and 0.05 ng/mg for norket- amine. The LOQs obtained were 0.5 ng/mg for ketamine and 0.1 ng/mg for norketamine (Table 2). These values were considered as the lower limit ofquantification (LLOQ). The upper limit of quantification (ULOQ) was considered as a value 100 times greater than the LOQ, being 50 ng/ mg for ketamine and 10 ng/mg for norketamine (Table 2). These two limits defined the analytical measuring range. Replicate quantifica- tions of LOQ concentration were within 10% in precision (RSD) and 6% of error in accuracy (bias) (Table 2). These results meetthe criteria previously established for the LOD and LOQ [32].

Accuracy, precision an uncertainty

An acceptable precision was obtained, measured as the relative standards deviations (RSD (%)), since comprised between 8.4% and 16.6% in all cases (Table 2). A good accuracy,

assessed by means of the bias, was obtained from about 7% to 9% for ketamine, and about 2% to 7% for norketamine. The uncertainty (Uexp (%)) associated to the internal standard method, used through the study of added samples, was comprised between 17.4% and 19.7% for ketamine and 21.0% and 34.6% for norketamine.

All the uncertainties values obtained in the initial validation (Table 2), with an exception for norketamine at medium level (1 ppm = ng/mg), were lower for traces than accepted by the organizer and therefore acceptable. In the last case, the acceptance value obtained for two times $(RSD)_{Hortwitz}$ must be 32% and the obtained values was close (35%).

The different proficiency tests (which uses certificated refer- ence material) have been performed by this method, during at the last three years (n = 9). Table 3 shows the results relative to standard inter-day RSD (T), accuracy error (%) and uncertainty Uexp (%). Each reported value was compared by pairs of values. The results from interlaboratory comparisons are used in the same way as a reference material [34]. A laboratory should analyze or participate in at least 6 times within a reasonable time interval.

These results are significantly better than those obtained in the initial method validation (Table 2), in particular for norketamine, so they demonstrate its validity through this other certified reference material.

Table 3. Global results of proficiency tests performed at the INTCF (n = 9). Relative standard inter-day $RSD_{(T)}$, accuracy error (%) and uncertainty Uexp (%) values for ketamine and norketamine.

Analyte	$RSD_{(T)}$ (%)	Accuracy (%)	U exp (%)
Ketamine	10.5	−2.7	21.7
Norketamine	8.8	−3. 4	18.8

Quality assurance

Quality assurance was made by two types of controls: internal quality and external quality controls (Proficiency Test, which is a certified reference material in matrix). For the everyday workflow analysis with real samples, two internal quality controls (spiked always on blank hair) should be passed: (1) the quality control is either a negative sample or a blank hair sample spiked only with the internal standard; and (2) controls at three concentration levels (low, medium and high). In a rotatory analysis, the methods accuracy and the precision were confirmed. The external quality control was analyzed as a routine sample, without any previous washing.

Internal quality controls

During this period, different quality controls were performed at three different concentration levels: low (LOQ), medium, and high (ULOQ) of the method. Results obtained over time presented reasonable dispersion values and bias throughout the studied period. These results are given as Supporting information (Figs. 1S– 3S). In all over the last fifteen months, it can be observed that, performing at least (n > 40) for each level, we did not observe any tendency or significant bias.

External quality controls: proficiency test

After the validation and routine implementation of the method, the results obtained in the different inter-laboratories analysis were satisfactory, with a maximum value of z-score around 1, which was acceptable according to the organizer (Arvecon). The acceptation criteria were z-score ≤ 2, while when z-score ≥ 2 and ≤ 3, the result was questionable. If z-score >3 it is an outlier.

The results of all the proficiency tests for ketamine and norketamine (n = 4 in this period fifteen months) were accepted by the evaluator (Arvecon) with a z-score between 1.18 and 0.68 and an accuracy for the target error (%) between 19% and 10%, as shown in Table 4. Different types of the pre-analytical phase were compared in their results, in addition to the hydrolysis of the validated method, ultrasonic and other sample preparations.

In addition, it should be highlighted that only less than one third of the participant laboratories reported a quantitative or qualitative result to the norketamine metabolite (28%), despite than almost two thirds of the laboratories showed these results for ketamine (61%).

Table 4. Proficiency tests and GTFCh drugs in hair (Arvecon).

PT (Arvecon)	Compound	Target value (ng/mg)	Test result (ng/mg)	Accuracy error (%)	Evaluation of organization PT			Number of labs		
					Range accepted (ng/mg)	Z-score	Pass	Total N	Testing n	Outlier n
DHF 2/17	Ketamine	2.05	2.09	2	1.45– 2.65	0.13	Yes	68	41	1
DHF 3/17		0.88	0.71	−19	0.592–1.168	−1.18		74	48	1
DHF 1/18		2.22	2.44	10	1.58– 2.86	0.68		80	48	2
DHF 2/18		1.65	1.53	−7	1.15– 2.15	−0.48		73	43	1
DHF 2/17	Norketamine	0.53	0.57	8	0.340–0.712	0.47	Yes	68	19	1
DHF 3/17		0.68	0.57	−16	0.446–0.910	−0.93		74	21	0
DHF 1/18		0.49	0.48	−2	0.312–0.660	0.6		80	22	4
DHF 2/18		0.562	0.51	−9	0.364–0.76	−0.52		73	22	0

This type of Proficiency Test material (which is certified reference material in matrix) is very valuable it allows to check the correct construction of new calibration curves with another batch independent of the reference materials used for calibration.

Application of the method in judicial hair samples

Due to the adequate analytical performance of the validated method using ketamine and norketamine standards, this method was applied for determining those drugs in real hair samples from forensic cases. An evaluation of the results reported in the INTCF workgroup, since July 2017 to October 2018 (15 months) are discussed below. In this period, 1664 judiciary matters that contained hair samples were analyzed and N = 1189 cases (71.5%) were particularly tested for ketamine. All those data are summarized in Table 5. Interestingly, 62 samples were positive for ketamine, which represents the 5.2% of the total.

Comparing previous data from the same laboratory (INTCF) in 2015 [10], AMP (23.4%) and MDMA (19.6%) consumption maintains the previous trend, while the detected consumption of ketamine increases with this validated method, from 0.7% to 5.2%. The presence of MDA is due to being incorporated as a metabolite of MDMA.

Table 6 presents detailed information of the 62 samples that resulted positive for ketamine. Concentrations for ketamine and norketamine lower than 0.5 ng/mg and 0.1 ng/mg, respectively, were not reported or listed in the report because they were lower than LOQ. Concentrations higher than 50 ng/mg and 10 ng/mg, for ketamine and norketamine respectively, were reported higher than ULOQ. The poly-consumption of ketamine with

different drugs as MDMA, amphetamine and methamphetamine has been also studied.

The medians (ng/mg) obtained for their respective concentrations were 2.65 (ketamine) and 0.77 (norketamine) and 0.152 (ratio). The mode obtained for their respective ranges were 1–2 ng/mg ketamine and 0.1–0.5 ng/mg norketamine, and for the ratio between them was 0.05–0.1, as shown in Fig. 2. The average value of the NK/KT (0.235) agrees to those reported in the literature (0.14–0.56) [20]. Although, this comparison is somewhat limited since up to ten different studies were carried out with different

Table 5. Positive (n) for amphetamines (amphetamine, methamphetamine, MDA, MDMA y MDEA) and ketamine consumption in judicial hair samples. Samples analyzed N = 1189.

	Amphetamines				Ketamine	Norketamine
	AMP	MAMP	MDMA	MDA	KT	NK
Samples analyzed (N)	1189	1189	1189	1189	1189	1189
Positive (n)	258	10	224	179	62	62
Positive (%)	21.7	0.8	18.8	15.5	5.2	5.2

number of cases, from n = 1 to n = 51. The frequency distribution of the positive consumption cases is shown in Fig. 2, for Ketamine (A), norketamine (B) and norketamine/ketamine ratio (C).

The results obtained in the INTCF (median values of 2.65 and

0.77 ng/mg for ketamine and norketamine, respectively) are fully comparable with those reported by Salomone et al. [20] (median values of 0.26 and 2.75 ng/mg for ketamine and 0.04–0.25 ng/mg for norketamine), taking into consideration that their analysis were

done on a very different population in type (driving relicensing), years (2013–2014), country (Italy), and number of cases. The age range of the drug users (in n = 8) was between 17– 32 years, and their age average was approximately 24 years, which is similar to our results shown in Table 6.

The validated method of this work was successful and it was implemented in the INTCF routine. Table 7 shows a comparation between the results obtained previously and those obtained with the validated method, although in different periods and cases. Interestingly, it has been possible to determine more reliable values of ketamine consumption as shown in Table 7.

In a similar period, even with a greater number of samples studied with the previous method used in the INTCF, it has been able to detect about six times more positive consumption of ketamine and norketamine using the new validated method. Applying the previous methodology, only cases of high consump- tion were detected, which resulted a much less effective detection. Ketamine median changed from 18.55 to values 2.65, which was

Table 6. Statistical of the set of 62 judicial hair samples that were positive in ketamine or poly-consumption within amphetamines.

	KT	NK	NK/KT
Positive (n)	62	62	–
Results <LOQ (%)	4.8	17.7	–
Percentile 25% (ng/mg)	1.48	0.21	0.070
Median (ng/mg)	2.65	0.77	0.152 Mean (0.235)
Percentile 75% (ng/mg)	12.99	3.27	0.313
Results >ULOQ (%)	14.5	14.5	–
Mode(range ng/mg)	1–2	0.1-0.5	0.05-0.1
Age average (years)	29.5	–	–
Age median (years)	29	–	–
Age mode (years)	21–25	–	–
Mono-consumption (without amphetamines) (%)	12 (19.3%)	–	–
Poly-consumption with MDMA (%)	48 (77.4%)	–	–
Poly-consumption with AMP (%)	37 (59.7%)	–	–
Poly-consumption with MAMP (%)	3 (4.8%)	–	–
Poly-consumption with AMP + MDMA (%)	35 (56.4%)	–	–
Poly-consumption with AMP + MDMA + MAMP (%)	3 (4.8%)	–	–

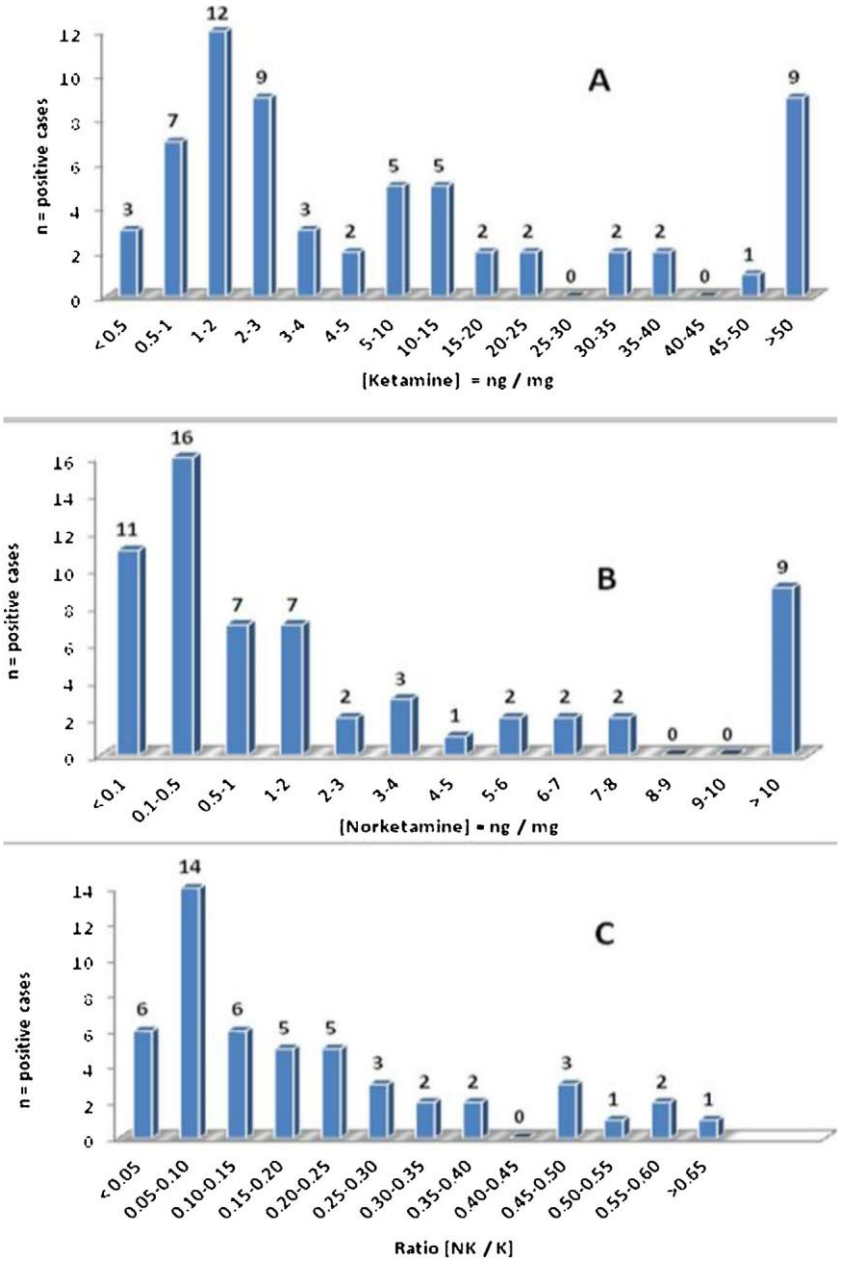

Fig. 2. Frequency distribution of positive cases versus their intervals of concentration (ng/mg) of ketamine (A), norketamine (B), and the norketamine/ketamine (NK/KT) ratio (C).

Table 7. Comparisons of analytical working methods, involving different sample pretreatment and same instrumentation for the determination of ketamine and norkeramine, applied to real judicial cases of hair analysis, in the INTCF.

Comparative	Period	Sample preparation (pretreatment)	Technique (instrumentation)	Subjects analyzed (N)	Positive subjects (n)	Ketamine median (ng/mg)	Norketamine median (ng/mg)	Ref.
Previous method	2015 (12 months)	Methanolic incubation	GC–MS	1550	10	18.55	Qualitative (positive)	[10]
Validated method ketamine/norketamine	2017–2018 (15 months)	Hidrolisis (basic digestión)	GC–MS	1189	62	2.65	0.77	Present work

probably due to only those high — consumers were detected. However, it should also be considered that the comparison comprises two different periods and can imply the change in the consumption of drugs, or different population profiles.

To evaluate whether the new method satisfies the analytical needs of the INTCF, it would have been suitable to compare the number of users of ketamine positively detected from the number of real users of ketamine, or analyze the different cases by both methods. Due to workload and efficiency in its resolution, the latter has not been possible. However, the validated method achieves a significant improvement in its detection capability making lower the real ketamine consumers not detected.

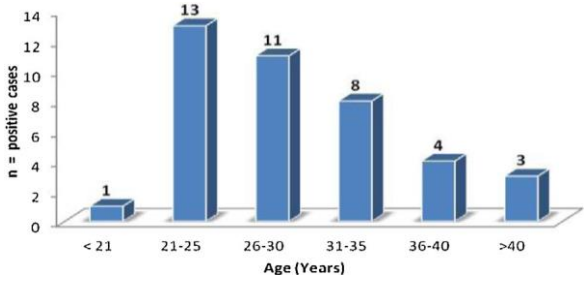

Fig. 3. Frequency distribution of intervals of age in positive cases to ketamine.

According to the data shown in Table 7, subject detection lower than 1% (0.7%) with the previous threshold has increased to more than 5%, as well as the ketamine concentration median decreases in both periods (from 18.55 to 2.65), taking into account the aforementioned limitations of this comparison.

In addition, other authors [13,15,20,11] and our experience through the different cases analyzed, suggest that this type of consumers are young consumers, poly-drug users associated with the consumption of raves or disco music, and different leisure. Fig. 3 shows the age distribution ranges of positive consumers of ketamine. The age interval between 21 and 30 years is prevalent with 24 cases of the 40 cases considered. In 22 of the cases there is no age in the analysis request form.

The age range of these consumers does not match with other consumption profiles analyzed in the INTCF laboratory, such as the consumption of cannabis, cocaine or heroin. For those drugs, the average ages are 33, 36 and 46 years, respectively [10]. While consumers of amphetamine, and especially MDMA, are closer to the average age (with 34 and 30 years, respectively), the results of methamphetamine give an average age of 30 years, although only there were 10 cases in the cited reference. Due to the similar values, both in age and consumption habits, and being contained in the same routine method of analysis, the relationship with poly-drug use of amphetamine and positive ketamine users has been evaluated. Frequency distribution of all the positive cases of

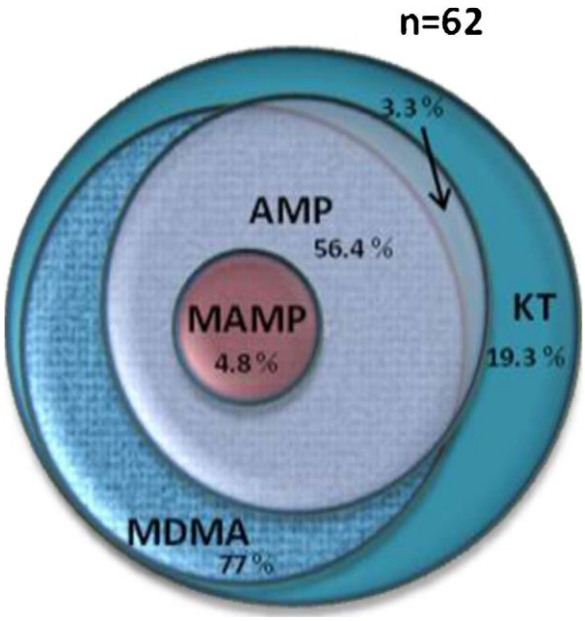

Fig. 4. Frequency distribution of all the positive cases of ketamines reported n = 62, where mono-consumption of ketamine (19.3%) is reflected as the polyconsumption of cases positives with amphetamines (80.7%).

ketamines reported (n = 62), as monoconsumption and polyconsumption with amphetamine-type stimulants is given in Fig. 4. Chronic or repetitive consumers of ketamine present a large percentage of poly-drugs use with other drugs of abuse, such as amphetamines, especially with MDMA, where there is a 77.4% of poly-consumers. On the other hand, the percentage of ketamine consume associated with methamphetamine is especially low (4.8%), but noting that it points to a poly-consumptions profile of different type of drugs of abuse. It is important to consider that part of these detected poly-consumptions might happen due to illicit preparations, as sometimes it has been observed more than one of the analyzed components, either for being structurally analogs

that come for a secondary reactions or inefficiency synthesis; or for the intentional addition of several substances, i.e. MDMA and ketamine. It should not even be rejected the possibility that in these illicit preparations (dust powder or tablet) may contain also New Psychoactive Substances (NPS). So, it is a group of consumers or population where we must be especially vigilant, to perform a retrospective analysis of NPS. Of course, it is very likely that the poly-consumption of several of the previously mentioned substances in different tablets or powders is the main cause.

CONCLUSIONS

This work has demonstrated that it is a valid method for determining ketamine, norketamine and amphetamines derivates in hair samples of forensic cases. This method was validated meeting the criteria of sensitivity and accuracy for detecting repeated consumption of ketamine according to the SWGDrug guideline [32], where the proposed cut-off for ketamine was 0.5 ng/mg [19]. The detection of norketamine allowed discriminating between active uses and external contamination.

The assessed method was applied for analyzing 1189 hair samples of judicial interest received in the INTCF along 15 months, obtaining 62 positives in ketamine consumption. This means a 5.2% of positivity for chronic consumption of ketamine. This percentage is higher than the initially expected according to the historical records of this forensic institution with the previous routine method of analysis. Therefore, this validated method satisfies the INTCF analysis needs, due to detection capability increased significantly. In addition, the accuracy and precision of the validated method have allowed to achieve better conclusions about the quantitative results and the poly-consumption, that have

enabled a better characterization of the different groups of the analyzed population, the type of case, the age and geographical area, and tendency to poly-drug use of other substances.

Ketamine consumers present a profile of young age (21–30 years old) and poly-consumption with synthetic substances, mainly MDMA and amphetamine. As consumer collective, prone to consume new psychoactive substances, requires special attention because they show a consumer profile with a higher prevalence in MDMA than amphetamine, which indicates that ketamine consumers belong to a subgroup with a different profile within the INTCF casuistry.

CRediT authorship contribution statement

J.M. Matey: Conceptualization, Formal analysis, Data curation, Investigation, Validation. M.D. Moreno de Simon: Conceptualization, Data curation, Formal analysis. C. García-Ruiz: Supervision, Writing - review & editing. G. Montalvo: Supervision, Writing - review & editing.

REFERENCES

[1] F. Pragst, M.A. Balikova, State of the art in hair analysis for detection of drug and alcohol abuse, Clin. Chim. Acta 370 (2006) 17–49.

[2] C. Jurado, M.P. Giménez, M. Menéndez, M. Repetto, Simultaneous quantifications of opiates, cocaine and cannabinoids in hair, Forensic Sci. Int. 70 (1995) 165–174.

[3] F. Musshoff, K. Lachenmeier, D. Lichtermann, B. Madea, Cocaine and opiate concentrations in hair from subjects in a heroin maintenance program in comparison to a methadone substituted group, Int. J. Leg. Med. 123 (2009) 363–369.

[4] M.J. Burgueño, A. Alonso, S. Sánchez, Amphetamines and cannabinoids testing in hair: evaluation of results from a two-year period, Forensic Sci. Int. 265 (2016) 47–53.

[5] C. Jurado Montoro, Análisis de drogas de abuso en muestras de pelo. Diagnóstico del consumo crónico, F (2004), Forensic Sci. Int. 145 (2004) 161– 166.

[6] Clemens Kirschbaum, Stresses from head to toe: steroid hormone levels in hair as markers of chronic stress, 22th Annual Meeting of Society of Hair Testing Cardiff, Walws, UK (2017).

[7] T. Kieliba, O. Lerch, H. Andresen-Streichert, M.A. Rothschild, J. Beike, Simulta- neous quantification of THC-COOH, OH-THC, and further cannabinoids in human hair by gas chromatography-tandem mass

spectrometry with electron ioniza- tion applying automated sample preparation, Drug Test. Anal. 11 (February (2)) (2019) 267–278.

[8] Elena Lendoiro, Ana de Castro, Cristian Jimenez-Morigosa, Xose A. Gomez- Fraguela, Manuel Lopez-Rivadulla, Angelines Cruz, Usefulness of hair analysis and psychological tests for identification of alcohol and drugs of abuse consumption in driving license regranting, Forensic Sci. Int. 286 (2018) 239–244.

[9] C. Jurado Montoro, T. Soriano Ramón, Limitaciones de los análisis de pelo para determinar las circunstancias que atenúan o eximen de la responsabilidad criminal, Cuad Med Forense 22 (1–2) (2016) 46–48.

[10] J.M. Matey Cabañas, S. Sanchez Perez, M.D. Moreno de Simón, A. Vicente García, Drugs of abuse in chronic consume in hair. National Institute of Toxicology and Forensic Sciences (INTCF) Spain. Results of the Drg of abuse department in 2015, 22th Annual Meeting of Society of Hair Testing Cardiff, Walws, UK (2017).

[11] E. Han, N.J. Kwon, L.Y. Feng, J.H. Li, H. Chung, Illegal use patterns, side effects, and analytical methods of ketamine, Forensic Sci. Int. 268 (2016) 25–34.

[12] D. Favretto, S. Vogliardi, M. Tucci, I. Simoncello, R. El Mazloum, R. Snenghi, Occupational exposure to ketamine detected by hair analysis: a retrospective and prospective toxicological study, Forensic Sci. Int. 265 (2016) 193–199.

[13] P. Xiang, M. Shen, X. Zhuo, Hair analysis for ketamine and its metabolites, Forensic Sci. Int. 162 (2006) 131–134.

[14] Order SAS/2712/2010, of October 13, which includes the substance Ketamine in Annex I of Royal Decree 2829/1977, of October 6, which regulates the manufacture, distribution, prescription and dispensation of psychotropic substances and preparations. Spain (Available in: https://boe.es/boe/dias/ 2010/10/21/pdfs/BOE-A-2010-16025.pdf).

[15] G. Miolo, M. Tucci, L. Menilli, G. Stocchero, S. Vogliardi, S. Scrivano, M. Montisci,

[16] D. Favretto, A study on photostability of amphetamines and ketamine in hair irradiated under artificial sunlight, Brain Sci. 8 (6) (2018) 96.

[17] Society of Hair Testing (SoHT). https://www.soht.org/. (Consulted 30/10/2018).

[18] Gesellschaft für Toxikologische und Forensische Chemie (GTFCh). https:// www.gtfch.org/cms/. (Consulted 30/10/2018).

[19] Substance Abuse and Mental Health Services Administration (SAMHSA). https://www.samhsa.gov. (Consulted 30/10/2018).

[20] A. Salomone, L. Tsanaclis, R. Agius, P. Kintz, M.R. Baumgartner, European guidelines for workplace drug and alcohol testing in hair, Drug Test. Anal. 8 (2016) 996–1004.

[21] A. Salomone, E. Gerace, P. Diana, R. Romeo, V. Malvaso, D. Di Corcia, M. Vincenti, Cut-off proposal for the detection of ketamine in hair, Forensic Sci. Int. 248 (2015) 119–123.

[22] Y.H. Wu, K.L. Lin, S.C. Chen, Y.Z. Chang, Simultaneous quantitative determina- tion of amphetamines, ketamine, opiates and metabolites in human hair by gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 887–897.

[23] M. Pieri, L. Castiglia, N. Miraglia, et al., Study of the fragmentation pattern of ketamine-heptafluorobutyramide by gas chromatography/electron ionization mass spectrometry, Rapid Commun. Mass Spectrom. 24 (2010) 49–56.

[24] H. Choi, S. Baeck, M. Jang, S. Lee, H. Choi, H. Chung, Simultaneous analysis of psychotropic phenylalkylamines in oral fluid by GC–MS with automated SPE and its application to legal cases, Forensic Sci. Int. 2011 215 (2012) 81–87.

[25] K.W. Leung, Z.C.F. Wong, J.Y.M. Ho, et al., Determination of hair ketamine cut- off value from Hong Kong ketamine users by LC–MS/MS analysis, Forensic Sci. Int. 2015 259 (2016) 53–58.

[26] Y.H. Wu, Kl. Lin, S.C. Chen, Y.Z. Chang, Integration of GC/EI-MS and

GC/NCI-MS for simultaneous quantitative determination of opiates, amphetamines, MDMA, ketamine, and metabolites in human hair, J. Chromatogr. B 870 (2008) 192–202.

[27] Y.H. Wu, K.L. Lin, S.C. Chen, Y.Z. Chang, Simultaneous quantitative determina- tion of amphetamines, ketamine, opiates and metabolites in human hair by gas chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 887–897.

[28] P. Xiang, Q. Sun, B. Shen, M. Shen, Disposition of ketamine and norketamine in hair after a single dose, Int. J. Leg. Med. 125 (2011) 831–840.

[29] A. Salomone, G. Gazzilli, D. Di Corcia, E. Gerace, M. Vincenti, Determination of cathinones and other stimulant, psychedelic, and dissociative designer drugs in real hair samples, Anal. Bioanal.Chem. 408 (2016) 2035–2042.

[30] J.Y. Kim, S.H. Shin, J.I. Lee, M.K. In, Rapid and simple determination of psychotropic phenylalkylamine derivatives in human hair by gas chromatography–mass spectrometry using micro-pulverized extraction, Forensic Sci. Int. 2009 196 (1) (2010) 43–50.

[31] D. Favretto, S. Vogliardi, G. Stocchero, A. Nalesso, M. Tucci, C. Terranova, S.D. Ferrara, Determination of ketamine and norketamine in hair by micro- pulverized extraction and liquid chromatography–high resolution mass spectrometry, Forensic Sci. Int. 226 (1) (2013) 88–93.

[32] Society of Forensic Toxicologists/American Academy of Forensic Sciences (SOFT/AAFS), Forensic Toxicology Laboratory Guidelines, (2006) .

[33] Scientific Group For Forensic Toxicology (SWGTOX), Scientific Working Group For Forensic Toxicology (SWGTOX), Standard practices for method validation in forensic toxicology, J. Anal. Toxicol. 37 (2013) 452–474.

[34] M. Concheiro, M. Castaneto, R. Kronstrand, M.A. Huestis, Simultaneous determination of 40 novel psychoactive stimulants in urine by liquid chromatography–high resolution mass spectrometry and library matching, J. Chromatogr. A 1397 (2015) 32–42.

[35] Bertil Magnusson, Teemu Näykki, Håvard Hovind, Mikael Krysell, NT technical report. Handbook for Calculation of Measurement Uncertainty in Environ- mental Laboratories. TR 537 Ed. 3.1. Approved 2012-11.

[36] W. Horwitz, Evaluation of analytical methods used for regulation of foods and drugs, Anal. Chem. 54 (1) (1982) 67A–76A.