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Short communication

Detection of ketamine and its metabolites in urine by ultra high pressure liquid chromatography–tandem mass spectrometry

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ABSTRACT

Current analytical methods used for screening drugs and their metabolites in biological samples from victims of drug-facilitated sexual assault (DFSA) or other vulnerable groups can lack sufficient sensitivity. The application of liquid chromatography, employing small particle sizes, with tandem mass spectrometry (MS/MS) is likely to offer the sensitivity required for detecting candidate drugs and/or their metabolites in urine, as demonstrated here for ketamine. Ultra-performance liquid chromatography–mass spectrometry (UPLC–MS/MS) was performed following extraction of urine (4 mL) using mixed-mode (cation and C8) solid-phase cartridges. Only 20 μL of the 250 μL extract was injected, leaving sufficient volume for other assays important in DFSA cases. Three ion transitions were chosen for confirmatory purposes. As ketamine and norketamine (including their stable isotopes) are available as reference standards, the assay was additionally validated for quantification purposes to study elimination of the drug and primary metabolite following a small oral dose of ketamine (50 mg) in 6 volunteers. Dehydronorketamine, a secondary metabolite, was also analyzed qualitatively to determine whether monitoring could improve retrospective detection of administration. The detection limit for ketamine and norketamine was 0.03 ng/mL and 0.05 ng/mL, respectively, and these compounds could be confirmed in urine for up to 5 and 6 days, respectively. Dehydronorketamine was confirmed up to 10 days, providing a very broad window of detection.

1. Introduction

Ketamine [2-(2-chlorophenyl)-2-methylaminocyclohexan-1-one] (Fig. 1) is a N-methyl-D-aspartate receptor antagonist used legitimately for its dissociative anaesthetic properties [1,2]. Recreational use of ketamine has gained popularity worldwide over the last 10 years [3,4], with users experiencing hallucinations and a cataleptic state called the 'K-hole' [5,6].

The cataleptic state brought on by administration of ketamine can also produce stupor and sedation, that together with possible amnesia and difficulty in fighting off an assailant, has led to its recent implication in drug-facilitated sexual assault (DFSA) [7,8]. DFSA incidents are often reported later than 24 h after the alleged assault, when very little drug will remain in the victim. Similarly, late samples are often collected from children where suspicion of non-accidental poisoning may only come to light after routine clinical evaluation has been performed. This has led to several groups reporting the use [9,10] and need [11,12] for highly sensitive assays that can provide sufficient retrospective detection for ketamine together with other classes of fast acting sedative drugs.

Many procedures have been described for detecting ketamine and its active metabolite norketamine in human urine, with varying limits of detection, ranging from 0.5 ng/mL to 25 ng/mL for both compounds [13–19] but only one group describe a method with an LOD of 0.05 ng/mL for norketamine, which was used to assay samples following intravenous infusion of ketamine for therapeutic purposes [13]. Despite these investigations on the analysis of ketamine, there is a paucity of data regarding the urinary elimination of ketamine and norketamine following oral administration.

We developed a highly sensitive quantitative assay for ketamine and its primary metabolite, norketamine, in urine and evaluated its applicability for forensic purposes by performing a low-dose elimination study. Dehydronorketamine was not available as a standard at the time of investigation but as Cheng et al. [20] indicated that it may be an important diagnostic marker, we chose to perform qualitative analysis for this metabolite.

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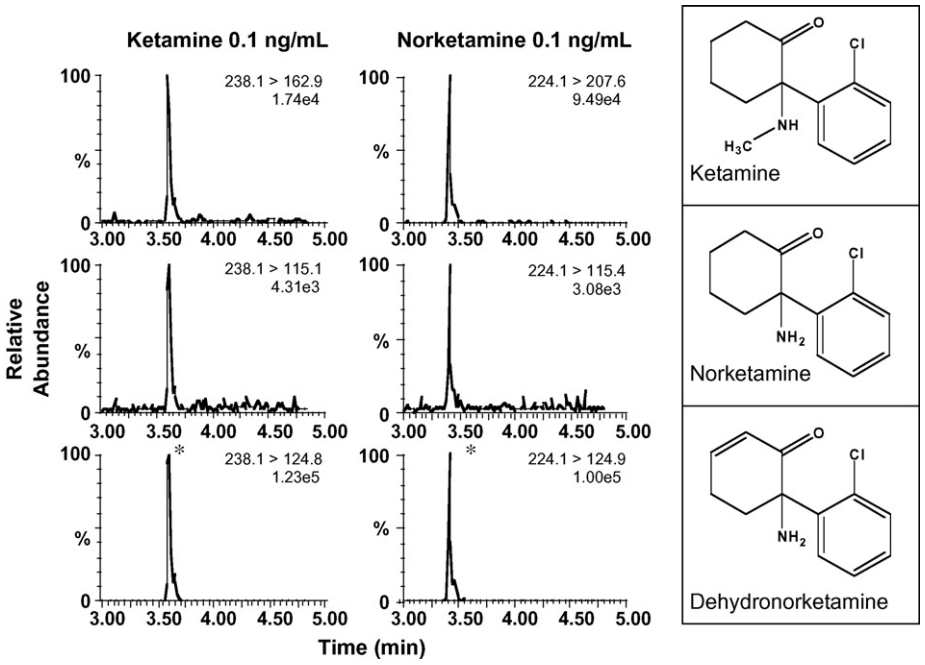

Fig. 1. Selected ion chromatograms from a extracted urine calibrant of the quantifying (*) and two qualifying ions of ketamine and norketamine at 0.1 ng/mL. This concentration represents the limit of quantification for these analytes. The structures for ketamine and its metabolites norketamine and dehydronorketamine are also shown.

2. Experimental

2.1. Chemicals, standards and reagents

Ketamine hydrochloride was obtained from Sigma, UK (purity >99%, w/w). Primary methanolic certified standard solutions of (±)-norketamine hydrochloride (as the free base; 1 mg/mL in methanol; 99%, w/w), (±)-ketamine-d_4 hydrochloride (as the free base; 100 μg/mL in methanol; 98%, w/w) and (±)-norketamine-d_4 hydrochloride (as the free base; 100 μg/mL in methanol; 99%, w/w) were obtained from LGC Promochem, UK. Synthesized dehydronorketamine was kindly donated as a gift from the National Bureau of Controlled Drugs, Department of Health, Taiwan. The dehydronorketamine was not pure and contained around 40% ketamine (w/w). Analytical grade potassium hydroxide, potassium dihydrogen orthophosphate, HPLC grade acetonitrile, dichloromethane and propan-2-ol were obtained from Fisher Scientific, UK. High purity analytical grade formic acid (98–100%, v/v), acetic acid and 25% (v/v) ammonia solution were obtained from BDH. Ultra pure water was prepared on an Elga Purelab Maxima HPLC water purification system supplied by Elga.

2.2. Calibrators and quality control samples

Seven urine calibrators containing ketamine and norketamine (as the free base) at the following concentrations: 0.0 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 10.0 ng/mL, 50.0 ng/mL and 100.0 ng/mL were prepared each day of analysis using drug-free donor urine. The 0.0 ng/mL calibration standard was used only to check for interferences in the drug-free urine and was not part of the calibration curve. Quality control (QC) working solutions of ketamine and norketamine (0.4 ng/mL, 4.0 ng/mL and 40.0 ng/mL, as the free base) were stored at −40 °C until required. A working internal standard solution (100 ng/mL) of deuterated ketamine and norketamine in water was prepared fresh daily from deuterated primary stock solutions.

2.3. Volunteer study administration specimens

Six healthy (3 male, 3 female), non-drug-using, volunteers aged 20–27 years were recruited. Approval by the Research Ethics Committee of King's College London and informed written consent was obtained in accordance with our institutional procedures. Each volunteer was initially screened for good general health. No volunteer was receiving any drug treatment and had not recently been involved in other drug studies.

Subjects were administered a small (50 mg) oral dose of a pharmaceutical preparation of ketamine (Ketalar®, Pfizer) with water. Urine samples were collected over 2 h periods from 0 h, 2 h, 4 h, 6 h, 8 h and then spot collections at exactly 12 h, 24 h, 30 h and at day 2 (48 h), 3 (72 h), 4 (96 h), 5 (120 h), 6 (144 h), 7 (168 h), 8 (192 h), 9 (216 h) and 10 (240 h). Specimens were kept frozen until analysis.

2.4. Sample preparation and extraction procedure

6 mL of phosphate buffer (10 mmol/L; pH 6) with 200 μL of the working internal standard solution were mixed with 4 mL of each of the calibrators, quality controls and volunteer study urine samples. Varian (Oxford, UK) Bond Elut Certify I mixed-mode (cation exchange and C8) solid-phase extraction (SPE) cartridges (130 mg) were preconditioned by slowly passing under vacuum 2 mL of propan-2-ol followed by 2 mL of phosphate buffer. The buffered urine samples were loaded onto SPE cartridges and allowed to slowly pass through the bed without vacuum. Empty urine tubes were washed with 1 mL of phosphate buffer and this wash was also added to the cartridge. Loaded SPE cartridges were then rinsed sequentially with 2 mL of water and 2 mL of the acetate buffer (0.1 mol/L; pH 4) slowly under vacuum. Retained drug and metabolites were eluted under gravity with 4 mL (added 1 mL at a time) dichloromethane/propan-2-ol/ammonium hydroxide (78:20:2, v/v). The resulting eluent was evaporated under nitrogen at 60 °C in a Zymark TurboVap LV Evaporator (Cheshire, UK). The dried extracts were reconstituted in

250 μL of water/acetonitrile/formic acid (89.9:10:0.1, v/v) and vortex mixed for 30 s before transferral to a chromatographic vial. A 20 μL portion of each extract was injected into the chromatographic system incorporating an ultra-performance liquid chromatography (UPLC) column coupled to a triple-quadrupole mass spectrometer.

2.5. HPLC (UPLC) conditions

Separation was performed on a Waters (Hertfordshire, UK) Acquity UPLC system using a 150 mm × 2.1 mm i.d., 1.7 μm particle size BEH C_{18} column maintained at a temperature of 45 °C. Gradient separation was performed with a binary mobile phase consisting of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 350 μL/min. The gradient used was as follows: initial 10% B for 30 s, increased to 50% B in 7 min, decreased to 10% B over 30 s. The total run time was 8 min.

2.6. MS detection

Detection was performed on a Waters Premier triple-quadrupole mass spectrometer with electrospray ionization working in positive ionization mode. The following optimized conditions were used: spray voltage of 2.8 kV; source temperature of 80 °C; desolvation temperature of 300 °C. The gas flow for desolvation was set at 900 L/h (nitrogen) and the cone gas was set at 10 L/h (nitrogen). Argon with a pressure of 3.5×10^{-3} mbar was used as the collision cell gas. All analytes and deuterated internal standards were monitored using selected reaction monitoring (SRM) and Table 1 lists the SRM transitions and LC–MS/MS instrumental conditions. The three most abundant transitions were optimised for confirming the presence of ketamine, norketamine and dehydronorketamine. The purity of the dehydronorketamine was unknown so quantitative analysis was restricted to ketamine and norketamine. Quantification was performed by calculating the peak area ratio of the product ion for ketamine (m/z 124.8) to its tetra-deuterated analogue (m/z 128.8) and likewise for norketamine (m/z 124.9) to norketamine-d_4 m/z 211.0), in calibrants, constructing a calibration line and then interpolating unknowns. The ion at m/z 211.0 was selected for norketamine-d_4 as this was slightly more abundant then the fragment at m/z 128.8. To assist with analyte identification, ion abundance ratios for ketamine and norketamine were measured relative to their respective internal standard ion transitions, the ion ratios for dehydronorketamine were measured relative to the norketamine-d_4 transition. The values obtained from a portion of the study specimens from the six volunteers were compared to calibration standards containing similar analyte concentrations and the results were evaluated according to accepted guidelines [21].

2.7. Validation procedures for quantification of ketamine and norketamine in urine using UPLC–MS

Selectivity, specificity, linearity, limit of detection, limit of quantitation, precision, recovery and dilution integrity for ketamine and its primary metabolite norketamine were evaluated according to accepted guidelines [21,22]. Specificity for each analyte was evaluated by analyzing 12 blank urine samples, which were obtained from healthy volunteers who had not taken any ketamine and were fortified with deuterated internal standards prior to analysis.

Assay linearity for ketamine and norketamine was determined by constructing calibration curves from urine to which no or six different concentrations of ketamine and norketamine and the deuterated internal standards had been added. A linear regression model with a weighting function of $1/x$ was applied. Quantitative accuracy for each calibrator was required within 20% of target and linearity should be achieved with $r^2 \geq 0.995$.

Limit of detection (LOD) and limit of quantification (LOQ) for ketamine and norketamine were evaluated. The LOD was based on the presence of the quantifying ion with satisfactory chromatography together with acceptable retention time (within 1% of average calibrator time) and was defined as the lowest concentration with a mean signal to noise ratio ≥3 based on the peak height of all three ion transitions. LOQ for each analyte was defined as the lowest concentration in the calibration curve at which the mean analyte peak height to baseline noise ratio was 10 for the quantifying ion and at least 3 for the 2 qualifying ions.

The intra-day ($n = 12$) and inter-day ($n = 3$) precision and accuracy of the assay was determined using three QC samples containing ketamine and norketamine at low, medium and high concentrations (0.4 ng/mL, 4.0 ng/mL and 40.0 ng/mL).

Matrix effects that can cause ion suppression or enhancement were investigated by injecting 5 different sources of blank urine into the LC–MS/MS whilst aqueous standard solutions (50 μg/mL) containing either ketamine or norketamine were infused into the electrospray source, via a connecting mixing tee, using the built in Harvard syringe pump on the mass spectrometer at a flow rate of 10 μL/min.

Recovery for ketamine and norketamine by SPE was determined by comparing two sets of urine samples. One set consisted of three urine samples with low, medium and high concentrations (0.5 ng/mL, 5.0 ng/mL and 50.0 ng/mL) of ketamine, norketamine and dehydronorketamine added before extraction, evaporation and reconstitution with mobile phase. The second set consisted of blank urine treated in the same manner, except that the equivalent concentrations of the two analytes were added prior to LC–MS/MS analyses following extraction and evaporation. Recovery, as a percentage, was calculated by comparing the absolute signal intensities of the urine set with the analytes added prior to extraction and evaporation with the set with the analytes added following extraction and evaporation.

Dilution integrity was determined as it was expected that some of the early urine collections (2 h, 4 h and 6 h) from the volunteer study would exceed the upper calibration standard. Urine samples containing 1 μg/mL and 2 μg/mL ($n = 3$) of ketamine and norketamine were diluted 20-fold and 40-fold, respectively, with blank urine. Deviations from the nominal concentrations were calculated.

Potential carryover was investigated by injecting aqueous blank samples following the injection of standards used for the calibration curve and the QC samples.

2.8. Qualitative analysis of dehydronorketamine in urine

A solution containing approximately 10 μg/mL was prepared and infused directly into the mass spectrometer to obtain the instrumental conditions outlined in Table 1. The total chromatogram showed one major peak along with a minor one corresponding to ketamine at 8% of the peak height of dehydronorketamine. To confirm that the major peak was consistent with that of dehydronorketamine a MS/MS scan was performed (m/z 222.1 precursor) yielding a spectrum consistent with what was expected for dehydronorketamine. A solution of approximately 1 ng/mL of dehydronorketamine in urine was prepared and injected onto the chromatographic system and the same gradient elution for ketamine and norketamine was used to obtain the retention time data shown in Table 1.

Table 1
Retention times, chosen transitions and MS conditions for selected analytes.

Compound	Retention time (min)	SRM transition[a] (*m*/*z*)	Cone (V)	Collision energy (V)	Dwell (s)
Ketamine	3.59	**238.1 → 124.8**	28.0	29.0	0.10
		238.1 → 115.1	28.0	47.0	0.10
		238.1 → 162.9	28.0	26.0	0.10
Ketamine-d_4	3.58	**242.2 → 128.8**	27.0	28.0	0.10
Norketamine	3.44	**224.1 → 124.9**	25.0	16.0	0.10
		224.1 → 115.4	25.0	42.0	0.10
		224.1 → 207.6	25.0	27.0	0.10
Norketamine-d_4	3.41	**228.2 → 211.0**	22.0	29.0	0.10
Dehydronorketamine	2.95	222.1 → 141.2	23.0	33.0	0.10
		222.1 → 114.8	23.0	45.0	0.10
		222.1 → 204.8	23.0	18.0	0.10

[a] Ion transitions used for quantification are in bold.

Table 2
LOD, LOQ and linearity.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Calibration range (ng/mL)	Calibration equation[a]	r^2[a]
Ketamine	0.030	0.1	0.1–100	$y = 0.348(0.0063)x - 0.0274(0.0402)$	0.997(0.004)
Norketamine	0.050	0.1	0.1–100	$y = 0.300(0.0369)x - 0.0189(0.0138)$	0.998(0.002)

[a] $n = 3$, data are mean (SD).

3. Results and discussion

3.1. UPLC–MS/MS

Details of the retention times, chosen transitions and MS conditions for selected analytes are given in Table 1. Fig. 1 shows the selected ion monitoring chromatograms of the quantifying and two confirmation ion transitions from a 0.01 ng/mL extracted urine standard solution for ketamine and norketamine, clearly showing a S/N ratio $\gg$ 10. Total analysis time was 8 min and all three analyte peaks were baseline separated with very sharp peaks (4 s average at half peak height).

3.2. UPLC–MS/MS method validation for ketamine and norketamine

Urine samples from twelve healthy volunteers that had not taken ketamine showed no interfering ions above baseline noise, indicating acceptable method specificity for ketamine and norketamine.

Linearity for ketamine and norketamine over the calibration range gave correlation coefficients (r^2) $\geq$0.997 (Table 2). Limits of detection for ketamine and norketamine were very low at 0.03 ng/mL and 0.05 ng/mL, respectively, and limits of quantification were 0.1 ng/mL for both analytes.

Accuracy and precision data for ketamine and norketamine analysis are summarized in Table 3. Within and between run precision for both ketamine and norketamine were <12% and accuracy ranged from 83% to 104% for ketamine and from 82% to 103% for norketamine.

Average extraction recovery of analytes by SPE was 72% and 65% at 0.1 ng/mL, 70% and 61% at 1 ng/mL, 68% and 62% at 50 ng/mL for ketamine and norketamine, respectively.

The mean percentage deviation from the nominal concentration for the 20-fold dilution of ketamine and norketamine was 7.4 ± 3.9% (mean ± RSD, $n = 3$) and 10.4 ± 6.2% (mean ± RSD, $n = 3$), respectively. For the 40-fold dilution of ketamine and norketamine the mean percentage deviation was 15 ± 0.6% and 3.1 ± 0.7%, respectively. Volunteer study urine samples that were expected to have a concentration greater than the highest calibrant were diluted 20-fold.

We chose to use UPLC for chromatographic separation over conventional HPLC as we observed it gave a 2–3-fold increase in sensitivity compared to HPLC. These modest gains in sensitivity are similar to results obtained by others using this approach for small molecule analysis [23,24]. This methodology employing UPLC–MS is easily transferable to HPLC–MS and thus could aid other toxicology or forensic laboratories in the detection of compounds that are implicated in DFSA type cases that do not have access to UPLC equipment.

Over the past 30 years a large number of analytical methods for the detection of ketamine in human biological samples have been developed. Recently, sensitive GC–MS and LC–MS/MS methods have been developed [13–17,19,20,25]. To the best of our knowledge this is the first validated UPLC–MS/MS method for the detection and quantitation of ketamine and norketamine in urine

Table 3
Intra and inter assay CVs for urine QCs enriched with ketamine and norketamine.

Analyte	Actual concentration (ng/mL)	Day 1 within run (mean, ng/mL) CV (%) ($n = 12$)	Day 2 within run (mean, ng/mL) CV (%) ($n = 12$)	Day 3 within run (mean, ng/mL) CV (%) ($n = 12$)	Between run (mean, ng/mL) CV (%) ($n = 3$)
Ketamine	40	(35.7), 4.1	(41.7), 5.8	(36.2), 2.1	(37.8), 8.8
	4	(3.4), 10.0	(3.6), 3.6	(3.4), 12.1	(3.5), 3.3
	0.4	(0.33), 4.2	(0.41), 5.3	(0.39), 6.5	(0.38), 11.1
Norketamine	40	(37.7), 6.8	(32.6), 2.2	(33.4), 3.8	(34.6), 7.9
	4	(3.8), 9.8	(3.3), 9.9	(3.5), 6.2	(3.6), 7.1
	0.4	(0.39), 14.6	(0.38), 9.4	(0.41), 8.1	(0.39), 3.8

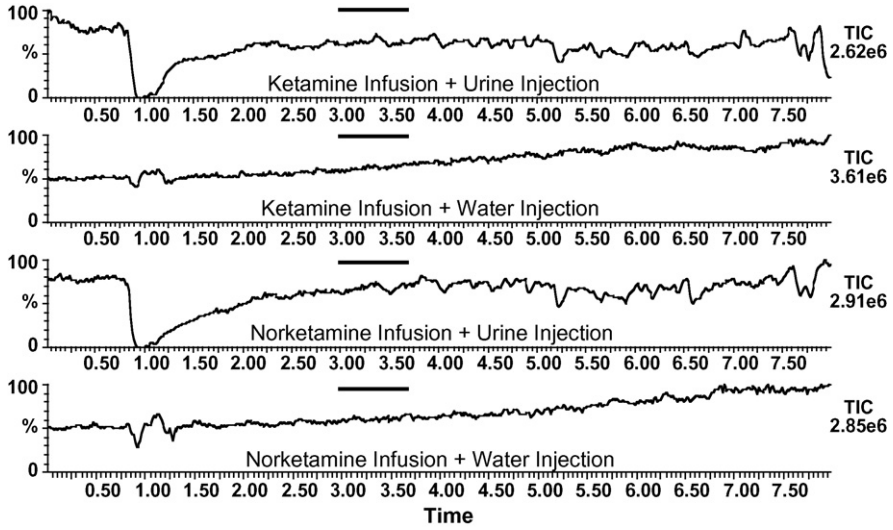

Fig. 2. Ion suppression profiles for ketamine and norketamine following the injection of an extracted blank urine sample and water. The area below the black line is the region that the analytes elute.

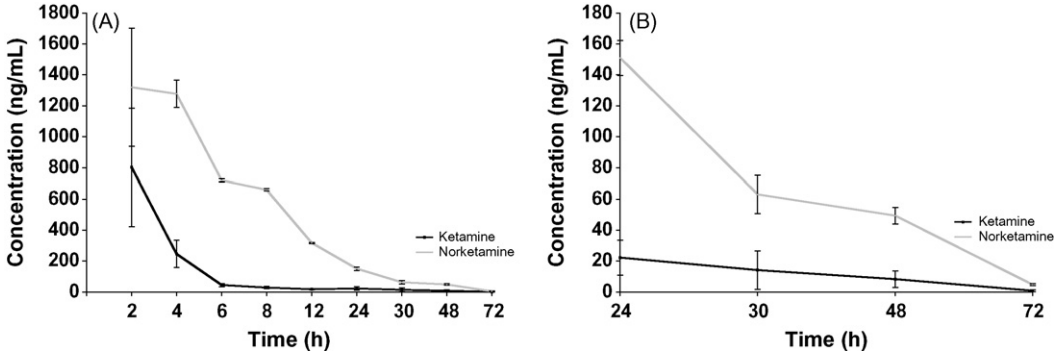

Fig. 3. (A) Concentration of ketamine and norketamine in urine from the six volunteers collected over the first 72 h. (B) The later samples from A and on a larger scale.

as well as the most sensitive analytical method for both of these analytes to date. Intra-assay accuracy and precision results were satisfactory with CVs < 15% and accuracy data for high, medium and low concentrations within 20% of target. Furthermore, linearity was achieved over three orders of magnitude.

Ion suppression can have adverse effects on quantitative data in LC–MS analysis [26]. Post column infusions of ketamine and norketamine showed no ion suppression in terms of a change in signal intensity when 5 blank urine samples were injected. The signal intensities were compared to an injection of purified water and the intensities were similar as shown by the example in Fig. 2.

No considerable loss was observed in the mixed mode SPE sample extraction procedure. A relatively large portion of urine (4 mL) was used for extraction but since only 20 μL of the 250 μL from the reconstituted phase was injected on column then the rest could be injected for other LC–MS/MS assays (i.e. to include other compounds implicated in DFSA cases).

3.3. Volunteer study

The average urine concentrations of ketamine and norketamine are displayed in Fig. 3, showing the relatively rapid elimination of ketamine. Samples collected after 72 h post-administration contained ketamine and norketamine at concentrations less than 1 ng/mL. Even so, our method could detect the parent drug between 3 and 5 days and norketamine between 4 and 6 days when applying strict LOD conditions as shown in Table 4 (all three ion transitions mean signal to noise ratio ≥3:1).

The relative ion ratios for the volunteer study specimens were compared to urine calibrants containing similar concentrations. Specimens near the limit of detection, containing 0.1 ng/mL or less of ketamine, were compared to the 0.1 ng/mL calibrant and the ratios were found to match to within ±27% ($n = 8$). Specimens near the middle of the calibration curve containing between 1 ng/mL and 50 ng/mL ketamine were compared to the 50 ng/mL calibrant and these values were within ±33% ($n = 12$). Specimens between 50 ng/mL and 100 ng/mL ketamine were compared to the 100 ng/mL calibrant and were within ±35% ($n = 8$). For norketamine,

Table 4
Detection period for ketamine and metabolites in volunteers.

	Ketamine maximum time detected[a] (days)	Norketamine maximum time detected[a] (days)	Dehydronorketamine maximum time detected[a] (days)
Volunteer 1	3	5	10
Volunteer 2	3	4	6
Volunteer 3	3	5	10
Volunteer 4	3	6	6
Volunteer 5	4	6	7
Volunteer 6	5	5	7

[a] Maximum time detected based on all three ion transitions present at three times the signal to noise.

specimens near the limit of detection containing 0.1 ng/mL or less were compared to the 0.1 ng/mL calibrant and the ratios matched to within ±33% (n = 12). Specimens near the middle of the calibration curve containing between 1 ng/mL and 50 ng/mL norketamine were compared to the 50 ng/mL calibrant and these values were within ±5% (n = 12). Specimens between 50 ng/mL and 100 ng/mL norketamine were compared to the 100 ng/mL calibrant and were within ±33% (n = 12). The SOFT/AAFS Forensic Laboratory Guidelines recommend ion ratios for LC–MS analysis to be within ±30% and most of our values are close to this range. Based on the volunteer study specimens where ketamine is known to have been administered, a less rigid but more realistic acceptance limit of ±35% should be considered for the positive identification of ketamine and norketamine.

Monitoring dehydronorketamine increased the retrospection of detection for the six volunteers further. This metabolite appears to be excreted in urine for a longer duration than ketamine or norketamine. Applying the strict LOD conditions, dehydronorketamine could be detected up to 10 days in two out of the six volunteers and up to a week in the remaining samples.

These data indicate that dehydronorketamine is also a useful diagnostic metabolite, given that it can be detected in urine for about 1 week after the administration of a modest dose of ketamine, using the approach we developed. We have been informed that dehydronorketamine is to become available as a reference standard (Ceriliant, USA), and this should help underpin its identification for evidential purposes.

4. Conclusions

In conclusion the results of this investigation demonstrate that the targeting of selected metabolites of ketamine can be particularly useful in evidential analysis, such as in investigations concerning DFSA. Data following oral administration of ketamine has not been previously reported. We recommend that laboratories performing analytical toxicology consider adding norketamine and dehydronorketamine to their drug screens employing LC–MS/MS.

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