1) Title Page

Use of Human Microsomes and Deuterated Substrates; An Alternative Approach for the Identification of Novel Metabolites of Ketamine by Mass Spectrometry

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2) Running title page

**Running title:** ketamine metabolite identification by mass spectrometry

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**Nonstandard abbreviations:** LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SPE, solid-phase extraction; UGT, UDP-glucuronosyltransferase; UPLC, ultra performance liquid chromatography; HPLC, high performance liquid chromatography; SRM, selected reaction monitoring; eV, electron volt; RF, radio
frequency; FT-ICR-MS, fourier transform-ion cyclotron-mass spectrometry; NMR, nuclear magnetic resonance
3) Abstract

**Abstract**

*In vitro* biosynthesis using pooled human liver microsomes was applied to help identify *in vivo* metabolites of ketamine by LC-MS/MS. Microsomal synthesis produced dehydronorketamine, seven structural isomers of hydroxynorketamine, and at least five structural isomers of hydroxyketamine. To aid identification, stable isotopes of the metabolites were also produced from tetra-deuterated isotopes of ketamine or norketamine as substrates. Five metabolites (three hydroxynorketamine and two hydroxyketamine isomers) gave chromatographically resolved components with product ion spectra indicating the presence of a phenolic group, phenolic metabolites being further substantiated by selective liquid-liquid extraction following adjustments to the pH. Two glucuronide conjugates of hydroxynorketamine were also identified. Analysis by LC-coupled ion cyclotron resonance mass spectrometry gave unique masses in accordance with the predicted elemental composition. The metabolites, including the phenols, were subsequently confirmed to be present in urine of subjects following oral ketamine administration, as facilitated by the addition of deuterated metabolites generated from the *in vitro* biosynthesis. To our knowledge, phenolic metabolites of ketamine are here reported for the first time, including an intact glucuronide conjugate. The use of biologically-synthesized deuterated material for use as an internal chromatographic and mass spectrometric marker is a viable approach to aid the identification of metabolites. Those metabolites that are particularly of diagnostic value can be selected as candidates for chemical synthesis of standards.
4) Introduction

Introduction

The development of procedures in analytical, clinical and forensic toxicology is usually restricted to compounds that are commercially available as analytical standards. As many drugs are extensively metabolised, it is desirable to target metabolites in the hope of improving sensitivity and specificity of suspected drug ingestion. Custom synthesis of metabolites of interest, known or putative, to aid drug metabolism studies, can be prohibitively expensive. Alternatively, in-house synthesis of metabolites in laboratories dedicated to analysis is usually hindered by a lack of expertise and equipment. The use of human microsomes to produce metabolites on a small-scale, which can then be characterised, may offer a simple, rapid and economical solution. Whereas this strategy is no substitute for authenticated chemically synthesised material, it does offer an important intermediate solution until such material becomes commercially available. In addition, it provides the prospect of using analogues of the drug as a substrate, particularly those labelled with stable isotopes that can then be converted to labelled metabolites suitable for use as internal ‘standards’ in analysis by mass spectrometry.

Ketamine was chosen as a model substrate as not all of its reference metabolites are available. In addition, having been used as an anaesthetic since the 1960s, its uses are widespread and include veterinary medicine and paediatrics. Recreationally, it is used as a ‘club drug’ (Degenhardt et al., 2005; Kenyon et al., 2005; Leong et al., 2005) and there is a concern that ketamine may be used to facilitate sexual assault. Methods developed to analyse ketamine and norketamine in various biological matrices are widespread (Bolze
and Boulieu, 1998; Yanagihara et al., 2003; Lua and Lin, 2004; Adamowicz and Kala, 2005; Leong et al., 2005; Negrusz et al., 2005; Wang et al., 2005; Apollonio et al., 2006; Xiang et al., 2006) but the analysis of the secondary metabolites dehydronorketamine and hydroxylated metabolites (both intact and conjugated) is much less common. (Adams et al., 1981; Bolze and Boulieu, 1998; Hijazi et al., 2001). At the time of this work, current lack of commercial standards hindered analysis of these metabolites, whose identification requires custom-synthesis (Huang et al., 2005; Wu et al., 2007). Procedures for identifying novel metabolites of ketamine are of interest, not least for forensic purposes.

Often, the enzymes responsible for metabolism are unknown and this hinders the use of recombinant enzymes to produce specific metabolites (Peters et al., 2007). The N-demethylation of ketamine is performed by CYP2B6 (Yanagihara et al., 2001) but the enzymes for other stages of metabolism have not been characterised. However, the in vivo metabolism of a drug can be mimicked by the use of microsomes. Human microsomes are used in drug discovery for in vitro assays to elucidate metabolic pathways, predict drug-drug interactions, determine inhibition or induction potential of enzymes by the new drug, and to help identify possible toxic metabolites.

Here, we report the approach of using commercial human microsomes to produce metabolites of ketamine and norketamine which, for the first time, have been used as analytical markers in their crude form. Furthermore, microsomes were also employed to synthesize metabolites of deuterated substrates to assist in the assignment of analytical properties of the metabolites, such as retention time and MS/MS spectra. Using this in-vitro approach, we have identified novel metabolites, these being phenolic isomers of hydroxyketamine and hydroxynorketamine, and two phase II metabolites. This, in turn,
led to the identification of metabolites in urine following ketamine administration to volunteers, using the synthesized deuterated metabolites as reference compounds.
5) Methods

Materials and Methods

Chemicals and Reagents. Methanolic standards of ketamine-HCl, norketamine-HCl (1 mg/mL as the free base) and 3,4,5,6-tetradeterophenyl-ketamine-HCl and 3,4,5,6-tetradeterophenyl-norketamine-HCl (0.1 mg/mL as the free base), were purchased from LGC Promochem (Teddington, U.K.). Infusions of these standards into a quadrupole mass spectrometer yielded protonated molecules that were consistent with these compounds. The only impurities observed were trideuterated ketamine in the tetradeuterated ketamine, and trideuterated norketamine in the tetradeuterated ketamine, these being 14 % and 3 % respectively (comparing peak heights of the pseudomolecular ions). Water was purified (resistivity 18.2 M cm) by an Elga Maxima HPLC ultra pure water system (High Wycombe, U.K.). Acetonitrile, acetic acid, propan-2-ol (GLC-pesticide residue grade), dichloromethane (HPLC-grade), hydrochloric acid (analytical reagent-grade), sodium hydroxide pellets, potassium hydroxide pellets, potassium dihydrogen orthophosphate, di-sodium hydrogen orthophosphate dihydrate and sodium dihydrogen orthophosphate dihydrate were from Fisher Scientific (Loughborough, U.K.). Ammonia solution was obtained from BDH Chemicals (Poole, U.K.). Formic acid was obtained from VWR International (Lutterworth, U.K.). 1-Chlorobutane (Chromasolv) was obtained from Sigma Aldrich (Poole, U.K.). ‘NADPH Regenerating System A’ consisting of 31 mM NADP+, 66 mM glucose-6-phosphate and 66 mM MgCl₂ in water, ‘NADPH Regenerating System B’ consisting of 40U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, ‘UDP Reaction Mix Solution A’ consisting of 25 mM UDPGA in water and ‘UDP Regenerating System B’ consisting of 250 mM Tris-
HCl, 40 mM MgCl₂, 0.125 mg/ml alamethicin in water, were obtained from BD Biosciences (Oxford, U.K.), as were the pooled human liver microsomes (20 mg/mL in 250 mM sucrose). Bond Elute Certify I (mixed mode; C8 with strong cation exchange) solid-phase extraction (SPE) cartridges were obtained from Varian (Walton-on-Thames, U.K.).

**In Vitro Metabolism of Ketamine or Norketamine by Microsomes.** For phase I metabolism, to either 10 µg ketamine, ketamine-d₄, norketamine or norketamine-d₄, the following was added and then mixed: 500 µL of 0.2 M phosphate buffer (pH 7.4), 50 µL of the microsome preparation (to give a final concentration of 1 mg/mL protein), 50 µL of ‘NADPH Regenerating System A’, 10 µL of ‘NADPH Regenerating System B’, and 390 µL of water to make up to a 1 mL volume in total. The combined components, with exception of the microsomes, were pre-incubated at 37 °C for 10 min, after which the microsomes were added to initiate metabolism. The mixture was subsequently incubated for 2 h at 37 °C, with vortex mixing every 30 min, after which metabolism was terminated with the addition of 250 µL acetonitrile, on ice, and centrifuged at 12000 g for 4 min to precipitate and remove the protein. Phase II metabolism was also performed in conjunction with phase 1, the methodology being that as described above, with the exception that instead of 380 µL water added, 125 µL of ‘UDP Reaction Mix Solution A’ and 200 µL ‘UDP Regenerating System B’ were added with 65 µL water. Ten incubations of each deuterated substrates were performed to synthesize a sufficient quantity of metabolites as our investigation progressed, this amounting in total to 100 µg of deuterated ketamine and 100 µg of deuterated norketamine.
**pH Dependent Extraction of Metabolites.** To help identify novel phenolic metabolites produced in microsomal incubates, the solution was extracted with 1-chlorobutane (3 x 5 mL) following pH adjustment to 8.4 using 0.1 M NaOH. After vortexing, shaking on a rotary mixer for 5 min and centrifugation at 2000 g for 5 min, the 1-chlorobutane layers were combined and evaporated at 60 °C under nitrogen and reconstituted in 250 µL acetonitrile:water (10:90 v/v). In addition, a separate triplicate extraction was performed following adjustment to pH 12 to confirm the absence of phenolic metabolites; as they are amphoteric (having a phenol and amine function) they should not be extracted at this pH. The aqueous portion of this extract was readjusted back to pH 8.4 and extracted again three times to aid phenolic metabolite identity.

**Ultra-High Performance Liquid Chromatography-Mass Spectrometry.** Analysis was performed by a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled to a Waters Quattro Premier triple quadrupole mass spectrometer (Elstree, U.K.). A Waters BEH C18 2.1 x 150 mm column with 1.7 µm particle size was used with gradient mobile phase conditions employing 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B) with a flow rate of 0.35 mL/min and a column temperature of 45 °C. A run-time of 8 min was employed starting at 10 % B for 0.5 min ramping to 50 % B over 7 min and ramping back down to the original conditions over 0.5 min. Incubates were injected in aliquots of 20 µL onto the HPLC system in either extracted or crude form.

Full scan MS/MS (m/z 50-260) experiments and selected reaction monitoring (SRM) were performed at cone voltages of 25 V. Total scan times were typically 1.2 s for MS/MS and SRM experiments. MS/MS experiments were typically performed using
collision energies of 15-22 eV. The most prominent ions obtained from MS/MS experiments were used for selected reaction monitoring (SRM) in positive electrospray ionisation mode following further optimisation of collision energies for each transition. The appropriate SRM transitions and the collision energies in eV (bracketed) employed were as follows; 238→115 (42), 238→125 (27), 238→163 (25) for ketamine, 242→129 (27) for ketamine-\textit{d}_4, 224→115 (42), 224→125 (27), 224→207 (16) for norketamine, 228→211 (42) for norketamine-\textit{d}_4, 222→125 (27), 222→177 (30) and 222→205 (30) for dehydronorketamine, 226→209 (30) for dehydronorketamine-\textit{d}_4, 240→125 (30), 240→151 (25), 240→177 (25), 240→195 (25), 240→205 (25) and 240→141 (30) for hydroxynorketamine isomers, 242→127 (30), 242→143 (25) and 242→179 (25) for \textit{^{37}}\text{Cl} isotopes of hydroxynorketamine isomers, 244→129 (25) and 246→131 (25) for hydroxynorketamine-\textit{d}_4 and its \textit{^{37}}\text{Cl} isotope (cyclohexanone ring-hydroxylated), 243→144 (30) and 245→146 (30) for hydroxynorketamine-\textit{d}_3 and its \textit{^{37}}\text{Cl} isotope (chlorobenzene ring-hydroxylated), 254→125 (30), 254→141 (22), 254→151 (25), 254→163 (25), 254→179 (25), 254→195 (25) for hydroxyketamine isomers, 256→127 (30), 256→143 (30) and 256→181 (25) for \textit{^{37}}\text{Cl} isotopes of hydroxyketamine isomers, 258→129 (30) and 260→131 (30) for hydroxyketamine-\textit{d}_4 and its \textit{^{37}}\text{Cl} isotope, 257→144 (30) and 259→146 (30) for hydroxyketamine-\textit{d}_3 and its \textit{^{37}}\text{Cl} isotope, 416→240 (20), 416→222 (35), 416→125 (25) and 416→207 (25) for hydroxynorketamine glucuronide, and 420→129 (20) for hydroxynorketamine glucuronide-\textit{d}_4. Electrospray, extractor and RF lens voltages were 3.5 kV and 4 V and 0 V respectively. MS experiments used the same conditions as mentioned above except for the collision energy, 0 eV, and entrance voltage, 50 V. All data were collected in centroid mode.
Volunteer Study and Collection of Samples. Six volunteers, 3 male and 3 female, were administered an oral dose of 50 mg ketamine in the form of a quarter-vial (5 mL) of a 200 mg Ketalar® dose (AAH Pharmaceuticals Ltd, Romford, U.K.), diluted with 125 mL water. The rationale for this dose was that, taking into account that the oral bioavailability of ketamine is around 20 %, a 50 mg oral administration would not be expected to cause a loss of consciousness and would have a minimal risk of psychedelic effects in volunteers with a normal body mass index. Indeed, with this dose, the volunteers were found to be only mildly sedated. Urine was collected 10 min prior to administration of the dose and at designated time points over a period of 11 days (0-2, 2-4, 4-6, 6-8, 8-12, 12-24 and spot collections at 30 h and 2-10 days post-dose with a.m. and p.m. samples collected on days 4, 7 and 10). The study was ethically approved of by our institution (King’s College Research Ethics Committee) and written informed consent was obtained from the volunteers.

Preparation and Extraction of Urine Samples from the Volunteer Study. To 4 mL of each urine sample, 6 mL of phosphate buffer (10 mmol/L; pH 6.0) together with 200 µL of deuterated incubate was added and vortex mixed for 30 s. Mixed-mode (cation exchange with C8) SPE cartridges 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 then loaded onto the 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 water and 2mL acetate buffer (0.1 mol/L; pH 4.0). Retained drug and metabolites were eluted under gravity with dichloromethane/propan-2-ol/ammonium
hydroxide (78:20:2 v/v) in 4 sequential aliquots of 1 mL (4 mL total). The eluent was evaporated under nitrogen at 60 °C. The dried extracts were reconstituted in 250 µL of water/acetonitrile/formic acid (89.9:10:0.1 v/v/v) and vortex mixed for 30 s and 20 µL was injected into the chromatographic system.

**Accurate mass measurement (LC-FT-ICR-MS).** The chromatographic conditions were operated using an Agilent 1200 Series Quaternary LC System (Wokingham, U.K.). A Waters XBridge C18 column with dimensions 150 x 2.1 mm, 3 μm particle size was employed, without temperature control, with a gradient programme involving acetonitrile and 0.1 % formic acid and water and 0.1 % formic acid as mobile phases A and B, respectively. The gradient involved maintaining a composition of 10 % B for 5 min followed by ramping up to 17 % over 7 min, maintaining this composition for 5 min and ramping up to 50 % B over 1 min, maintaining 50 % B for 3 min, ramping back down to 10 % B over 1 minute and re-equilibration for 19 min to give a total run time of 40 minutes. The flow rate was 0.25 mL/min and the volume of sample injected was 50 µL.

A Thermo Scientific LTQ fourier transform-ion cyclotron-mass spectrometer (FT-ICR-MS) was used to determine accurate masses of metabolites. Electrospray ionisation (ESI) source parameters were sheath gas flow rate, 25; auxiliary gas flow rate, 5; sweep gas flow rate, 0; electrospray voltage, 4 kV; capillary temperature, 275 °C; capillary voltage, 13 V; and tube lens voltage, 30 V. Data was collected in profile mode.
6) Results

Results

**In Vitro Metabolism of Ketamine and Norketamine and their Deuterated Analogues.** Incubation of ketamine and norketamine and their deuterated analogues with microsomes resulted in a number of metabolites. Metabolites of the substrates ketamine and norketamine from the microsomal incubate were identified by liquid chromatography-mass spectrometry. The structures of the metabolites are given in Table 1, as interpreted from the data presented here.

Following centrifugation of the incubate, a portion of the supernatant was analysed, which resulted in a number of peaks in the total ion chromatogram that were not observed in the absence of substrate. It was assumed that these peaks were dehydro- and hydroxylated metabolites and therefore extracted ion chromatograms were generated based on the predicted monoisotopic (containing the $^{35}$Cl isotope) protonated molecule [M+H]$^+$, as presented in Fig. 1. These were $m/z$ 222 for dehydronorketamine, $m/z$ 240 for hydroxynorketamine and $m/z$ 254 for hydroxyketamine. Isotopic doublets $^{35}$Cl/$^{37}$Cl of each metabolite were observed at the expected 3/1 ratio. An extracted ion chromatogram of $m/z$ 236 showed no peak, indicating dehydroketamine was not present in significant amounts. No evidence of hydroxylated dehydronorketamine ($m/z$ 238) or di-hydroxynorketamine metabolites ($m/z$ 256) was obtained from microsomal incubates of ketamine or norketamine.

When norketamine was used as a substrate, common metabolites to that of ketamine were identified but the yield was approximately three-fold higher, as judged by comparison of peak areas. This is not surprising as norketamine is considered to be the
primary metabolite of ketamine, from which secondary metabolites are formed (Wieber et al., 1975).

**Preliminary Detection of Phenolic and Alcoholic metabolites by Liquid Chromatography-Mass spectrometry.** The extracted ion chromatogram for m/z 240 showed 7 possible isomers, as shown in Figure 1 but only 4 alcoholic isomers of hydroxynorketamine have been described in the literature (Adams et al., 1981). Further, when tetradeuterated substrate was employed only 4 analogous peaks were visible at m/z 244 (compare panels 3 and 4 of Fig.1). Given this difference in the number of peaks, it was reasonable to consider the possibility of the formation of previously unreported phenolic metabolites, where substitution with a hydroxyl group on the chlorobenzene ring would result in the loss of a deuterium atom giving an [M+H]+ of m/z 243. Indeed, when an extracted ion chromatogram of m/z 243 was generated, this showed three additional peaks (Fig. 1, panel 5), the extracted ion chromatograms of m/z 243 and 244 displaying seven peaks in total with contemporaneous retention times to those observed when non-deuterated substrate was used. It should be noted that the early eluting peaks (retention times of 1.7, 2.2 and 2.4 min) correspond to alcoholic metabolites in the m/z 243 extracted ion chromatogram as a result of a tri-deuterated impurity, i.e. norketamine-d3 present in the tetradeuterated standard.

Hydroxyketamine metabolites were indicated by m/z 254 showing five dominant peaks. Only three peaks were observed of the same retention time and similar intensity when deuterated ketamine was used (m/z 258), indicating that these were alcoholic metabolites and m/z 257 showed two peaks, indicating the presence of phenolic metabolites.
Supportive Evidence of the Formation of Phenolic Metabolites by pH Dependent Extraction. To help confirm the presence of phenolic metabolites, a liquid-liquid extraction was performed with 1-chlorobutane after adjusting the pH of the microsomal incubates. It was assumed that the primary or secondary amine was conserved, making the phenolic metabolites amphoteric. The first extraction was at pH 8.4, a pH which would be expected to be favourable for extraction of both alcoholic and phenolic metabolites into 1-chlorobutane, based on 2, 3 or 4-chlorophenol having a pKa of 8.5, 9.0 and 9.4, respectively, and that the pKa of ketamine (secondary amine) is 6.7 and norketamine (primary amine) is 7.5 (Cohen and Trevor, 1974). Another separate extraction was performed after adjusting the incubate to pH 12, a pH at which phenolic metabolites would be expected to have a net negative charge and therefore remain favourably partitioned in the aqueous medium. To increase analytical sensitivity, MS/MS was performed on the precursor ions (for putative hydroxyketamine ($m/z$ 254 and its chlorine-37 isotope, $m/z$ 256) and putative hydroxynorketamine ($m/z$ 240 and 242) and the total product ion chromatograms were recorded. Analysis of the organic extract of the incubate adjusted to pH 8.4 resulted in four of the five peaks also recorded in Figure 1 for putative hydroxyketamine, as well as some smaller peaks (undetected in MS mode) and six of the seven peaks for putative hydroxynorketamine. Extraction at pH 12 showed only two of these peaks for hydroxyketamine and three for hydroxynorketamine, suggesting that the peaks absent from the chromatogram of the pH 12 extract were due to phenolic compounds being retained in the aqueous fraction. Readjustment of the remaining aqueous fraction from pH 12 to pH 8.4 followed by 1-chlorobutane extraction showed the ‘reappearance’ of these peaks. This data further supports the presence of at
least two phenolic isomers of hydroxyketamine and three phenolic isomers of hydroxynorketamine. Two metabolites present in the incubate, as analysed by injection of unextracted microsomal preparations (Fig 1, hydroxynorketamine 7 and hydroxyketamine 5), appeared not to be extracted into 1-chlorobutane at either pH (portion of chromatograms therefore not shown). It is likely, nevertheless, that these are hydroxylated metabolites, given the elemental composition obtained by FT-ICR-MS (see later). The lack of extraction into 1-chlorobutane infers that these metabolites are more polar, one speculative explanation being that the hydroxyl substituent is possibly adjacent to the keto function on the cyclohexane ring, favouring the formation of an enol tautomer to give a dihydroxy metabolite.

**Identification of Hydroxylated Metabolites by Tandem Mass Spectrometry.**

MS/MS fragmentation of the metabolites by direct injection of portions of the incubates gave product spectra consistent with dehydronorketamine and hydroxynorketamine, and also hydroxyketamine if ketamine was used as the substrate. The major fragment ions \((m/z)\) of monoisotopic norketamine \(^{35}\text{Cl isotope}\) were 207, 189, 179 125, which were consistent with published data, as described by Wang et al. (2004); other ions observed were 163, 151, 141. Product ions \((m/z)\) for \(^{35}\text{Cl-dehydronorketamine}\) were 205, 187 177, 170, 142 and 141. Fragmentation of \(^{37}\text{Cl isotopes}\) was used to help confirm the identity of the fragment ions containing \(^{35}\text{Cl isotopes}\); the presence of ions with a \(m/z\) 2 unit difference in a 3:1 ratio signifying a fragment containing chlorine.

MS/MS spectra of the metabolites thought to be hydroxynorketamine, fell into two classes, either displaying or not displaying a prominent product ion at \(m/z\) 141 (Fig. 2), with the possible structures of fragments of alcoholic isomers and phenolic
metabolites being displayed in Fig. 3(a) and 3(b) respectively. A dominant product ion \( m/z \) 141 is consistent with a fragment of a phenolic metabolite (Fig. 3b). The absence of a phenolic group would result instead in a chloromethylbenzene fragment and indeed an ion with an \( m/z \) 125 was prominent in the spectra not displaying a prominent \( m/z \) 141, indicating these metabolites were hydroxylated on the cyclohexanone ring rather than the benzene ring (Fig. 3a). Fragmentation of \( ^{37}\text{Cl} \) isotopes of the metabolites confirmed the presence of chlorine in both the \( m/z \) 125 and \( m/z \) 141 fragments (a mass shift of +2 was observed, giving \( m/z \) 127 and \( m/z \) 143). The product ion spectra for the alcoholic metabolites where the \( ^{35}\text{Cl} \) precursor was selected showed a weak \( m/z \) 141 but it must be emphasised that fragmentation of \( ^{37}\text{Cl} \) isotope showed no \( m/z \) 143, thus indicating that this \( m/z \) 141 fragment was not chlorinated and not the same structure as the prominent and diagnostic \( m/z \) 141 chlorinated fragment associated with the phenolic metabolites. Another difference was the common loss of water in alcoholic isomers to give \( m/z \) 222 from \( m/z \) 240, and \( m/z \) 179 from \( m/z \) 195, and similarly for the \( ^{37}\text{Cl} \) isotope. This was not seen in phenolic compounds where the loss of \( \text{NH}_3 \) was much more common to give the abundant product ion of \( m/z \) 223. Other fragment ions were common in mass to both phenolic and hydroxylated metabolites, i.e. \( m/z \) 205, 195, 179 and 67. The same differences were observed between alcoholic and phenolic hydroxyketamine isomers, as the same ions were produced but from a different precursor ion (i.e. \( m/z \) 254). Collectively, the chromatographic and mass spectrometry data, with that also gained from pH dependent extraction, is strong evidence to support the presence of phenolic as well as alcoholic metabolites. Differentiating between the phenolic isomers of hydroxyketamine, 

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Accurate Mass Determination of Metabolites of Ketamine and Norketamines and Deuterated Analogues. FT-ICR-MS was used to obtain the accurate masses of compounds suspected of being particular metabolites and further confirmed the results obtained by UPLC-MS/MS. In all cases the mass accuracy for both the molecular and fragment ions agreed with the theoretical value by less than 0.5 ppm (Table 1). The product ion at $m/z$ 141.01018 can be assumed to be the diagnostic product ion of the phenolic metabolites and the fragment with the elemental composition $\text{C}_7\text{H}_6\text{OCl}$, with a corresponding deuterated metabolite giving the expected $m/z$ 144.02899 ($\text{C}_7\text{H}_3\text{D}_3\text{OCl}$). These ions were exclusively obtained for metabolites thought to be phenolic and their deuterated equivalents. The hydroxynorketamine metabolite observed following injection of the microsomal incubate HNK7 (Fig. 1, panel 3) but not in the 1-chlorobutane extract, also gave an accurate mass consistent with an isomer of hydroxynorketamine, adding evidence to their proposed identity, despite this anomaly.

Deuterated Microsomal Metabolites as Internal Chromatographic Markers to Aid Detection of Metabolites in Man.

Phase I Metabolites: Deuterated metabolites generated following microsomal incubation of deuterated ketamine or norketamine were used as an internal chromatographic marker of in vivo urinary metabolites of ketamine, following an oral dose of ketamine (50 mg) to 6 volunteers. Analysis of these samples, following mixed-mode SPE, established the presence of all the metabolites found to be present in microsomal incubates. Figure 4 displays the ion chromatograms for the hydroxylated
metabolites, demonstrating the value of superimposing the transitions for the deuterated metabolites spiked into an elimination urine. Figure 4a shows the SRM transition \( m/z \) 240→125 superimposed with \( m/z \) 244→129 for alcoholic hydroxynorketamine isomers and Figure 4b shows the transitions \( m/z \) 240→141 and \( m/z \) 243→144 attributable to phenolic hydroxynorketamine metabolites. The same approach was used to establish the presence of urinary dehydronorketamine.

Alcoholic isomers of hydroxynorketamine were detected in urine samples for 3-4 days and phenolic isomers were detectable for 30 hours to 2 days. Alcoholic hydroxyketamine isomers were detected for 24-30 hours whereas phenolic isomers of hydroxyketamine were visible for 12-24 hours.

**Phase II Metabolites:** Microsomal incubation of deuterated analogues of norketamine was also helpful in the identification of novel phase II metabolites in urine. Following microsomal incubation of norketamine, two dominant peaks were observed in an extracted ion chromatogram (\( m/z \) 416), at retention times of 1.2 min and 5.3 min, this \( m/z \) being consistent with protonated molecules of hydroxynorketamine glucuronide. When tetradeuterated norketamine was used as a substrate, only one significant peak was observed in the \( m/z \) 420 ion chromatogram, this being at the latter retention time of 5.3 min, the data thus supporting the conjugation of glucuronic acid to a hydroxyl group on the cyclohexanone ring. Furthermore, MS/MS fragmentation of this conjugate showed similar spectral characteristics to alcoholic hydroxynorketamine, with a loss of a glucuronic acid neutral of 176. The other peak in the extracted ion chromatogram of \( m/z \) 416 at 1.2 min indicated a glucuronide conjugated on the chlorobenzene ring, as the
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product ion spectrum was similar to that observed for the phase 1 phenolic metabolites, including the presence of the diagnostic ions \( m/z \) 223 and 141. Furthermore, an intense analogous peak at \( m/z \) 419 was observed at this retention time when tetradeuterated norketamine was employed as the substrate and this was fragmented to \( m/z \) 243, a difference of one \( m/z \) unit from the other glucuronide, indicating hydroxylation at one of the deuterium sites and hence a conjugated phenolic metabolite.

Following the optimisation of collision energies of the glucuronide in microsomal incubates, SRM transitions were selected for the identification of these metabolites in volunteer urine. Only the alcohol conjugated glucuronide was observed, being present for up to 30 hours in samples from all six volunteers.
7) Discussion

**Discussion**

We report an effective approach that integrates the use of microsomal generated metabolites and their stable-isotope labelled analogues with LC-MS/MS to aid the identification of drug metabolites in man. Previously, enzymatic synthesis has been used successfully to generate novel compounds e.g., Peters et al., (2007). The current investigation is, nonetheless, distinct in that microsomes were used to generate several deuterated metabolites for addition in their crude form to a biological matrix, in this case urine, as a valuable tool for studying drug metabolism in man. Once metabolites are detected, MS/MS provides informative product ion spectra for identification purposes. Accurate mass determination, although not essential, is a valuable aid for confirmatory purposes and we recommend this option to also be considered, if suitable mass spectrometry instrumentation is available.

Superimposing chromatograms of urinary and deuterated microsomal metabolites can be extremely useful, as one simply has to locate isotopic differences in chromatographic peaks, i.e. isotopic doublets, to target metabolites in urine worthy of further investigation. In this respect, our approach may be compared to previous studies where the co-administration of drugs with their stable isotope analogues, to mammals, including ketamine (Leung and Baillie, 1989), aids the detection of metabolites in plasma or urine. It is not economically viable to co-administer the larger amounts of stable isotopes of ketamine required to perform such an investigation in man, and there are also ethical considerations regarding co-administration of unlicensed products. The alternative microsomal approach is economical in that only a small amount (100 µg) of stable
isotopes of ketamine and norketamine were required as substrate to generate sufficient metabolic markers to aid identification of metabolites in urine following administration of 50 mg of ketamine hydrochloride to six volunteers.

Several of the metabolites of ketamine, such as norketamine, dehydronorketamine and cyclohexanone-hydroxylated metabolites have been reported previously in vitro (Adams et al., 1981; Woolf and Adams, 1987) and in vivo (Chang and Glazko, 1974; Wieber et al., 1975; Leung and Baillie, 1989) but not hydroxylation of the chlorobenzene moiety, many investigations relying on derivatization and gas chromatography-mass spectrometry. The relatively recent availability of electrospay LC-MS permits the analysis of polar molecules without derivatization, and tandem MS confers increased sensitivity. This technique combined with microsomal generated deuterated analogues of metabolites facilitates detection of novel metabolites in biological matrices. Differential extraction further helped to discriminate between alcoholic and phenolic metabolites. Of interest, is that Adams et al (1981), as a small part of their elegant substantial investigation on ketamine metabolism by rat liver microsomes, employed tetradeuterated ketamine as a substrate but they comment, that because there was no loss of deuterium on formation of hydroxylated metabolites, that the site of hydroxylation could not be aromatic. Two possible explanations for the difference in findings are that this metabolic route exists in the human but not in the rat or that the phenolic metabolites are not readily amenable to the chemical derivatisation that these investigators employed. As an aside, the removal of deuterium upon hydroxylation in vitro (Rettie et al., 1988) and in vivo (Walle et al., 1983; Hecht and Young, 1981) of various labelled drugs have been described to help elucidate structural characteristics in metabolic studies.
There were also differences in the alcoholic metabolites identified in the current study and those by Adams et al., 1981 who reported four hydroxynorketamine metabolites with hydroxylation sites on the cyclohexanone ring but with only three carbon atoms being involved to give C4-, 5-, and 6- hydroxyketones. Two C5-hydroxylated metabolites were therefore proposed, and as the authors employed derivatization, these would have been converted to diastereoisomers that can be separated on a conventional GC stationary phase. In our investigation, no chemical derivatization was performed so chiral metabolites would not be separated by LC using a C18 phase but, nonetheless, we too observed four alcoholic metabolites for hydroxynorketamine. Logic dictates that another geometric isomer of hydroxynorketamine exists, possibly a 3-hydroxyketone that was not detected in that study because derivatization was sterically hindered by the close proximity of the chlorobenzene and amine moieties. It is reasonable to consider, therefore, that the four metabolites identified described herein represent all available carbon sites being hydroxylated on the cyclohexanone ring. We report also three alcoholic hydroxyketamine isomers, at least one more than in the previous study (Adams et al., 1981)

With respect to phase II metabolites, only one alcoholic glucuronide was observed, being present for up to 30 h in samples from all six volunteers. This metabolite was probably conjugated HNK 7 as judged by an increase of this metabolite following hydrolysis by both E. coli and H. pomatia ß-glucuronidases. The lack of further glucuronide conjugates could be a result of a less favoured conformational fit of other phase I hydroxylated metabolites with the active site of glucuronyl transferase. Even so, there was evidence of
the presence of a phenolic conjugate in the microsomal incubate that was not detected in urine. Most likely, positive electrospray ionization of the glucuronide conjugates is not as efficient as for the phase I metabolites and therefore they are less likely to be detected, as based on our experience with other drugs and their conjugates. With a detection window of 30 h in our administration study, it is unlikely the targeting of intact urinary hydroxynorketamine glucuronide will be of any benefit in increasing the retrospective detection of ketamine administration for forensic purposes, as dehydronorketamine could be detected for 6 to 10 days post-dose in the same volunteers (Parkin et al., 2008). Moreover, hydrolysis of urine samples collected 4 and 7 days post-administration with glucuronidase from *E. coli*, gave unremarkable results, indicating that the aglycones of hydroxylated metabolites are not likely to offer any advantage over targeting dehydronorketamine (data not shown for brevity). We also compared the data obtained following hydrolysis with glucuronidase from *E. coli* and *H. pomatia*, the latter which has aryl sulfatase activity, and an increase (5-7 fold) in the phenolic hydroxynorketamine isomers HNK 4 and 5 was observed only with the latter in the early stage of elimination (6 and 8 hours) indicating the presence of a sulphate conjugate. Other investigators report an indication that acid-labile N-glucuronide conjugates of ketamine, norketamine and dehydronorketamine are formed (Lin and Lua, 2004) but we found little indication to support this. Previously, glucuronides of alcoholic hydroxynorketamine have been reported in a slowly-migrating fraction of polar metabolites, using thin layer chromatography, and an increase in hydroxynorketamine was seen upon hydrolysis (Chang and Glazko, 1974).
The production of several metabolites and their stable isotopes simultaneously is advantageous in laboratories that are interested in multi-analyte approaches. Once metabolites of interest are identified, if there is a need, larger scale synthesis can be considered to generate enough metabolites for NMR analysis, either by chemical synthesis or using recombinant enzymes in various vectors including yeast strains and \textit{E. coli} (Mehmood et al., 1995; Anari et al., 2000; Arnell et al., 2007; Peters et al., 2007).

When using deuterium labelled substrates to generate stable isotope metabolites, due consideration should be given to possible deuterium isotope effects, greater energy being required for cleavage of a bond to deuterium than to hydrogen. In the current investigation, the deuterium was exclusively on the phenyl ring of the substrate, aromatic hydroxylation tending to be unaffected by deuterium isotope effects. By contrast, if the deuterium had been placed on the aliphatic carbons of the cyclohexanone moiety of the substrate, the formation of alcoholic metabolites may have been more difficult due to the P450 mechanism of aliphatic hydroxylation. All four hydrogen atoms on the phenyl ring of the stable isotopes of ketamine and norketamine were replaced with deuterium and thus substitution to form a phenol can only result in the loss of one of the deuterium atoms. Had the phenyl ring contained fewer deuterium atoms, hydroxylation of a carbon bonded to a deuterium atom is likely to have resulted in migration of the deuterium atom to an adjacent carbon originally bonded to a hydrogen atom, referred to as the ‘NIH shift.’ There are therefore important considerations with the use of metabolic substrates labelled at specific positions with deuterium and the interested reader is referred to the review article by Baillie (1981) for more information on the use of stable isotopes in pharmacological research.
From our perspective, this investigation was of practical value in generating metabolites and, in particular, their stable isotope analogues for the qualitative investigation of elimination of ketamine metabolites. As well as determining novel phenolic metabolites in vivo and performing LC-MS analysis on intact glucuronides for the first time, we revisited metabolites previously described in view of determining the relative windows of detection of urinary metabolites, which are often not analyzed for long periods of time in drug administration studies. This helped us to determine the most useful metabolite to target in a forensic context, this being dehydronorketamine (Parkin et al, 2008), which is supported by data showing that dehydronorketamine has a longer plasma half-life compared to ketamine and norketamine in intensive care patients (Hijazi et al, 2003). In conclusion, the application of microsomal synthesized compounds from stable isotope substrates for use as an internal chromatographic and mass spectrometric marker is a viable approach for facilitating the identification of metabolites in vivo.

8) Acknowledgements

Acknowledgements

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9) References

References


Huang MK, Liu C, Li JH and Huang SD (2005) Quantitative detection of ketamine, norketamine, and dehydronektoetamine in urine using chemical derivatization


10) Footnotes

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11) Legends for Figures

Figure 1:

Extracted ion chromatograms of selected metabolites obtained from microsomal incubates; ketamine ($m/z$ 238), norketamine ($m/z$ 224), hydroxynorketamine ($m/z$ 240), tetradeuterated hydroxynorketamine ($m/z$ 244), trideuterated hydroxynorketamine ($m/z$ 243), hydroxyketamine ($m/z$ 254), tetradeuterated hydroxyketamine ($m/z$ 258), trideuterated hydroxyketamine ($m/z$ 257) and dehydronorketamine ($m/z$ 222). Dotted lines are drawn through the peaks discussed in the main text.

Figure 2:

MS/MS spectra obtained from fragmenting alcoholic or phenolic hydroxynorketamine. $^{35}$Cl and $^{37}$Cl isotopes were targeted and used to help elucidate metabolite structure. a) and b) show the typical spectra produced respectively for alcoholic and phenolic metabolites from $^{35}$Cl isotopes whereas c) and d) show the fragmentation of the $^{37}$Cl isotopes. Prominent fragments above 5% of the intensity of the base peak are labelled or ions that become dominant at other collision energies.

Figure 3:

Postulated fragments for the MS/MS spectra shown in Figure 2. Part (a) displays possible structures of fragments of alcoholic isomers of the hydroxylated metabolites while (b) shows the possible structures for phenolic metabolites. Of importance is $m/z$ 125 (alcohols) versus $m/z$ 141 (phenols) and $m/z$ 222 (alcohols) compared with $m/z$ 223 (phenols) as shown in this figure.
Note: The benzyl group is drawn as a tropylion in this figure and elsewhere although no evidence of a structure has been determined (McLafferty and Winkler, 1974).

**Figure 4:**

Chromatograms of a urine sample spiked with deuterated metabolites prior to extraction, showing isomers of hydroxynorketamine a) superimposed chromatograms for the transitions $m/z$ 240/125 and 244/129 for alcoholic hydroxynorketamine and hydroxynorketamine-$d_4$ showing overlap at peaks 1-3 and 7 and b) superimposed chromatograms for the transitions $m/z$ 240/141 and 243/144 for phenolic hydroxynorketamine and hydroxynorketamine-$d_3$ showing overlap at peaks 4-6.
12) Tables

**Table 1:**

Properties of ketamine and metabolites that were monitored in microsomal preparations and in urine

Retention times and m/z ratios targeted for the metabolites of ketamine showing all the isomers of hydroxynorketamine and hydroxyketamine. Accurate masses are shown where obtained.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Abbreviation</th>
<th>LC&lt;sub&gt;t_R&lt;/sub&gt; (min)</th>
<th>m/z (deuterated analogue)</th>
<th>Accurate m/z (observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ketamine</td>
<td>K</td>
<td>3.7</td>
<td>238 (242)</td>
<td>238.09923 (0.084)</td>
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<tr>
<td>norketamine</td>
<td>NK</td>
<td>3.5</td>
<td>224 (228)</td>
<td>224.08365 (0.089)</td>
</tr>
<tr>
<td>dehydronorketamine</td>
<td>DHNK</td>
<td>3.0</td>
<td>222 (226)</td>
<td>222.06801 (0.045)</td>
</tr>
<tr>
<td>hydroxyketamine; alcohol</td>
<td>HK 1</td>
<td>2.0</td>
<td>254 (258)</td>
<td>254.09418 (0.275)</td>
</tr>
<tr>
<td></td>
<td>HK 2</td>
<td>2.6</td>
<td>254 (258)</td>
<td>254.09418 (0.275)</td>
</tr>
<tr>
<td></td>
<td>HK 5</td>
<td>5.8</td>
<td>254 (258)</td>
<td>*</td>
</tr>
<tr>
<td>hydroxyketamine; phenol</td>
<td>HK 3</td>
<td>3.2</td>
<td>254 (257)</td>
<td>254.09418 (0.275)</td>
</tr>
<tr>
<td>Metabolite</td>
<td>M/Z</td>
<td>M/Z 2 (244)</td>
<td>M/Z 3 (243)</td>
<td>Ref.</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>Hydroxynorketamine; alcohol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK 4</td>
<td>3.4</td>
<td>254 (257)</td>
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<td>*</td>
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<tr>
<td>HNK 1</td>
<td>1.7</td>
<td>240 (244)</td>
<td>240.07858</td>
<td>(0.042)</td>
</tr>
<tr>
<td>HNK 2</td>
<td>2.2</td>
<td>240 (244)</td>
<td>240.07857</td>
<td>(0.042)</td>
</tr>
<tr>
<td>HNK 3</td>
<td>2.4</td>
<td>240 (244)</td>
<td>240.07858</td>
<td>(0.042)</td>
</tr>
<tr>
<td>HNK 7</td>
<td>5.8</td>
<td>240 (244)</td>
<td>240.07858</td>
<td>(0.042)</td>
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<tr>
<td>Glucuronide conjugate</td>
<td>5.3</td>
<td>416 (420)</td>
<td>416.11075</td>
<td>(0.192)</td>
</tr>
<tr>
<td>Hydroxynorketamine; phenol</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HK 4</td>
<td>2.8</td>
<td>240 (243)</td>
<td>240.07863</td>
<td>(0.208)</td>
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<tr>
<td>HNK 5</td>
<td>3.1</td>
<td>240 (243)</td>
<td>240.07858</td>
<td>(0.042)</td>
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<tr>
<td>HNK 6</td>
<td>3.3</td>
<td>240 (243)</td>
<td>240.07870</td>
<td>(0.499)</td>
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<tr>
<td>Glucuronide conjugate</td>
<td>1.2</td>
<td>416 (419)</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

*Insufficient metabolite for accurate mass determination
Figure 2