Original Paper

Determination of Ketamine in Human Brain and Liver Tissues

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ABSTRACT

Ketamine hydrochloride is administered as an anaesthetic for human and veterinary use. Several methods have been suggested for the estimation of ketamine. Conventional method for determination of ketamine hydrochloride is non-aqueous titration. Other methods available in the literature are direct UV-visible spectrophotometry, gas chromatography-mass spectrometry and liquid chromatography. The work presents determination of ketamine and its metabolite, norketamine, from liver and brain tissues along with pyrovalerone, another drug used during administration of anaesthesia. The development and validation of the new method for determination of unchanged ketamine from tissues incorporated the use of the internal standard - phenacetin. The new method is fast and sensitive, and also enabled the qualitative determination of other drugs present in the matrix along with ketamine. The extraction procedure involved the modification of Stas-Otto method and the ammonium sulphate method used for extraction of basic drugs in the laboratories.

Key Words: Gas chromatography-Mass spectrometry; GC-MS; Ketamine; Phenacetin; Pyrovalerone

INTRODUCTION

Ketamine hydrochloride or 2-(2-chlorophenyl)-2-(methylamino)cyclohexanonehydrochloride is widely used as an anaesthetic drug for human and veterinary use, which stimulates N-methyl-D-aspartate (NMDA) receptors on neuronal cells. Ketamine blocks NMDA receptors that is responsible for its potent anaesthetic and analgestic properties. It occludes the open channel by binding to a site located within the channel pore.[1] Inhibition of potassium channels in central neurons underlies some excitatory effects and emergence phenomena observed with ketamine.[2]

Several methods have been suggested for the estimation of ketamine in tissue samples.[3–5] The conventional method is non-aqueous titration. Other methods mentioned in the literature include direct UV–visible spectrophotometry,[6] gas chromatography–mass spectrometry (GC-MS)[7,8] and liquid chromatography.[9] Chronic administration of ketamine or repeated exposure to the drug, such as occurs with radiotherapy or burn patients, can lead to the development of tolerance to the analgesic effects.[10] Ketamine also maintains self-administration behaviour similar to central nervous system (CNS) depressant drugs.[11] Often called "K" or "Special K," ketamine produces effects similar to phencyclidine (PCP) with the visual effects of LSD. The appearance of anecdotal accounts of ketamine abuse in teenagers' reports of "rave parties," increasing arrests for "driving under the influence" involving ketamine, etc, are evidence of increasing abuse.[12]

This work presents determination of ketamine and its metabolite, norketamine, from liver and brain tissues along with pyrovalerone, another drug administered during anaesthetic procedures. The development and validation of this new method for determination of unchanged ketamine from tissues incorporated the use of the internal standard - phenacetin. This method is rapid and sensitive,

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and enables the qualitative determination of other drugs present in the matrix along with ketamine. The extraction procedure involves the modification of Stas-Otto method, and the ammonium sulphate method, used for extraction of basic drugs in various laboratories.

MATERIALS AND METHODS

Materials: The standard ketamine was supplied by Neon Pharmaceuticals Pvt. Ltd. Phenacetin was chosen as the internal standard and was supplied by Pfizer. All solvents used were high-performance liquid chromatography (HPLC) grade, unless otherwise stated. Solvents used were acetonitrile, cyclohexane, 5% acidified ethyl alcohol, petroleum ether, rectified spirit and methanol. All other reagents were supplied by Merck. Ketamine samples, calibrators (4, 8 and 12 mcg/L) were prepared in methanol.

Extraction Procedure

Brain: Stas-Otto method[13,14] was modified for the extraction of ketamine for quantitative analysis. 50 g of brain was minced, mixed with 150 mL of rectified spirit in a flask and acidified with acetic acid. The mixture was heated on a steam bath for 4 hrs with thorough shaking at frequent intervals. The filtrate was evapourated and the residue was again extracted with 5% ethyl alcohol, filtered and washed several times with hot rectified spirit. The combined filtrates were evapourated in a china dish and a dark viscous extract was obtained. 100 mL of rectified spirit was added very slowly with constant stirring. It was warmed with occasional stirring for about half an hour and filtered. The combined alcoholic extracts were evapourated almost to dryness. The extract was further made upto 2 mL with cyclohexane. The organic (top) layer was transferred to clean centrifuge tubes. The extract was acidified with 0.2N H_2SO_4 and centrifuged for 5 min. The top layer (organic) was discarded and 4 drops concentrated KOH was added to the remaining aqueous layer followed by 4 mL cyclohexane. The tubes were once again rotated for 5 min, followed by 5 min centrifugation. The organic layer was transferred to glass test tubes, followed by evaporation to dryness in a 56°C waterbath under a gentle stream of nitrogen. The residue was reconstituted in 50 µL of methanol, vortexed and stored for analysis.

Liver: About 50–100 g of liver along with gall bladder were cut into small pieces, macerated, mixed with 100 mL of 5% acetic acid and taken into a reflux flask. Solid ammonium sulphate was then added to it by frequent shaking to make a saturated solution. About 20 g of solid ammonium sulphate was added in excess. The mixture was then refluxed for 4 hrs. The mixture was cooled slightly and filtered through Buchner funnel. The residue on the funnel was again extracted with two portions of 100 mL of 5% acetic acid and filtered as before. The filtrates were combined and taken into a 500 mL separating funnel. The ether fraction was added to the aqueous acidic extract in the separating funnel and shaken for 5 min and separated. 100 mL of ether was again added to the acidic layer, shaken for 5 min and separated. The ether layers were combined. The aqueous solution remaining in the separating funnel after separation of acidic drugs was made alkaline by adding liquid ammonia and extracted three times with 100 mL portions of cyclohexane:petroleum ether (3:1). The organic layers after separation were combined and washed with 50 mL of water. 10% sulphuric acid was added to the organic layer and was centrifuged for 7–8 min. The organic layer was discarded, and to the aqueous layer, 50 mL mixture of cyclohexane:petroleum ether (1:3) was added. Dilute ammonium hydroxide solution was added to make the solution alkaline and shaken for 5 min. The organic layer was separated, and washed and dried with nitrogen for analysis. 50 µL of 0.1 mg/mL phenacetin (internal standard) was added to all tubes. The medium was made slightly basic for better phase separation by adding 2 drops of 1% ammonium hydroxide and vortexed for 10 min.

Instrumentation: The analyses were carried out on a Perkin Elmer Clarus 600 Gas Chromatograph coupled with a Clarus 600 S Mass Spectrometer with turbomass 5.3.0 and a split–splitless injector. Samples were injected in splitless mode, using normal injection port. Separation of the analytes was obtained on a ZB-5MS column using helium as a carrier gas. The GC injection port and interface transfer line were maintained at 250 and 270°C, respectively. The oven temperature was initially held at 180°C for 30 sec, then increased to 230°C at 35°C/min, and then finally to 270°C at 10°C/min, and held for 2 min. The injection port was kept at 250°C with a split flow of 35 mL/min. The mass spectrometer was operated in positive electron ionization (EI) mode with electron energy of 70 eV. The source temperature was 150°C. The mass scan was done in EI mode between 40–400. A solvent delay of 2 min was set to protect the filament from oxidation.

Method Validation: The methods were validated for linearity, recovery, accuracy and precision. Calibration

curves were generated by linear regression analyses of the peak area of lidocaine and bupivacaine against the concentration. Precision [expressed as % relative standard deviation (RSD)] and accuracy (expressed as % error) were calculated for four QC samples. Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision. Relative recoveries from the blood method were calculated for spiked samples at between 1 and 20 mcg/mL (n=3) by dividing the peak area for the drugs by peak area for an equal concentration of ketamine in deionized water. The method was validated accordingly as per ICH guidelines. The chromatographic parameters such as retention time for both ketamine and phenacetin are shown in **Table 1**. The limit of detection (LoD), limit of quantification (LoQ) and linearity range and coefficient correlation all revealed that the recovery of this method was found to be better than 99%.

RESULTS AND DISCUSSION

Chromatographic Parameters: Figs 1–6 show chromatograms of brain tissues spiked with 1 mg/mL of ketamine, extract of brain tissue, extract of liver tissue, and MS spectra of ketamine, phenacetin, and pyrovalerone. The chromatographic parameters for ketamine are listed in **Table 1**. Retention time for ketamine was 8.11, and for internal standard i.e., phenacetin was 9.77. Metabolite of ketamine was detected at 5.28 and a sharp peak of pyrovalerone drug was detected in brain and liver tissues at 12.74. The quantifier ion for ketamine was found to be at 180 (**Fig 4**).

Table 1 Chromatographic Parameters for Ketamine and Phenacetin (I.S.)

Parameters	Ketamine	Phenacetin
Retention time (min)	8.11	9.77
Relative retention time	0.6	0.1
Width (min)	0.006	0.017
Number of plates	55,6780	23,5650

Method validation: The method was validated according to the ICH guidelines for validation of analytical procedures.[15]

Linearity: The linearities between the amount of ketamine and the peak area in the mass chromatogram in the EI mode were obtained. The minimum detectable concentration and the lowest quantifiable level were determined for ketamine. The LoD and LoQ for ketamine are 4 ng/mL and 13 ng/mL, for which the relative standard deviation (measure of precision and accuracy) are to be less than 20%. The standard curve (**Fig 7**) of ketamine was linear over the concentration range of 0.2–13 mcg/mL (three different concentrations were used, and standard was injected five times). The equation of the standard curve based on the ratio of the peak heights of ketamine/internal standard to the ketamine/internal standard concentration was Y=81397X – 8261.9, R^2=0.9974.

Precision: The intra-day precision was determined by analyzing three different preparations at concentrations of 4, 8 and 12 mcg/mL for ketamine on the same day. The interday precision was studied by comparing the assays on three different days (**Table 2**). The obtained values showed a suitable precision for the analytical method.

Accuracy: Accuracy was achieved by determining the recovery percentage of 3 different preparations of lidocaine and bupivacaine solutions in concentration levels of 50, 100 and 150% of the given drug levels for diluted samples (3 replicates of each concentration). The accuracy of the assay was determined by comparing the found concentration.

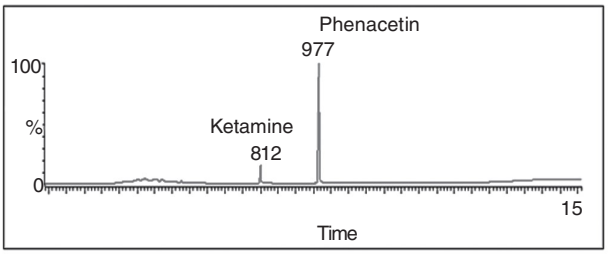


Fig 1: Brain Tissue Spiked with 1 mg/mL of Ketamine/ Standard Curve of Ketamine

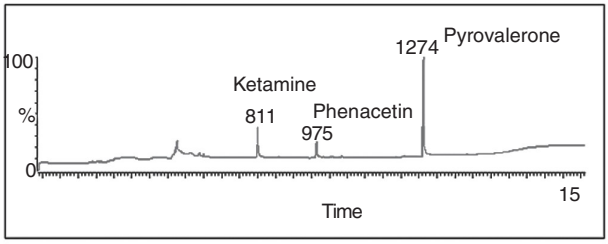


Fig 2: Chromatogram for Extract of Brain Tissue

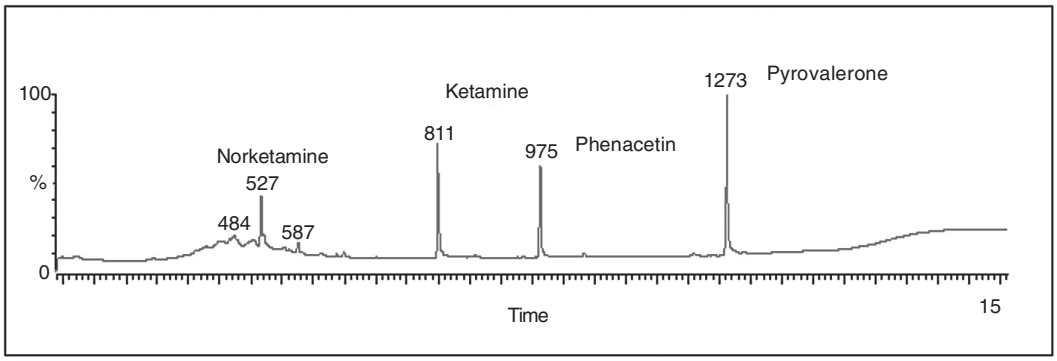


Fig 3: Chromatogram for Extract of Liver Tissue

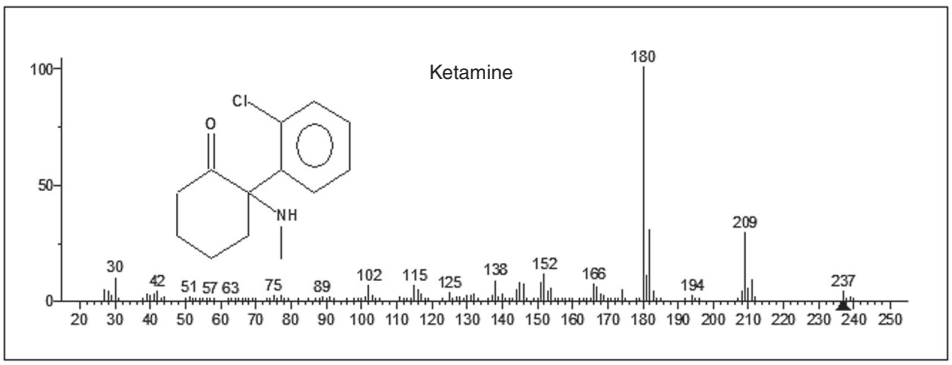


Fig 4: MS Spectra of Ketamine

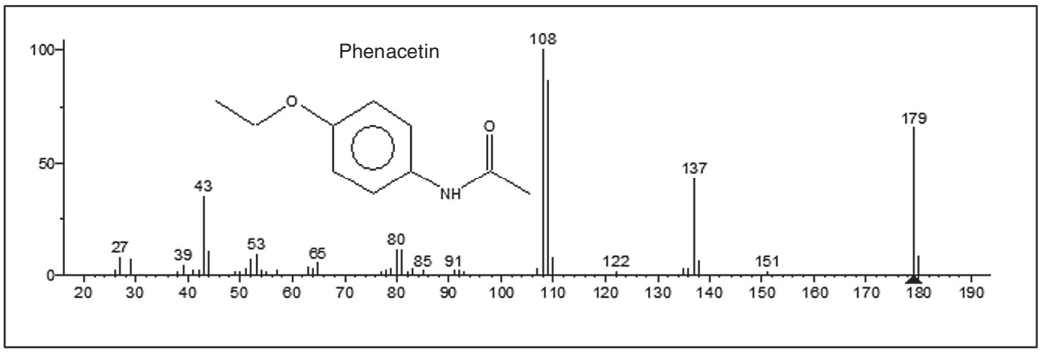


Fig 5: MS Spectra of Phenacetin

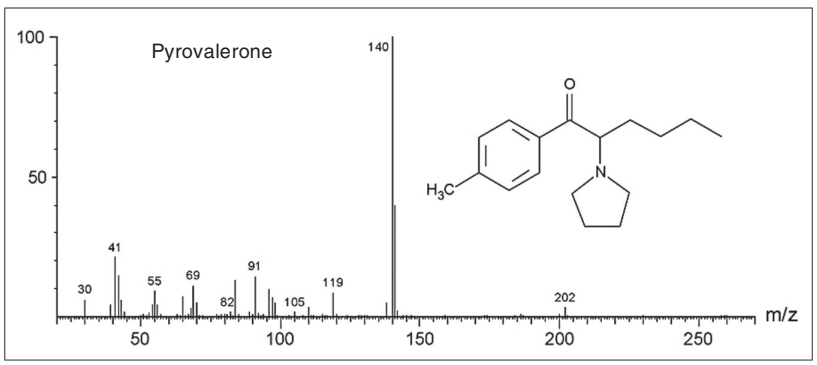


Fig 6: MS Spectra of Pyrovalerone

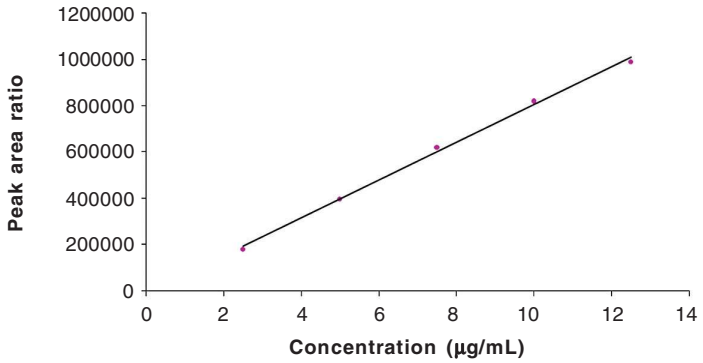


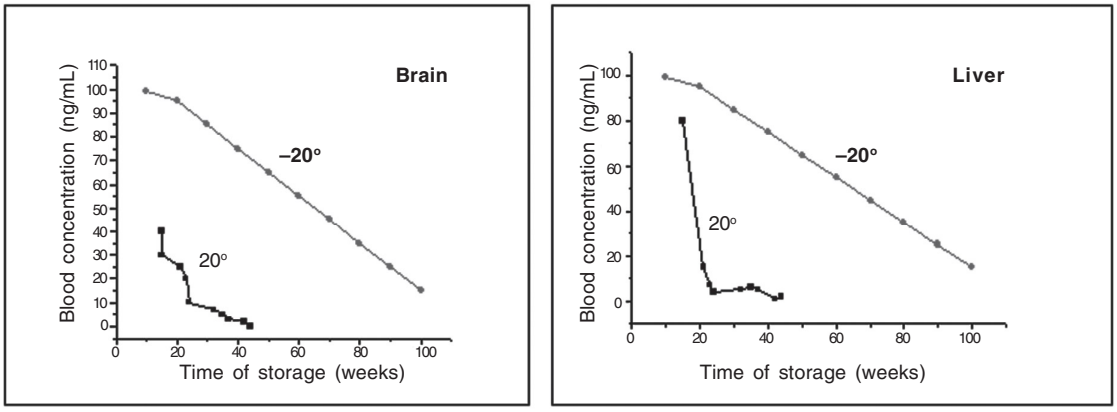
Fig 7: Calibration Curve of Ketamine Hydrochloride

Table 2 Intraday and Interday Reproducibility Data

Compound	Amount added (mcg/ml)	Intraday reproducibility		Interday reproducibility	
		Average amount found (mcg/mL)	±SD (n=6)	Average amount found (mcg/mL)	±SD (n=3)
Brain tissue	4	3.54	0.63	3.42	1.16
	8	7.17	1.12	7.35	0.82
	12	10.37	0.94	10.16	1.94
Liver tissue	4	3.26	1.65	3.12	1.63
	8	7.0	1.37	7.21	1.21
	12	10.11	0.87	10.26	1.06

Stability Studies of Ketamine: The stability studies were carried out for spiked samples of ketamine in brain and liver samples. Ketamine (1 mg/mL) was spiked in liver and brain samples at the following conditions: room temperature (25°C) under light exposure and light protection and +8°C in its original container (glass) after it was opened and sealed with Teflon, and –20°C in six glass containers (1 mL in each one). Samples were taken at appropriate time intervals, and analyzed in duplicate by GC-MS to determine the ketamine concentration, after being thawed for 1 hr at room temperature when they were preserved at –20°C. The percentage of ketamine remaining after each time interval was determined by comparing the concentration at that time with the initial ketamine concentration, measured before storage at different conditions. **Fig 8** shows the ketamine concentration profiles in brain (a) and liver (b) samples (n=10), stored at room temperature and at 20°C. The degradation patterns differed from each other due to the presence of enzymes in liver tissues. Also, it was seen that ketamine degradation rate was more in liver than in brain. Thus the major factors affecting degradation were temperature and biological matrix.

Ketamine concentrations did not change significantly during the first month in liver at –20°C, but changed significantly at 20°C after four days of analysis. A remarkable decline in ketamine concentration was observed after a 3-month period when the drug concentration fell to 20% of the first determined value. The ketamine concentrations in brain stored at –20°C were variable during the observed period. Their values were (mean±SD) 18±2.5 ng/g. The degradation pattern of ketamine shows that the stability of the analyte is influenced not only by the storage conditions but by the biological matrix itself. Ketamine can undergo degradation by enzymatic deacetylation or hydrolyses. Enzyme activities in liver increase with temperature. Hence, the rate of degradation was found highest in liver at 20°C.



(a) Brain tissues at two different temperatures **(a) Liver tissues at two different temperatures**

Fig 8: Stability studies of ketamine

CONCLUSION

With this study, a new quantification method for ketamine in brain and liver tissues was developed. Good separation was achieved between ketamine and the internal standard - phenacetin. Phenacetin was used as an internal standard for the first time for the analysis of ketamine. The developed method covered the calibration range from 3 ng/mL to 15 ng/mL. In addition, there was a good relationship of the quantification values between the developed method and widely used GC-MS method. The modified methods of extraction enabled better extraction and high sensitivity. This method could be useful for routine casework and toxicological analysis as well.

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