Original Article

ANALYTICAL STUDY OF KETAMINE FROM DIFFERENT AQUEOUS MEDIUM FOR FORENSIC DETERMINATION

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ABSTRACT

Ketamine is widely used as a drug of abuse. A study was conducted to develop a simple and rapid gas chromatography- flame ionization detector (GC-FID) method for the analytical study of ketamine from different aqueous medium sample. The developed method was selective as chromatogram showed no interferences and overlapped peak eluted at the retention time where ketamine eluted. A linear relationship of peak area ratio versus concentration (µg/mL) was obtained in the range of ketamine concentration 31.25 µg/mL - 500.00 µg/mL with r^2 = 0.994. The precision and accuracy of the method were within the acceptable limits. Various sample recovery rates were established with Coca-cola drink showing the highest recovery among the drinks tested. The urine sample spiked with ketamine also gave reasonable recovery value, though higher recovery rate from urine might be obtained through other extraction strategy.

INTRODUCTION

Ketamine is *N*-methyl-D-aspartate (NMDA) receptor - antagonist and chemically known as 2-(2-chlorophenyl)-2-(methylamino)-cyclohexan-1-one, with empirical formula $C_{13}H_{16}ClNO$ and with molecular weight of 237.73 g/mol [1]. Physically, ketamine can appear as free base or hydrochloride salt. Ketamine hydrochloride salt is a white crystalline powder and is easily dissolved in water forming a free base [1]. Its structure is shown in Figure 1**.**

In response to its anaesthesiology properties, ketamine was initially developed in early 1960s for the clinical purposes especially during surgeries [2, 3]. However, ketamine was later reported to be abused as a 'date rape' drug and also a drug-facilitated sexual abuse (DFSA) [4]. Ketamine was namely as such due to the reason that it is frequently used by the assailants to commit sexual assaults and other criminal offences [5]. Ketamine is colourless, odourless and tasteless, therefore it can be easily added and dissolved in drinks without being easily noticed by the victim [6, 7].

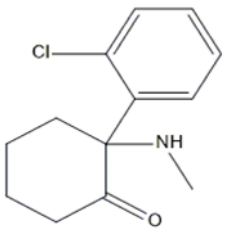

Figure 1: Chemical structure of ketamine

In Malaysia, ketamine is categorised as a Schedule I and placed under Amphetamine-Type Stimulant (ATS) drug in the Dangerous Drug Act (1952) [8]. This means that unauthorised possession of it is illegal. A statistic from Royal Malaysia Police reported by Singh, Chawarski [9] indicated that, the number of ketamine users have considerably increased from year 2006 to 2012. The drugs were sold in the original form or in the latest scenario, added into energy drink, soft drink, flavoured drink and alcoholic drinks [10].

The purpose of the present study was to develop a validated method for the recovery of ketamine in various aqueous medium for the forensic determination. The preliminary study involved the

development of method followed by optimisation, validation and recovery study on drinks and urine samples. The validation of the method was assessed by performing the selectivity, linearity, precision (reproducibility and repeatability) and accuracy study, limit of detection (LOD) and limit of quantification (LOQ) and recovery study using GC-FID method [11-14]. The recovery study was performed on five selected drink samples; Coca-Cola, Red bull, Livita, 100 plus, Nescafe® original and one biological sample, pooled human urine.

MATERIALS AND METHODS

Reagents and chemicals

Analytical grade dichloromethane (DCM) was purchased from Merck KGaA, Germany.Standards, including ketamine hydrochloride (98%), methamphetamine,*3,4-methylenedioxy-methamphetamine* (MDMA) and caffeine were supplied by Chemistry Department of Malaysia.Internal standard (IS) n-Octadecane (99.6%) was sourced from Dr Ehrenstorfer GmbH (Augsburg, Germany).

Instrumentation

GC-FID

The analysis was performed using Agilent 7890A gas chromatograph coupled with flame ionization detector (FID). The liquid samples were injected into GC-FID by means of auto sampler. A volume of 1 µL of sample was introduced into the injection port. The GC automation and data analysis on the system was completed via Chemstation software (revision B.04.02) (Agilent Technologies, Santa Clara, CA). The GC-FID has a split-splitless inlet system operated in the splitless mode and the injector temperature was set to 250^{o}C, pressure at 7.3267 psi, septum purge flow 3 mL/min and purge flow to split vent is 40 mL/min at 0.75 min.

The chromatographic separation was accomplished on an Agilent J & W 19091J-413 HP5 fused-silica capillary column (30m length x 0.320 mm i.d. x 0.25 µm film thickness) purchased from Agilent Technologies. The purified nitrogen gas with 99.999% purity was used as the carrier gas at 1.0 mL/min at a constant flow rate mode. The oven temperature program was set up as follows: first held at 150^{o}C/ min for 1 min, ramp at 10^{o}C/min until the temperature reached 220^{o}C and finally ramping at 90^{o}C/min until 290^{o}C/min, held for 3 minutes. The total analysis time was 11.778 min. The resulting peak in the chromatogram obtained were identified and analysed.

GC-MS

Agilent Technologies GC equipped with an Agilent5977A mass selective detector (MSD), an Agilent 7693 autosampler and an Agilent Mass Hunter for data acquisition. The GC-MS had a split-splitless inlet system operated in the splitless mode. The analytical column was an Agilent DB-5MS silica capillary column (30 m length x 0.250 mm i.d. x 0.25 µm film thickness) using helium gas (99.999% purity) as the carrier gas at 1.0 mL/min. The mass spectrometer operates at a full scan mode. With the identical column and chromatographic condition settings, the total ion chromatographic (TIC) obtained was recorded and compared.

Sample collection

Regular Coca-cola, Red Bull, Livita, 100 Plus Original Isotonic and Nescafe® original drinks were purchased from local groceries and pooled human urine was collected from non-ketamine user volunteers of two males and two females The ethics for the human biological sample was approved by Human Research Ethics Committee of USM (HREC) coded USM/JEPeM/16050194.

Preparation of the standard solutions and validation samples

Ketamine hydrochloride stock solution at concentration 1000 µg/mL was prepared by weighing 10.0 mg of ketamine hydrochloride powder accurately using an analytical balance and then transferred into a 10 mL volumetric flask. DCM was added into the volumetric flask up to the mark and homogenised by gently inverting the flask to ensure that the ketamine white powder was completely dissolved in the solvent.

Preparation of Internal Standard (IS) Stock Solution

n-Octadecane (C-18) at concentration 100 µg/mL was used as an internal standard (IS) stock solution. Ten miligrams of C-18 was accurately weighted using analytical balance and dissolved in 100 mL of DCM in a 100 mL volumetric flask. The IS solution was homogenised by gently inverting the volumetric flask.

Preparation of Working Standard Solutions

A working standards were prepared from the 1000 µg/mL stock solutions with a serial dilution techniques. These working standards were prepared in seven serial dilutions with a total volume of 10 mL each and in decreasing concentration ranged from 500 µg/mL - 7.81 µg/mL.

For the preparation of working standard at concentration 500 µg/mL, 5 mL of stock solution was aliquoted into a 10 mL volumetric flask. The flask containing 5 mL of stock solution was topped up with 5 mL of DCM to make up a final volume of 10 mL and homogenized. For the preparation of working standard at second concentration level i.e., 250 µg/mL, 5 mL of the first working solution at 500 µg/mL was transferred into another 10 mL volumetric flask and topped up with DCM to the mark. The same dilution procedure was applied for the next concentration of working standard. Table 1 summarises the measurements used to prepare the working standards.

Table 1: Preparation of the working standards at seven concentrations.

Working standard	Concentration of working standard (µg/mL)	Volume of working std at previous concentration level (mL)	Volume of DCM (mL)	Total volume of working standard (mL)
Working std. 1	500	5	5	10
Working std. 2	250	5	5	10
Working std. 3	125	5	5	10
Working std. 4	62.5	5	5	10
Working std. 5	31.25	5	5	10
Working std. 6	15.63	5	5	10
Working std. 7	7.81	5	5	10

Preparation of 1.0 M Sodium Hydroxide (NaOH)
Sodium hydroxide (NaOH) was used during sample extraction to achieve optimum pH for ketamine recovery study. NaOH (1.0 M) was prepared by dissolving 4 g of NaOH pallette into 100 mL of deionised water in a 100 mL volumetric flask. The palette was stirred and then transferred into a bottle, capped, labelled and kept at room temperature.

Optimization of the extraction method
The extraction procedure was optimised as a two-steps liquid-liquid extraction (LLE) technique using DCM as an extraction solvent. A sample volume of 10 mL was aliquoted into an assembly of glass tubes and the pH were adjusted to alkaline condition ranging from pH 10-13 by the addition of NaOH solution. Two mililitres of DCM was added into the samples and shaken for 10 minutes. The samples were then briefly vortexed and centrifuged at 5000 rpm for 5 minutes. The lower layer of the mixture containing the analyte of interest was pipetted out and transferred into a new tube. After that, the second extraction step was carried out by the addition of 2 mL DCM into the sample. The process of extraction was repeated. The lower organic layer of the second extraction was combined into the same tubes and then evaporated. When the solvents reached near dryness, 500 µL of the solvents were aliquoted into the chromatographic vial and reconstituted with 500 µL of IS stock solution. The vial is then place in the GC sample rack and run by auto injection programme set in the GC-FID.

Validation
Selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy as well as sample recovery were investigated for validation studies. Method selectivity study was determined by spiking five known standards; methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), caffeine, ketamine standard and internal standard n-Octadecane (C-18) in DCM and analysed by GC-FID for peak separation studies. Separated peak was then confirmed by injecting the same sample in GC-MS where the retention time and identity of the compounds were established by mass spectral analysis. For LOD and LOQ, a range of ketamine working standards as prepared in Table 1 were analysed at highest concentration until to the lowest concentration that were no longer detectable by the GC-FID. The LOQ was calculated as three times of LOD value.

The linearity study was established by preparing a standard solution at five concentration levels ranged from 500 µg/mL to 31.25 µg/mL. A total of six injections of every standards concentration were performed using GC-FID. The results obtained were further calculated for the peak area ratio of the standards using Equation 1.

$$\text{Peak area ratio} = \frac{\text{peak area of ketamine standard}}{\text{peak area of IS}}$$

...Equation 1

A calibration curve was plotted using Microsoft Excel®. A linear regression equation, the intercept and the r-squared (r^2) were determined. The precision study (repeatability and reproducibility) was implemented by analysing ketamine standards at three nominal concentration levels; low (50 µg/mL), medium (200 µg/mL) and high (400 µg/mL). Seven injections of standards were run at each concentration level. For repeatability, the measurement for all standards concentration were taken within the same day using the same instrument and within the same period meanwhile, for reproducibility, the same standards concentration levels were analysed at three injections on three consecutive days. The precision was expressed as % RSD.

The percentage of accuracy were determined at three replicates for each concentration level and analysed at three consecutive days. For sample recovery study, the spiked standard samples were prepared at two different concentration levels; 500 µg/mL and 100 µg/mL with three replicate samples for each spiked sample. After prepared, these two concentration levels of spiked samples were analysed for the target analyte. The results obtained were tabulated in tables and the recovery was reported in percentage.

RESULTS

Selectivity study

The peak of a known standard must be well separated and specifically identified in the chromatogram. Thus, in this analytical method development study, the selectivity testing was conducted to ensure that a peak of a known standard was not interfered with other analytes potentially present together. Figure 2 below shows the chromatograms of a separation of mixed standard containing methamphetamine, MDMA, IS, caffeine and ketamine standard dissolved in DCM analysed using GC -FID.

Limit of detection (LOD) & Limit of Quantification (LOQ)

From this experimental study, it was determined that the LOD obtained was 7.82 µg/mL. The calculated LOQ was determined as 23.46 µg/ mL.

Intra-day and inter-day precision

Intra-day and inter-day precision were recorded as in Table 2 and Table 3.

Recovery study

The samples chosen for recovery study consists of five types of beverages and one type of biological sample namely Coca-Cola, Livita, Red Bull, Nescafe® original and 100 plus isotonic drink and pooled human urine. The results of recovery study for these samples as tabulated in Table 4.

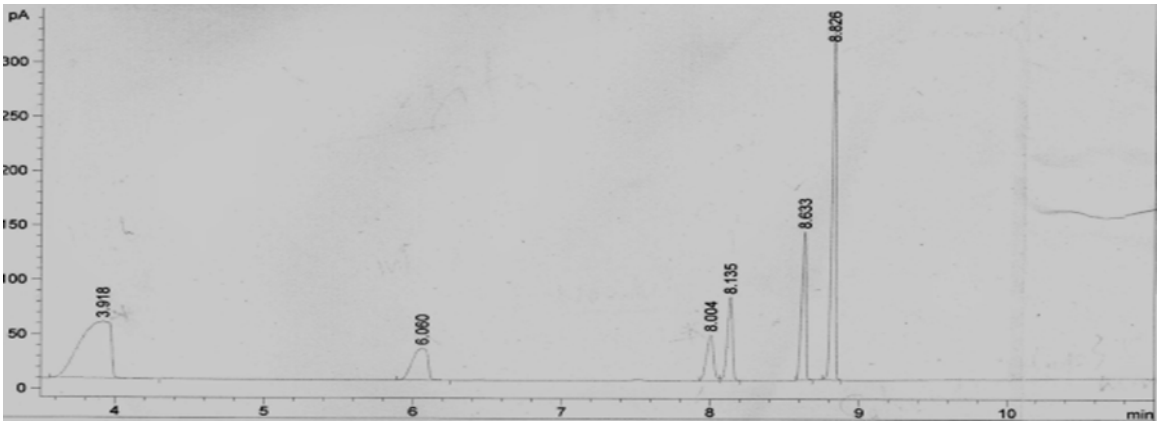

Figure 2: The chromatogram of a known standards spiked in DCM analysed using GC-FID.

Table 2: Intra-day precision study and accuracy of ketamine standard

Level (µg/mL)	Mean ± SD		RSD (%)
50	47.96 ± 1.20		2.51
200	188.97 ± 2.22		1.17
400	383.19 ± 2.30		0.60

Table 3: Inter-day precision and accuracy study of ketamine standard

Level (µg/mL)	Mean ± SD	Accuracy (%)	RSD (%)
50	53.97 ± 0.43	-7.94	1.45
200	189.33 ± 2.85	5.33	1.51
400	408.95 ± 4.13	-2.24	1.01

Table 4: Recovery value of a spiked standards in multi medium samples.

No.	Sample	Recovery(%) ± SD, (n=3)	
		Low concentration of spiked standard (100 µg/mL)	High concentration of spiked standard (500 µg/mL)
1	Coca-cola	80.35 ± 3.83	80.40 ± 10.51
2	Red Bull	72.73 ± 8.81	65.22 ± 11.43
3	Livita	65.67 ± 4.68	66.17 ± 4.66
4	100 Plus	62.15 ± 7.92	72.53 ± 6.84
5	Urine	26.33 ± 0.41	12.52 + 3.52
6	Nescafe® (original)	Unable to extract	Unable to extract

DISCUSSION

The selective properties of one method was tested and the peak of a known standard must be well isolated and specifically identified in the chromatogram. As in Figure 3.1, the resulting peak on the chromatogram for known standards spiked in DCM showed good peak separation. It is clearly indicated that all eluted peaks neither interfere nor overlapped with each other indicating good compound separation under the experimental parameter used. Consequently, the method was specific and selective enough for the determination of analyte of interest in the sample. GC-MS has proved to be able to provide an identification of a compound of every peak discernable on the chromatogram, but varied slightly and shifted in the retention time as may have occurred where there is slight change in the instrument conditions. Using the mass spectrum, all the compounds in the mixed samples were identified, based on the destructive mass spectral features as well as the mass spectra library match.

The GC-FID method was still able to detect the ketamine standard concentration at 7.82 µg/mL but this was very close to the noise level and however could still be confidently distinguished from the adjacent peaks. Therefore from this experimental study, the LOD was determined to be 7.82 µg/mL, meanwhile the calculated LOQ was determined as 23.46 µg/ mL. The calculated LOQ was obtained as the LOD 7.82 µg/ mL multiplied by three given the result of 23.46 µg/ mL

A linear regression equation and correlation coefficient, r-squared value was obtained from the development of a calibration curve. The linear regression equation obtained from the calibration curve was y = 0.0057x - 0.1103. The high r^2 obtained (>0.99) indicates that the data fits in a straight line and the calibration curve is good for response in a linear manner in the range of concentration 31.25 µg/ mL to 500 µg/mL.

Precision is a measure of the dispersion or closeness of results when an analytical procedure is repeated on one sample. Precision reflects the random error which frequently happens in analytical method. There are two common sets of precision study so-called repeatability (intra-day precision) and reproducibility (inter-day precision). Referring to table 3.1 and 3.2, the %RSD for intra-day precision study ranged from 0.60% to 2.51%, whereas for inter-day precision the %RSD ranged from 1.01% - 1.51%.

Among the drinks, Coca-cola drink gave the highest recovery of about 80% at two different concentrations indicating that the matrix effect from Coca-cola drink is minimal. In Red Bull and Livita drinks, the recovery at low concentration was more than 65% while Red Bull suffered from slightly lower recovery for high concentration spiked samples and this reversed from 100 Plus drink. The Nescafe® original drink, did not give any response on GC-FID indicating severe matrix effect that had interfere the sample recovery using the experimental parameters. Urine sample did not give high recovery percentage due to biological sample matrix effect, to have given only about 10% to 30% recovery.

CONCLUSION

This study applied a direct approach for the analytical study of ketamine from different aqueous medium. Drink samples has been chosen due to an increasing trend of 'club drugs' or 'date rape' drugs spiked in beverages especially during special occasions like rave parties, concerts or dance clubs. This analytical study covered an analytical method development, method optimisation and method validation. When compared to UNODC guidelines, all the validation parameters were within the acceptable limits with the exception of recovery value of ketamine in urine.

One of the more significant findings emerging from

this study is that the optimised chromatographic condition was suitable for simultaneous determination of many analytes. Without sample derivatisation, the peak of analytes of interest were still able to be retrieved. Overall, this study strengthens the idea that with direct and simple spiking method with the exception of sample hydrolysis and derivatisation, the recoveries in selected drinks samples and in urine were well performed. It is important to note that a direct recovery from drink samples provide a simple, rapid and reproducible result of analysis in forensic laboratories which routinely receive samples for analysis.

In brief, this is the first time that ketamine has been used to explore in a selected drinks in Malaysia. Hence this would be part of the exploratory study for the recovery of ketamine in such drinks. Despite its exploratory scheme, this study offers some insight into the investigation whether ketamine can be used as a surrogate compound for further drug of abuse analysis.

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