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Research article

Determination of Ketamine, Nor-Ketamine, Hydroxynor-ketamine, and Dehydronor-Ketamine by LC-MS/MS in Plasma with the Application of Green Analytical Chemistry Principles

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ABSTRACT

Introduction: Ketamine (KT) is a medication used in veterinary, pediatric, and ophthalmological medicine primarily as an anesthetic. In recent years, it has also been used recreationally and occasionally as a crime-facilitating drug. Additionally, KT exhibits effects similar to classical antidepressant drugs, making it a consideration in depression treatment. In the area of forensic toxicology, sample preparation is a relevant part of the analytical procedure; however, it is essential to consider aspects such as environmental responsibility, the use of clean methods, and sustainable development.

Objective: To optimize and validate a Liquid-Liquid Extraction (LLE) technique and a bioanalytical method based on the principles of Green Analytical Chemistry (GAC) to determine ketamine and some of its biotransformation products (norcetamine, hydroxynorcetamine, and dehydronorcetamine) in human plasma samples using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS).

Materials and methods: The study involved optimizing a bioanalytical method based on GAC principles for the determination of the analytes of interest, utilizing Butyl Acetate (BuAc) as the extracting solvent in LLE.

Results and discussion: The study's results indicate the effectiveness of the developed extraction technique and bioanalytical method, validated by LC-MS/MS, in quantifying plasma levels of KT, NK, HNK, and DHNK. The applicability to human plasma samples was confirmed by the effectiveness and satisfactory confidence limits in determining the analytes of interest. The findings align with conventional literature data, underscoring the method's viability for toxicological analyses. Furthermore, the study emphasizes the importance of environmental responsibility and sustainable development, emphasizing the need for more responsible practices to reduce negative environmental impacts from human activities and preserve natural resources for future generations.

Conclusion: Regarding the application of GAC principles, the extraction technique and bioanalytical method achieved 5 out of its 12 basic principles and can therefore be considered a valid alternative for toxicological analyses.

Keywords: Analytical toxicology; Drug abuse; Liquid-liquid extraction; Liquid chromatography; Mass spectrometry

BACKGROUND

The use of psychoactive substances dates back to the history of civilization, with the earliest human experiences occurring through the consumption of plants. Since the 19th century, there has been the isolation of active plant principles such as morphine, cocaine, and ephedrine. However, it was only in the late 20th century, with the emergence of amphetamines, that a psychoactive substance was entirely produced in the laboratory [1].

Currently, the synthetic drug market is expanding, giving rise to a variety of New Psychoactive Substances (NPS). These substances,

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often designed to circumvent legal regulations, pose unique challenges for toxicological analysis. In this context, attention to environmental responsibility and sustainable development becomes even more relevant, emphasizing the importance of innovative and sustainable analytical methods to address these growing complexities [1].

Regarding the group of NPS, Ketamine (KT) stands out, synthesized from phencyclidine hydrochloride and widely used in veterinary medicine, ophthalmology, and pediatrics. Known as "K," "Special-K," and "Kit-Kat," KT produces effects similar to those observed with phencyclidine use, including visual effects comparable to Lysergic Acid Diethylamide (LSD). Due to its psychedelic effects, the abuse potential of KT is significant and should be considered in drug use and abuse policies [2].

KT gained popularity at a festival in Europe in the 1990s and soon spread to the United States, often combined with other drugs, showing a high prevalence of simultaneous exposure to KT and other substances. Classified as a Crime-Facilitating Drug (CFD) [1].

In 1970, KT was officially approved by the Food and Drug Administration (FDA) as a human anesthetic and is currently used in veterinary and human medicine. Recent discoveries highlight its multiple clinical applications, including the treatment of treatment-resistant depression [3-6]. Preclinical studies show antidepressant effects similar to classic drugs in rodents [3].

KT also demonstrates antidepressant action in patients with bipolar depression [6,7]. (S)-ketamine, administered intravenously and intranasally, has shown efficacy as an antidepressant [8,9]. Additionally, KT is of interest as a therapeutic substance for pain treatment, presenting potential without contraindications and dependence, especially compared to opioids [3].

The global use of Novel Psychoactive Substances (NPS) has increased significantly, driving the constant need to develop methods for the quantification and identification of these analytes in various biological samples. However, the literature lacks information on the pharmacokinetics of Ketamine (KT), especially drug concentrations and its metabolites, Norketamine (NK), Hydroxynorketamine (HNK), and Dehydronorketamine (DHNK), in plasma samples [10].

The importance of developing sustainable analytical methods to ensure human and environmental safety is emphasized [11]. The concept of "green analytical chemistry," introduced since the 1990s, emphasizes reducing environmental impact in chemical analyses and promoting the responsible use of chemicals [12]. Techniques such as miniaturized extraction have been developed in this context.

Sample preparation for analyte determination varies according to the biological matrix, requiring specific considerations for each. The use of less toxic solvents is essential to reduce environmental impact and protect the health of analytical professionals [10]. Additionally, there is a solvent selection guide aligned with the Globally Harmonized System (GHS) and European regulations, assisting in choosing more sustainable solvents for toxicological analyses [13].

Regarding biological samples, for therapeutic drug monitoring, serum and blood plasma are the most common biological matrices, with whole blood being avoided due to concentration differences in red blood cells and plasma [14]. The use of plasma samples in analytical toxicology is preferred due to the availability of references [15].

Despite the development of various methodologies for ketamine analysis in plasma, none of them addresses the simultaneous detection of KT, NK, HNK, and DHNK by LC-MS/MS [16-25]. In this context, the present study aims to optimize and validate a bioanalytical method for the quantification of KT, NK, HNK, and DHNK by LC-MS/MS in human plasma.

MATERIALS AND METHODS

Materials

Standard solution, reagents, and solvents: The HPLC-grade solvents methanol (CAS: 67-56-1, ≥ 99.9%) and acetonitrile (CAS: 75-05-8, ≥ 99.9%) were obtained from J. T. Baker. The HPLC-grade solvent MTBE (tert-butyl methyl ether) (CAS: 1634-04-4, 99.8%) was obtained from Scharlau. The HPLC-grade solvent ethyl acetate (CAS: 141-78-6, ≥ 99.7%) was obtained from Tedia (United States of America), and chloroform (CAS: 67-66-3, 98.8%) was obtained from Exodus Scientific. The standards for KT, NK, DHNK, HNK, and KT-d4 were acquired from Sigma-Aldrich (Saint Louis, USA).

Samples: The plasma samples were provided by the Toxicological Information and Assistance Center of Campinas (CIATox) at the Faculty of Medical Sciences of the State University of Campinas (UNICAMP). In total, two (2) samples were obtained (from intoxication cases) to test the bioanalytical method validated in the present study.

The samples were collected in plastic tubes containing sodium fluoride and EDTA and stored at -20°C from the collection to the analysis. The tubes were identified with a numerical code to preserve the identities of those involved in the research. After collection, the samples were centrifuged at 3,000 RPM for 10 minutes, and a minimum of 1.5 mL of plasma was obtained and transferred to a cryogenic tube, with the remainder stored in another cryogenic tube as a duplicate sample (backup). All samples were stored in a freezer at -20°C until the day of analysis.

Application of the method: The collected samples were subjected to analysis using the validated method, identifying the target analytes when present. The analysis was conducted at the Nucleus of Toxicopharmacological Studies and Research (NEPET) laboratory and at LABFAR-Multiuser Laboratory for Chemical and Biological Analysis for Development and Innovation, at the School of Pharmacy of the Federal University of Goiás. The quantification of Ketamine (KT) and its biotransformation products (NK, HNK, and DHNK) was performed by LC-MS/MS.

Methods

Sample preparation: The Eppendorf-type microtubes were properly identified and organized. First, 200 µL of plasma was added, followed by 100 µL of sodium hydroxide (NaOH) and 1250 µL of ethyl acetate (EtOAc). Afterward, the microtubes were vortexed for 30 seconds and centrifuged at 10,000 revolutions per minute (RPM) for 5 minutes.

Following this step, 1000 µL of the supernatant was transferred to a new microtube, which underwent evaporation under a stream of air at a temperature of 45°C for 30 minutes. After drying, the analytes were reconstituted with 200 µL of mobile phase and vortexed for an additional 30 seconds. Subsequently, centrifugation was performed at 14,000 RPM at 8°C for 10 minutes to eliminate any residues that could interfere with the analysis. Right after, 150 µL

of the supernatant from each tube was collected, transferred to the insert, and taken for analysis using LC-MS/MS.

LC-MS/MS: For the validation of the bioanalytical method, the equipment employed was a Liquid Chromatograph (LC) coupled to a tandem mass spectrometer (MS/MS), QTRAP® 5400 model from SCIEX, available at the Multiuser Laboratory of Chemical and Biological Analysis for Development and Innovation (LABFAR) located at the Pharmacy School (FF) of the Federal University of Goiás (UFG). The software used for system control functions, including chromatographic peak integration, determination of calibration curve equations, and quantification of quality controls and samples, was Analyst® 1.7 (Sciex Singapore).

Chromatographic conditions: For the validation of the bioanalytical method, a C-8 chromatographic column (150 × 4.6 mm, 5 µm) from the ACE brand was used at a temperature of 35°C. The mobile phase consisted of a solution containing 10 mM ammonium formate with 0.01% ammonium hydroxide (pH 8.0): Acetonitrile (65:35, v/v), flowing at a rate of 1 mL/min in isocratic mode, and the total run time was 13 minutes. The injection volume for each analyte was 50 µL at concentrations of 1, 2.5, 5, 25, 50, 100, and 200 ng/mL.

Method validation: After development and optimization, the method was validated based on the establishment of the following parameters: Upper Limit of Quantification (ULoQ), Lower Limit of Quantification (LLoQ), intra- and inter-assay precision, linearity, precision/accuracy, recovery, specificity, residual effect, and verification of processed sample stability. The method was validated following Resolution No. 27, dated May 30, 2012, from ANVISA (Brazilian Health Regulatory Agency).

RESULTS AND DISCUSSION

Determination and quantification of analytes by LC-MS/MS

Standard infusion in mass spectrometer: Initially, the analytical method development was carried out on the Mass Spectrometer (MS), where infusion of KT, NK, HNK, DHNK, and NK-D-4 standards (internal standard) was performed at a concentration of 500 ng/mL. From this infusion, the chromatographic peak profiles, retention times, and transitions (m/z) for each analyte were established (Table 1). Additionally, certain MS parameters were defined. Table 2, displays some of the established parameters.

Table 1: Precursor ions, quantifiers, and qualifiers for ketamine, norcetamine, hydroxynorcetamine, dehydronorcetamine, and deuterated norcetamine.

Analytes	Quantifier ions	Qualifier ions
Ketamine (KT)	238	207,0-220,0
Norketamine (NK)	224,1	207,0-125,1
Hydroxynorketamine (HNK)	240,2	151,3-125,1
Dehydronorketamine (DHNK)	222,1	205,1-177,0
Norketamine-D4 (NK-d4)*	228,0	183,0-211,1

Note: *Internal standard. Source: Own authorship (2024).

Table 2: MS Parameters for the substances KT, NK, HNK, DHNK, and NK-d-4.

Analytes	DP	EP	CE
Ketamine (KT)	50	10	15
Norketamine (NK)	50	10	20
Hydroxynorketamine (HNK)	50	10	35
Dehydronorketamine (DHNK)	50	10	20
Norketamine-D4 (NK-d4)*	50	10	20

Note: *Internal standard. Source: Own authorship (2024).

Bioanalytical method validation

The validation of the bioanalytical method was conducted, and Figures 1-5 depict the chromatograms obtained for each evaluated analyte.

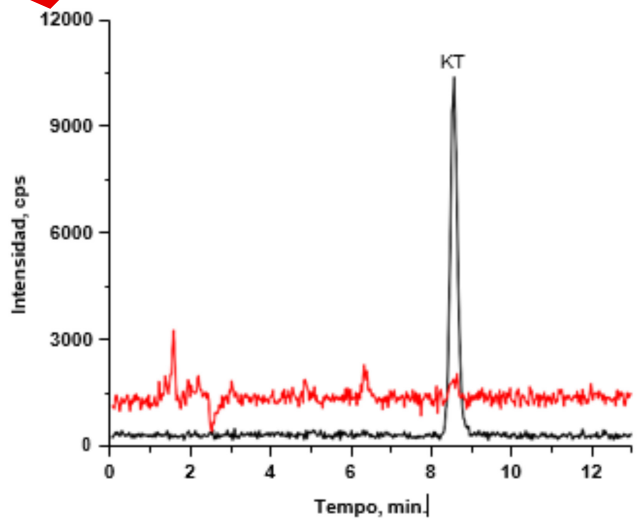

Figure 1: Chromatogram referring to the analyte KT.

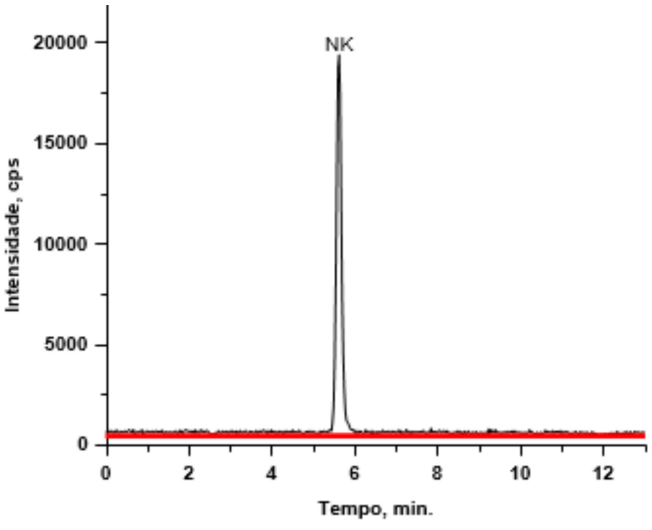

Figure 2: Chromatogram referring to the analyte NK.

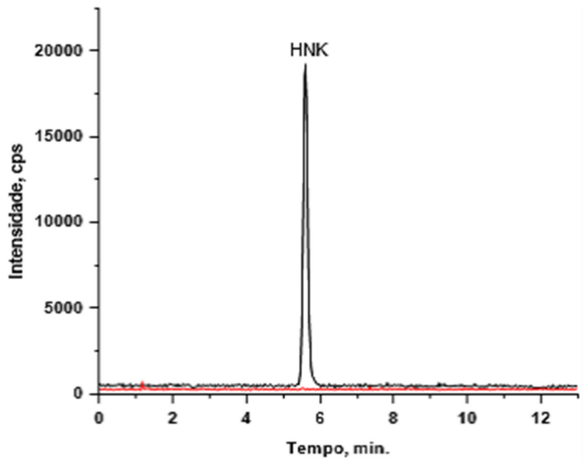

Figure 3: Chromatogram referring to the analyte HNK.

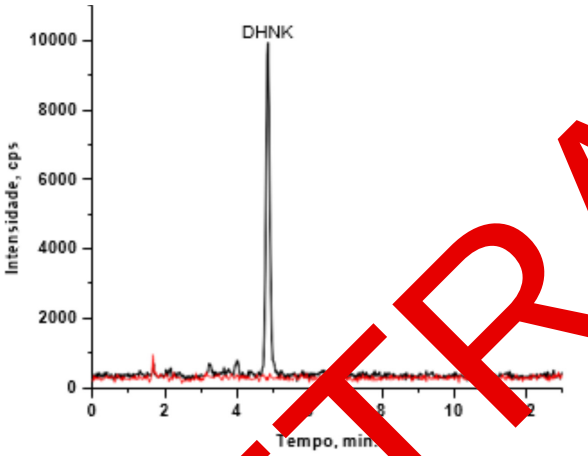

Figure 4: Chromatogram referring to the analyte DHNK.

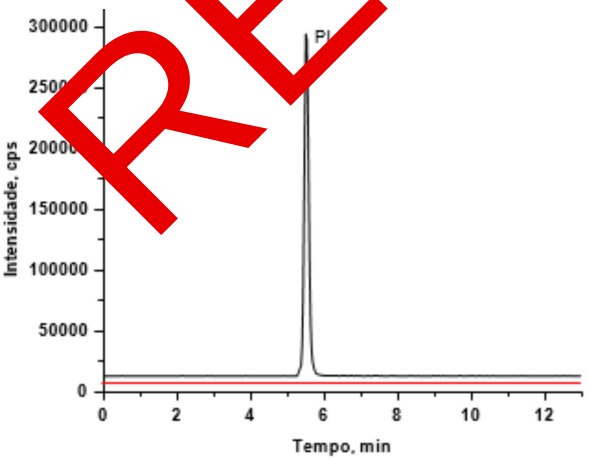

Figure 5: Chromatogram referring to the internal standard (PI).

The confidence limits of the bioanalytical method for the determination of KT (Ketamine), NK (Norketamine), HNK (Hydronorketamine), and DHNK (Dehydronorketamine) were established in accordance with the National Health Surveillance Agency (ANVISA) Resolution (RDC No. 27, May 17, 2012), which outlines the minimum requirements for the validation of bioanalytical methods used in studies for the registration and post-registration of drugs. The validated parameters in the bioanalytical method included recovery, precision and accuracy, stabilities, selectivity, linearity, matrix effect, and residual effect.

Recovery: The choice of the extracting solvent was made through the analysis of the recovery rate of some extracting solvents from liquid-liquid extraction. The solvents that achieved a better recovery rate were Ethyl Acetate (EtOH) and Tert-Butyl Methyl Ether (MTBE), with recovery rates of 72% and 56%, respectively, for ketamine after extraction from human plasma. A study [26], reported similar results in their investigation of Ketamine (KT), Nor-Ketamine (NK), and Dehydronorketamine (DHNK) in human plasma, where a combination of dichloromethane and ethyl acetate as solvents yielded an approximately 90% recovery rate.

Figure 6, presents a comparative chromatogram of Liquid-Liquid Extraction (LLE) technique for determining KT in human plasma, using two organic solvents (ethyl acetate and MTBE).

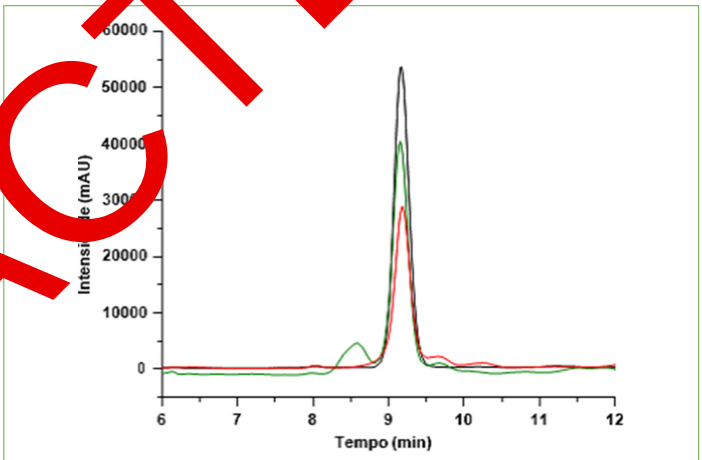

Figure 6: Overlaid chromatograms comparing the extraction technique using two different organic solvents with a standard KT solution (10 µg/mL) of KT in human plasma. **Note:** (–) Solution; (–) Ethyl Acetate; (–) MTBE

Therefore, the chosen extracting solvent for determining KT and its biotransformation products in human plasma was ethyl acetate. The recovery study for the extraction procedure using this extracting solvent at a basic pH resulted in values close to 100% yield for KT, NK, and HNK. For DHNK, the yield was 49%. In a quantitative study [27], of KT and its biotransformation products in human urine samples using the Liquid-Phase Microextraction (LPME) technique, the recovery value for the analyte DHNK was also lower (69.7%) compared to KT and NK.

Precision and accuracy: For the establishment of method accuracy, it is necessary to determine its limit of variation and specificity, in addition to its linearity. To achieve this, a minimum of nine determinations should be performed, covering the range of variation of the procedure, with three concentrations (low, medium, and high) carried out in triplicate. Tables 3-6, present the analytical parameters for precision and accuracy validation (intra and inter-day) for the qualification of KT, NK, HNK, and DHNK, respectively, in human plasma. All obtained results fell within the limits and specifications recommended by RDC No. 27/2012, ensuring proper precision and accuracy of the present bioanalytical method. Intra- and inter-assay precision and accuracy values resulted in coefficients of variation below 15% for the controls, deemed satisfactory for the bioanalytical method. The result obtained in the LLOQ (Lower Limit of Quantification) was also satisfactory, as RDC No. 27/2012 allows values equal to or less than 20% for this parameter.

Table 3: Analytical validation parameters (intra and inter-day precision and accuracy) in LC-MS/MS for the quantification of KT in human plasma.

		Intra-day (n=5)			Inter-day (n=5)	
Level	[ ] ng/mL	Batch	Precision (CV%)	Accuracy (CV%)	Precision (CV%)	Accuracy (CV%)
LIQ	1	1	11.0	100.5	13.[illegible]	101.7
		2	19.1	93.4		
		3	7.7	111.[illegible]		
CQB	3	1	5.3	[illegible]9.7	9.3	103.6
		2	12.9	[illegible]6.8		
		3	6.0	104.[illegible]		
CQM	80	1	2.0	97.5	5.4	100.1
		2	7.0	101.2		
		3	5.7	102.0		
CQA	160	1	[illegible]	95.4	6.8	99.8
		2	[illegible]	97.4		
		[illegible]	3.8	108.3		
CQD	320	1	3.5	93.7	7.5	99.1
		2	8.7	99.2		
		3	4.8	105.7		

Note: KT: Ketamine; CV: Coefficient of Variation; [ ]: Concentration; LIQ: Lower Limit of Quantification; CQB: Low-Quality Control; CQM: Medium-Quality Control; CQA: High-Quality Control; CQD: Diluted Quality Control. Source: Author's own (2024).

Table 4: Analytical validation parameters (intra and inter-day precision and accuracy) in LC-MS/MS for the quantification of NK in human plasma.

		Intra-day (n=5)			Inter-day (n=5)	
Level	[ ] ng/mL	Level	[ ] ng/mL	Level	[ ] ng/mL	Level
LIQ	1	1	8.4	101.9	8.1	105.2
		2	8.0	101.5		
		3	4.1	112.2		
CQB	3	1	5.2	102.7	6.4	104.8
		2	5.9	100.8		
		3	4.1	110.8		
CQM	80	1	3.1	99.1	3.9	102.0
		2	4.2	102.1		
		3	2.5	104.9		
CQA	160	1	2.2	99.8	3.7	101.8
		2	4.5	101.2		
		3	2.6	104.9		
CQD	320	1	1.8	98.7	5.5	98.5
		2	6.2	94.1		
		3	3.0	103.8		

Note: KT: Ketamine; CV: Coefficient of Variation; [ ]: Concentration; LIQ: Lower Limit of Quantification; CQB: Low-Quality Control; CQM: Medium-Quality Control; CQA: High-Quality Control; CQD: Diluted Quality Control. Source: Author's own (2024).

Table 5: Analytical validation parameters (intra and inter-day precision and accuracy) in LC-MS/MS for the quantification of HNK in human plasma.

		Intra-day (n=5)			Inter-day (n=5)	
Level	[ ] ng/mL	Level	[ ] ng/mL	Level	[ ] ng/mL	Level
LIQ	1	1	9.4	105.2	9.2	104.6
		2	12.5	105.1		
		3	7.0	103.5		
CQB	3	1	12.9	104.7	9.7	104.8
		2	9.5	99.2		
		3	2.8	110		
CQM	80	1	9.3		9.4	104.6
		2	11.3	112.0		
		3	2.2	98.7		
CQA	160	1	5.2	99	4.1	99.6
		2	4.4	8		
		3	3.	99.6		
CQD	320	1	5.1	98.7	7.7	98.0
		2	1	98.3		
		3	3.4	97.0		

Note: KT: Ketamine; CV: Coefficient of Vari on; [ ]: Co tration; LIQ: Lower Limit of Quantification; CQB: Low-Quality Control; CQM: Medium-Quality Control; CQA: High-Quality Con CQD: Dilute uality Control. Source: Author's own (2024).

Table 6: Analytical validation par eters (intra and i -day precision and accuracy) in LC-MS/MS for the quantification of DHNK in human plasma.

		Intra-day (n=5)			Inter-day (n=5)	
Level	[ ] ng	Level	[ ] ng/mL	Level	[ ] ng/mL	Level
LIQ		1	10.0	108.3	9.6	104.6
		2	7.0	105.1		
		3	11.1	98.9		
CQB	3	1	10.7	104.2	9.1	104.8
		2	8.5	98.2		
		3	8.3	105.1		
CQM	80	1	4.0	104.0	9.4	97.5
		2	6.8	93.0		
		3	7.7	95.6		
CQA	160	1	5.0	113.9	4.1	106.4
		2	4.4	103.4		
		3	8.2	100.9		
CQD	320	1	2.9	110.1	7.7	100.9
		2	6.0	93.7		
		3	6.3	98.6		

Note: KT: Ketamine; CV: Coefficient of Variation; [ ]: Concentration; LIQ: Lower Limit of Quantification; CQB: Low-Quality Control; CQM: Medium-Quality Control; CQA: High-Quality Control; CQD: Diluted Quality Control. Source: Author's own (2024).

Stability: The stability of KT, NK, HNK, and DNK was assessed using Low and High-Quality Control (CQ) levels. In order to ensure the stability of the samples throughout the study, the deviation between the theoretical and experimental values found in different sample processing was calculated.

CQ samples were analyzed in triplicate using a newly prepared calibration curve, following various storage conditions (0h, 3h-Room Temperature (RT), 10h, and 12 months) and concentration levels (CQB=3 ng/mL and CQA=160 ng/mL). For each experiment, the variation in the average concentration should be<15% of the nominal value. Table 7, presents the stability parameters of the bioanalytical method, as well as the respective times and temperature conditions.

Table 7: Stability parameters of the bioanalytical method for the determination of KT, NK, HNK, and DHNK in human plasma.

Analyte	[ ] (ng/mL)	Short duration (TA) 0 h to 3 h	Freezing/ Thawing (3 cycles)	Post-processing (15°C) 10 h	Long duration (-20°C) 12 months
KT	3	109.9	114.6	101.1	98.1
	160	94.4	91.4	107.3	99.7
NK	3	106.5	116.7	112.5	90.2
	160	100.5	97.7	112.9	87.[illegible]
HNK	3	94.9	106.9	105.8	90.5
	160	97.3	93.6	[illegible].9	[illegible]03.0
DHNK	3	124.3	106.8	[illegible].7	102.9
	160	138.0	[illegible]	109.7	86.2

Note: Ketamine: KT; Norke[illegible]mine: NK; [illegible]ydroxynorketamine: HNK; Dehydronorketamine: [illegible]HNK; RT: Room [illegible]emperature; [ ]: Concentration. Source: Ow[illegible] authorsh[illegible] (2022).

The stabilities of the anal[illegible]KT, NK, [illegible]d HNK were ensured and remained wi[illegible] deviati[illegible] not [illegible]ceeding 15% of the mean concentration obtain[illegible] compa[illegible] to the nominal value, as required by [illegible]DC No[illegible]7/2012 [27]. In one study [28], KT was shown to be s[illegible]le [illegible] plasma samples stored at -20°C for up to 3 months. In anoth[illegible] study [29], it was reported that KT, NK, and DHKN were stable [illegible] plasma samples stored at -20°C for up to 2 months. In the present study, an attempt was made to cover a longer time interval for long-term stability analysis (12 months), and the results were satisfactory, as all analytes remained stable at -20°C, even after a period up to 6 times longer compared to the aforementioned studies.

A group of authors [23], observed that KT, HNK, and DHNK remained stable in plasma at room temperature for at least 24 h and at 4°C for 48 h. The samples also remained stable in the autosampler rack when kept at 15°C for 48 h and after three freeze/thaw cycles, conditions similar to those to which the samples in the present study were subjected.

In the present study, only the short-term stability (0 to 3 h) of DHNK was found to be failed, as higher concentrations were found compared to the results obtained under other evaluated conditions. There is no similar information in the literature regarding an increase in the concentration of this metabolite under similar ambient conditions. It is emphasized that this parameter was redone and reassessed to rule out the possibility of contamination during the analyst's solution handling, but this hypothesis was discarded as DHNK levels remained high.

For the purpose of result comparison, little has been reported on the stability of these biotransformation products in human blood samples, and therefore, a more in-depth analysis of the occurrence of increased concentration only for DHNK in short-term stability is compromised.

Selectivity: The ability of the bi[illegible]nalytical met[illegible]d to assess the analyte, distinguishing it [illegible]rom [illegible]er endoge[illegible]ous substances present in the biological [illegible]trix, was [illegible]luated [illegible]or this purpose, different blank plasma [illegible]mples (n[illegible]rmal, [illegible]lyzed, lipemic) from six distinct sources w[illegible] used [illegible]xtracted from standard solutions and subsequently [illegible]bject[illegible] the esta[illegible]shed extraction process.

The method [illegible]monstrated [illegible]ct[illegible]ity/specificity for NK, HNK, and DHN[illegible], as [illegible]re were no [illegible]nterfering peaks of endogenous substances at the re[illegible]ntion time of the compound of interest. Ho[illegible]ver, the method [illegible]d not show selectivity for KT, requiring a[illegible]ditional repetitions (Table 8).

Table 8: Interfer[illegible]ce Percentage (variation range, n=6) in the retention ti[illegible]s of each a[illegible]lyte and the internal standard, KT, NK, HNK, and DH[illegible] h[illegible]n plasma.

Analytes	Interference (%)
KT	25,3 (14,3-37,2)
NK	2,1 (1,0-3,2)
HNK	1,8 (0,5-3,6)
DHNK	1,0 (0,5-2,2)
NK-D4	0,1 (0,0 -0,1)

Note: Ketamine: KT; Norketamine: NK; Hydroxynorketamine: HNK; Dehydronorketamine: DHNK; NK-D4: deuterated norketamine. Source: Own authorship (2024).

According to RDC No. 27/2012 [30], interference of up to 20% of the analyte response and 5% of the internal standard response is considered acceptable. The interference percentage of the analyte KT in the sample exceeded the acceptable limit (25.3%). However, as shown in the range of variation for this analyte based on the results (14.3-37.2), some plasma samples obtained an acceptable interference value and met the resolution specifications, and these were used in the validation process. Additionally, according to the FDA's bioanalytical method validation guidance for industry regarding recommendations and acceptance criteria for bioanalytical method validation and conduct in studies, the interference limit may correspond to ± 20% of the lower limit of quantification.

Linearity: The method's linearity was successfully conducted over a concentration range of 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL, which were analyzed in triplicate. The linearity study within the proposed concentration range for the execution of the work showed an appropriate correlation coefficient (r>0.99) in the range of 1-200 ng/nL for KT, NK, HNK, and DHNK (Table 9).

Table 9: Parameters related to the calibration curve of the bioanalytical method for the determination of KT, NK, HNK, and DHNK in human plasma.

Analytes	Linearity (ng/mL)
Ketamine (KT)	Y=0,043x+0,146 (r^2=0,9942)
Norketamine (NK)	Y=0,043x+0,146 (r^2=0,9801)
Hydroxynorketamine (HNK)	Y=0,043x+0,146 (r^2=0,9698)
Dehydronorketamine (DHNK)	Y=0,043x+0,146 (r^2=0,9829)

Note: Ketamine: KT; Norketamine: NK; Hydroxynorketamine: HNK; Dehydronorketamine: DHNK; r^2: Correlation Coefficient. Source: Own authorship (2024).

Matrix effect: The Coefficient of Variation (CV%) for FMN relative to the samples should be less than 15%. In the present study, 8 plasma samples from different sources were analyzed, including 4 normal, 2 lipemic, and 2 hemolyzed samples. The plasma samples were processed, and subsequently, the analyte, internal standard, and solutions were added at the same concentrations as the CQB and CQA samples. Table 10, presents the results obtained for each analyte.

Table 10: Matrix effect results evaluated in the bioanalytical method for the determination of KT, NK, HNK, and DHNK in human plasma.

Analytes	Matrix effect (CV%) (ng/mL)
KT	6,4
NK	0,9
HNK	10,0
DHNK	1,1

Note: Ketamine: KT; Norketamine: NK; Hydroxynorketamine: HNK; Dehydronorketamine: DHNK; CV: Coefficient of Variation. Source: Own authorship (2024).

The CV% for the matrix effect of each analyte was less than 15%, and therefore, they are suitable for the determination of KT, NK, HNK, and DHNK. The matrix effect is a study aimed at investigating the presence of possible interferents from the biological matrix's own constituents, which can basically cause phenomena of either reduction or amplification of the analyte or internal standard response during the analytical run. For each sample, the Matrix Factor normalized by the internal standard (FMN) should be determined.

Residual effect: Three injections of the same blank sample were analyzed, one before and two immediately after the injection of one or more samples processed in LC-MS/MS. The results were compared with those obtained from samples processed in liquid (LIQ), and therefore, they were satisfactory.

Application of the method to real samples

In total, two post-mortem plasma samples were collected, originating from referrals for toxicological examination at the Forensic Toxicology Unit (NTF) of the São Paulo State Scientific Police Superintendence and the Forensic Toxicology Laboratory of the Public Security Department of Sergipe (LATOX). The results of the analysis of these samples are presented in Table 11.

Table 11: Result of the determination of KT, NK, HNK, and DHNK in the two plasma samples using the validated bioanalytical method.

Analytes	Sample 1 (ng.mL)	Sample 2 (ng.mL)
KT	140,8	2,6
NK	45,5	1,0
HNK	8,9	<LIQ
DHNK	7,1	<LIQ

Note: Ketamine: KT; Norketamine: NK; Hydroxynorketamine: HNK; Dehydronorketamine: DHNK; LIQ: Limit of Quantification. Source: Own authorship (2024).

As observed in Table 11, sample 1 exhibited concentrations above the LOQ for all evaluated analytes. On the other hand, sample 2 showed concentrations above the LOQ only for KT and NK, with levels of HNK and DHNK in the samples below the LOQ. Due to its more hydrophilic nature, DHNK has less affinity for the employed organic solvent and is consequently less effectively solubilized compared to other analytes.

NK was detected in both samples and represents the primary active biotransformation product of KT. Additionally, NK is responsible for 1/5 to 1/4 of KT's potency, and its detection in samples allows discrimination between active use and external contamination. KT undergoes extensive metabolism, initially being metabolized into NK, which is subsequently metabolized to form DHNK and HNK [31]. Thus, the presence of NK supports the understanding that the biological samples originate from active use.

Traditionally, biological samples collected from a victim with possible intoxication are analyzed to provide evidence of drug administration. However, the rapid metabolism of many drugs, coupled with delays in collection and analysis, can compromise the detection of such substances, which may have occurred with sample 2.

Possibly, sample 1 may have a shorter time interval between drug administration and sample collection, while sample 2 may have been collected over a longer time interval between drug administration and collection.

Nevertheless, even with a limited number of biological samples (n=2) for applying the method to real samples, the effectiveness of the developed bioanalytical method for the determination and quantification of KT, NK, HNK, DHNK in human plasma by LC-MS/MS was demonstrated. Thus, the described method has the necessary characteristics to monitor drug plasma concentrations after the administration of low doses of KT.

Furthermore, post-mortem changes can affect drug metabolism, varying in extent among different drugs. Heroin and cocaine, for example, are rapidly converted into their hydrolytic products during life and, after death, undergo rapid in situ bioconversion. Results from post-mortem specimens should be interpreted with caution.

In this regard, key factors include the state and quality of the specimen, drug stability, effects of tissue decomposition, and ambient temperature. Some drugs, like benzodiazepines, are converted by anaerobic bacterial action, impacting post-mortem detection. Others, such as cocaine and benzoylecgonine, degrade over time in blood samples.

Moreover, the stability of some drugs can be maintained if stored at low temperatures, while others, like morphine, show significant losses under inappropriate storage or post-mortem conditions. Variations in the ratio of morphine to morphine glucuronides among different blood collection sites indicate that concentrations may have changed substantially since death, especially in early stages of putrefaction or prolonged storage.

Application of GAC principles

In this study, the implementation of High-Performance Liquid Chromatography (HPLC) in conjunction with Liquid-Liquid Extraction (LLE) in the development of the bioanalytical method allowed the incorporation of GAC principles 1, 5, 6, 8, and 12 [10]. This approach encompassed waste prevention, the use of less toxic auxiliary solvents, the pursuit of energy efficiency, the avoidance of unnecessary derivatization, and the selection of more sustainable solvents. These decisions were made with the aim of minimizing the potential for chemical accidents, underscoring the study's commitment to the principles of green analytical chemistry.

A challenging aspect of the current study was achieving a balance between the quality of chemical analyses and environmental parameters. However, efforts were made to adhere as closely as possible to the principles established in the GAC [10]. In this regard, for the development of the current bioanalytical method, Ethyl Acetate (EtOAc) was chosen as the extracting solvent in the LLE.

CONCLUSION

From the results obtained in this study, it was possible to infer that the extraction technique and the developed and validated bioanalytical method proved to be efficient in quantifying the plasma levels of KT, NK, HNK, and DHNK by LC-MS/MS. Furthermore, the method can be applied to real samples, as it was effective and demonstrated satisfactory confidence limits in determining the analytes of interest in real human plasma samples. When compared, the results of this study are consistent with conventional literature data.

Many chemical processes can lead to significant environmental impacts. From this study, it is concluded that the appreciation of aspects such as environmental responsibility, the use of "green" alternatives, and sustainable development are of utmost importance to ensure a more sustainable and balanced future for the next generations. It is essential for companies and society as a whole to adopt more responsible and sustainable practices, aiming to reduce the negative environmental impacts caused by human activities and ensure the preservation of natural resources for future generations.

In the optimization process, this study can be considered a viable alternative for toxicological analyses, as principles of Green Analytical Chemistry (GAC) were applied. Thus, the bioanalytical method achieved 5 out of 12 basic principles of GAC, including: Avoiding waste production; using safer solvents; seeking energy efficiency; avoiding the formation of derivatives (derivatization); and using substances with lower toxic potential to reduce the potential for chemical accidents.

In summary, environmental education is essential to raise awareness among the population about the importance of environmental preservation and sustainable practices. Therefore, a change in mindset and behavior regarding environmental preservation and the use of cleaner alternative methods is necessary. Only through such changes can a more sustainable and balanced future be ensured for the next generations.

DECLERATION

Ethical considerations

This study was approved by the Research Ethics Committee of the Faculty of Pharmaceutical Sciences at the University of São Paulo (Opinion Number: 4.831.168) and by the Research Ethics Committee of the State University of Campinas (UNICAMP)–Campinas campus (Opinion Number: 4.76[illegible]).

Waiver of Informed Consent was requested, and as justification, post-mortem blood samples collected for toxicological examination at the Forensic Toxicology Unit (UTF) of the São Paulo State Scientific and Technical Police Superintendence and the Forensic Toxicology Laboratory of the State of Sergipe (LATOX) were utilized for method application with real samples.

Official examinations on the samples had already been concluded, and the respective forensic report had been issued before the samples were used in this study, at which point the samples were slated for disposal. As the identification data of patients, police report numbers, or judicial process information were not relevant to the study, no access was made to such information. Consequently, the study was conducted with assurance of confidentiality and anonymity for the cases analyzed.

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