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Simultaneous Quantitative LC-MS Method of Ketamine, Midazolam and their Metabolites (Dehydronorketamine, Norketamine and 1-Hydroxymidazolam) for its Application in Patients on Extracorporeal Membrane Oxygenation (ECMO) Therapy

Ankit Rochani^a, Edwin Lam^b, Julian Tanjuakio^b, Hitoshi Hirose^c, Walter K. Kraft^{b,c}, Gagan Kaushal^a

^aDepartment of Pharmaceutical Science, Thomas Jefferson University, Philadelphia, Pennsylvania, USA

^bDepartment of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, Pennsylvania, USA

^cDepartment of Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania, USA

Abstract

Ketamine (Ket) and midazolam (MDZ) are commonly administered drugs in the intensive care setting for analgesia and sedation. Ket and MDZ are metabolized to dehydro-norketamine (DHNK), nor-ketamine (NK) and 1-hydroxy midazolam (1HMDZ). Limited studies evaluating their pharmacokinetics exist in patients on extracorporeal membrane oxygenation (ECMO) therapy. Therefore, we developed a quantitative, high-performance liquid chromatography-mass spectrometry (with single ion monitoring) method to simultaneously detect Ket, MDZ and their (DHNK, NK and 1HMDZ) metabolites in human plasma. Considerable sensitivity was obtained for the analytes using a C₁₈ HILIC column operated by a high-performance liquid chromatography system coupled with a Thermo Exactive Orbitrap mass spectrometer. Calibration curves were developed for analyte molecules (n = 5) in the presence of carbamazepine (CBZ) as an internal standard. The lower limits of quantitation (LLOQ) for Ket and MDZ were 20 and 10 ng/mL, respectively with the LLOQ for DHNK, NK and 1HMDZ at 470, 320 and 150 ng/mL. Moreover, the percent coefficient of variance and precision for inter- and intra-day runs were within the standards set forth by the ICH and FDA guidelines. This method is sensitive and has been successfully applied to an ongoing pharmacokinetic study in patients on ECMO therapy.

Corresponding Author: Dr. Gagan Kaushal, Associate Professor, Department of Pharmaceutical Science, Jefferson College of Pharmacy, Thomas Jefferson University, Philadelphia, Pennsylvania (PA)-19107, United States, Phone: +1 (215) 503-2025, gagan.kaushal@jefferson.edu.

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⁷-Conflict of Interest
None

Graphical Abstract

Simultaneous quantitation of Ket, MDZ and their metabolites from human plasma and application of the method for studying kinetics of drugs in a critically ill patient on ECMO therapy.

Keywords

ECMO patients; ketamine; midazolam; metabolites; pharmacokinetics; LCMS; clinical trial

1. Introduction

Extracorporeal membrane oxygenation (ECMO) provides mechanical cardiopulmonary support in patients with refractory cardiopulmonary failure [1]. The utility of ECMO therapy is increasing nationally and internationally [2]. Due to critical illness, patients on ECMO are treated in the intensive care unit (ICU) where drugs for analgesia and sedation are used to promote pain relief and comfort. Analgesics and sedatives are selected based on institutional-specific protocols and are used to reduce pain from the surgical procedure, relieve anxiety and stress, and prevent agitation-related harm during mechanical ventilation and ECMO circuit. Centers that perform ECMO most frequently use morphine and fentanyl for pain management [3, 4]. Although opioids make up the backbone for pain management in critically ill adults, concerns about safety leading to worsening outcomes following ICU admission have led clinicians to seek alternative strategies to opioid use that can maintain adequate analgesia [5].

Ketamine (Ket) is a cyclohexylamine derivative and is a dissociative anesthetic capable of inducing analgesia at low doses. At high doses, the drug can produce both analgesia and anesthesia. It non-competitively antagonizes the N-methyl-D-aspartate receptors centrally producing amnesia and analgesia. Low-dose Ket is currently recommended as an adjunct to opioid therapy to reduce the doses of opioids [5]. In observational studies, low-dose Ket combined with standard care, reduced sedative and opioid requirements during ECMO therapy [6]. However, these results may be conflicting due to varying pain and sedation practices among institutions [7].

Midazolam (MDZ) is a sedative-hypnotic belonging to the class of benzodiazepines and is the most common sedative used among ECMO centers [3, 4]. MDZ activates the gamma-aminobutyric acid receptors, producing anxiolytic, sedative, hypnotic, and anticonvulsant effects.

Pain and sedation management in critically ill patients on ECMO is complex due to the altered pharmacokinetics of medicines used in this population [8]. There is substantial

interpatient variability as patients may present with varying degrees of organ impairment, fluctuating hemodynamics, and additional extracorporeal circuits (e.g. continuous renal replacement). Furthermore, drug adsorption and sequestration into the circuitry are considerable and impact the therapeutic effect of drugs given during ECMO [9, 10]. With limited evidence on the optimal dosing in this patient population pharmacokinetic studies are necessary in order to determine the impact of ECMO therapy on Ket and MDZ.

Developing a simultaneous quantitation method to estimate Ket and MDZ can be a bioanalytical challenge. There are reports about the development of high-performance liquid chromatography (HPLC)/ fast atom bombardment (LC-FAB MS) based methods for the quantitation of MDZ and its metabolite in human body fluids [11]. However, few studies report the quantitation of Ket and its metabolites using liquid chromatography-mass spectrometry (LCMS) based estimations [12, 13]. With the development of newer, sensitive LC-MS tandem mass spectrometer systems, it is possible to have sensitive detection at low concentration ranges. NosScir *et al* [14] reported a LC-MS based method for the simultaneous estimation of Ket, lorazepam, MDZ, and sufentanil. This is the best known method for the simultaneous estimation of Ket and MDZ in the presence of other drug molecules for therapeutic drug monitoring.

Ket and MDZ are metabolized in-vivo to their active metabolites through the cytochrome (CYP) P450 enzymes. There are limited studies that show the individual profile of these two drugs and their metabolites [14–16].

The purpose of this work was to develop a sensitive and robust LCMS method that is capable of simultaneously detecting and measuring Ket, MDZ and their metabolites (dehydronorketamine (DHNK), norketamine (NK) and 1hydroxymidazolam (1HMDZ)) in human plasma. As patients in the ICU are poly-medicated and limited in sampling blood volume, there is a need for a practical and economical method for simultaneous detection of these two compounds and their metabolites in human plasma. We describe the method development, its validation using human plasma, and its application to a prospective pharmacokinetic study in critically ill patients on ECMO therapy.

2.0 Materials & Methods

2.1 Chemicals & Reagents

Ket, MDZ and DHNK reference standards were procured from Sigma-Aldrich Corp. (Round Rock, TX, USA). NK and 1HDMZ standards were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA), Carbamazepine (CBZ) was obtained from Acros Organics (New Jersey, USA). LC-MS-grade acetonitrile (ACN), water (with and without 0.1% formic acid (FA)), and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Blank human plasma was obtained from Thomas Jefferson University Hospital Blood Donor Center (Philadelphia, PA, USA). Patient samples were obtained from patients for whom informed consent was provided in a clinical trial approved by the Thomas Jefferson University Institutional Review Board (IRB: 17D.617).

2.2 Liquid chromatography conditions

Analysis was performed using a Dionex Ultimate 3000 HPLC system (Thermo Fisher, Waltham, MA) attached with Thermo Orbitrap mass spectrometer. The mobile phase consisted of 0.1% aqueous formic acid and acetonitrile with 0.1% formic acid (70:30, %v/v). The injection volume was 5 μ L with a flow rate of 0.250 mL/min. The column temperature was maintained at 30°C. The data acquisition from mass spectrometry was delayed for 2 minutes followed by 8 minutes of data acquisition for a total of 10 minutes run time. The analysis was performed under a single ion monitoring (SIM) mode with a heated ion transfer capillary, Exactive (v.1.1SP6) software was used for mass spectrometry method development and data acquisition. Thermo Xcaliber (v. 3.0.63) was used for integration of Chromeleon, and Exactive was used for sample injections and LCMS data acquisition in .raw file format.

2.3 Mass spectrometry conditions

LC-MS analyses were performed on a Thermo Scientific Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). A scanning mass range of 200–345 m/z was used. All the scans were performed under positive ion mode and ionization was achieved using electron spray ionization. Sheath gas flow rate was maintained at 30 psi; auxiliary gas flow rate was maintained at 5 psi; electron spray voltage used for the ionization was kept at 3.5 kV; and capillary temperature was set to 375°C. Capillary, tube lens, and skimmer voltages were kept at 67.50 V, 130.0 V and 28 V, respectively. These parameters were selected and saved as direct infusion method for using Thermo Exactive (v. 1.1 Sp6) software of Thermo Exactive Orbitrap Mass spec. This method was integrated with HPLC method to create a single method for LCMS runs.

2.4 Sample preparation for Ket and MDZ

A primary stock solution containing Ket and MDZ was prepared in the mobile phase. A secondary stock was prepared in 70:30 water and acetonitrile with 0.1% FA at concentrations ranging from 220–11,000 ng/mL. The secondary stock was stored in –20°C for preparing plasma calibration standards for further analysis. These stocks were reconstituted with blank plasma to obtain the final calibration standards of 10–2,000 ng/mL. CBZ, used as an internal standard (IS), was added to each standard at a constant concentration of 1 μ g/mL.

Liquid-liquid extraction was used for the analysis of Ket and MDZ in plasma. Calibration samples consisted of Ket, MDZ, and CBZ in human plasma. Together with the test plasma samples, protein extraction was carried out using acetonitrile and 0.1% FA (1:3). Samples were vortexed for 30–40 seconds and centrifuged for 20 minutes at 15,700 g. Following this, 100 μ L of clear supernatant was used for carrying out LC-MS analysis. This sample preparation procedure was used for the calibration standards, quality control (QC) standards, and patient plasma samples.

2.5 Sample preparation for DHNK, NK and 1HMDZ

For developing linearity curve, stock of DHNK, NK and 1HMDZ were prepared in ACN 0.1%FA (10 μ g/mL) mix. This stock was spiked in human plasma for making DHNK (400–25,000 ng/mL), NK (300–25,000 ng/mL) and 1HMDZ (100–25,000 ng/mL) calibration

standards in human plasma. The calibration standards and patient samples were spiked with CBZ (1µg/mL) and extracted using standard protein precipitation method by using 1 part of plasma and 3 parts of ACN 0.1% FA. Samples and standards were centrifuged at 15,700 g, 15min and 100µl clear supernatant was subjected to LCMS runs.

2.6 Linearity for Ket, MDZ and their metabolites

The linearity of the standard calibration curve for Ket, MDZ, DHNK, NK and 1HMDZ was evaluated in the presence of CBZ. Linearity curves was established for Ket, MDZ (n = 4 runs) and their metabolites (n = 6 runs) for the freshly prepared samples and injected on two independent days. The calibration standard runs consisted of acetonitrile 0.1% FA, plasma blank extract, and calibrator points followed by a blank. The acceptable mean value of estimated concentration (or % accuracy) in calibration and validation standards was kept within ± 15 –20% of the theoretical value [17]. The accuracy around the mean value for the lower limit of quantitation (LLOQ) was set within $\pm 10\%$ of coefficient of variance (CV), whereas, for other concentration points beyond the LLOQ, the precision limit was set to $\pm 5\%$ CV [16]. At least 75% of the non-zero standards should meet these criteria and the linear coefficient (r^2) should be near to or more than 0.99 [16]. Linear regression ($y = mx + c$) equation, was obtained from concentration versus area for standards extracted from spiked plasma. This equation was used to determine concentration of Ket, MDZ, DHNK, NK and 1HMDZ in ECMO patients.

2.7 Validation & Stability for Ket, MDZ, DHNK, NK and 1HDMZ quantitation

Method validation was performed according to the ICH and FDA guidelines for bioanalytical method validation [17]. The method was validated for inter- and intra-day accuracy, precision, and stability. This was achieved using quality control (QC) samples with concentrations ranging from 20, 40, 900 and 1600 ng/mL for Ket and 30, 40, 900 and 1600 ng/mL for MDZ. Similarly, random validations standards for NK (320, 600, 900, 9000 and 21000 ng/mL), DHNK (470, 600, 900, 9000, and 21000 ng/mL) and 1HMDZ (150, 500, 1000, 9000 and 21000 ng/mL) were also measured. Readings were taken on the first and second day to estimate the inter- and intra-day precision and accuracy after an overnight freeze-thaw before LC-MS injections for both parent (n = 4 runs) and metabolite (n = 6 runs) standards.

2.8 Clinical Application of the Assay

This assay was successfully applied to a single-site observational pharmacokinetic study of Ket and MDZ in critically ill patients on ECMO therapy. A continuous infusion of Ket and MDZ was administered in accordance with institutional protocol for sedation and adjunct pain management, respectively. MDZ continuous infusion was initiated at 2 mg/hour with a total of 35 boluses given. Rate adjustments ranged from 0.75–14 mg/hour. Ket was administered as a 10 mg initial bolus followed by 75 mg/hour infusion for 19 days. Adjustments to the Ket infusion rate ranged from 3–160 mg/hour. Following informed consent, arterial blood sampling was obtained for analysis. Whole blood (4 mL) was collected into tubes coated with K2 EDTA and immediately placed on ice. Blood samples were then centrifuged at 2,200 rpm for 15 minutes at 4°C. The plasma supernatant was then

collected and stored at -80°C until analysis. The sampling period ranged from 1–552 hours beginning with the start to the end of the infusion during the patient's hospital course.

3.0 Results & Discussion

Ket and MDZ are water soluble. However, they have variable aqueous solubility characteristics that could be used for developing an efficient chromatographic method. In-silico analysis of Ket and MDZ (using Chem-Axon tools) show that they have pK_a values of 7.6 and 6.19 (shown in Supplementary figure 1), respectively. They also exhibit differences in their aqueous solubility in acidic pH (1 to 5) range. Hence, using an appropriate organic and aqueous solvent ratio with an appropriate column chemistry and flow rate could provide good separation of two molecules for clinical application.

3.1 Method Development for Simultaneous Qualitative Estimation of Ket and MDZ

Moorthy *et al* [16] developed an LC-MS-based quantitation assay for the estimation of MDZ and its metabolites in pediatric patients. This method was based on solid-phase extraction and it was established using a relatively less-sensitive mass spectrometer. Later, Nossier *et al* [14] developed an LC-MS/MS method for the quantitative estimation of Ket, MDZ, and Sufentanil in critically ill patients. As a next step, it was important to develop a simple and economical LC-MS-based quantitation method for Ket and MDZ using an HPLC-high resolution mass spectrometer (HRMS) that is comparable or perhaps better than the single reaction monitoring (SRM) method of triple quadrupole mass spectrometers [18]. In the present study, the LC-MS quantitation assay for Ket and MDZ was developed and applied to determine the pharmacokinetic profile in the plasma samples of critically ill patients on ECMO.

Initially, a qualitative method to determine Ket and MDZ was developed on Thermo Exactive Orbitrap mass spectrometer. Here, $1\mu\text{g/mL}$ of Ket and MDZ was prepared in ACN; H_2O (1:1) 0.1% FA and was injected by direct infusion with a flow rate of $5\mu\text{L/min}$. Electrospray ionization parameters under positive ion mode were optimized by auto-tuning function, taking parent ion (m/z) peaks of 238.1 and 326.1 for Ket and MDZ respectively.

The hydrophilic nature of Ket and MDZ makes it challenging to efficiently resolve these compounds using liquid chromatography and simultaneous detection using mass spectrometry [19]. Hence, C_{18} HILIC column was used with the aim of achieving maximum chromatographic separations. Previous reports showed that Ket and MDZ could be resolved with a difference of only 0.05 seconds [20]. Initial runs with flow rates of 0.3 mL/min and 0.250 mL/min for 70:30 Water: ACN (0.1% FA) were carried out to achieve optimum chromatographic separation. Fourfold chromatographic separation could be achieved with 70:30 Water: ACN (0.1% FA) at 0.250 mL/min compared to previously reported studies (as shown in the supplementary figure 2). This could be attributed to the low flow rates and a delicate aqueous-organic mobile phase balance, which can be observed from the previous study [20]. The extraction of both drugs was also optimized by classical liquid-liquid extraction followed by protein precipitation. ACN with 0.1% FA could provide extraction for both the drug molecule by giving optimum chromatographic peaks for Ket ($m/z = 238.1$), MDZ ($m/z = 326.1$) and CBZ ($m/z = 237.1$) as shown in figure 1 and 2. Use of ACN with

0.1% FA helped in preventing peak splitting for MDZ (as shown in figure 1d and 1e) that was observed with the use of ACN only. Furthermore, we also evaluated the effect of CBZ, the IS, on the elution profile of Ket and MDZ when extracted in the 1:3 ratio of spiked plasma to ACN 0.1%FA. It could be seen that CBZ could prove to be the most efficient IS as it gets resolved efficiently (figure 2 and table 1) and the elution profile (peak shape and RT) remains uninterrupted. This also indicates that the extraction protocol of 1:3 (spiked plasma: ACN 0.1% FA) showed optimum extraction of both the drugs and the IS.

3.2 Method Development for Simultaneous Quantitation of Ket and MDZ from Plasma

The present study reports a SIM method for simultaneous estimation of Ket and MDZ in the presence of CBZ as the IS. This method was established using an HPLC system coupled with a Thermo Orbitrap (Exactive) HRMS (LC-HRMS) system, which operates with a mass error in the range of 0.2 to 0.3 ppm. The chromatograms for the Ket and MDZ in plasma are shown in Supplementary figure 3a and 3b. The retention time (Rt) was observed to be 7.64 minutes, 7.83 minutes, and 8.41 minutes for Ket, MDZ, and CBZ, respectively. Extraction of CBZ as the IS was considerably consistent with all the calibration and validation standards. The calibration curves were developed in the linearity range of 10 ng/mL to 2,000 ng/mL for Ket and MDZ. The concentration of CBZ was kept constant at 1 µg/mL. The calibration curve provided linearity equations for Ket and MDZ as $y = 2326.8x$ ($r^2 = 0.9989$) and $y = 11946x$ ($r^2 = 0.9998$), respectively. The average percentage extraction (for inter and intraday run) recoveries for all the calibration standards from plasma was in the range of 115.4 ± 8.1 to 80.7 ± 1.3 for Ket and 102.8 ± 5.3 to 97.1 ± 4.2 for MDZ. The inter- and intra-day variations in % accuracy and coefficient of variance (%CV) are presented in table 2. The average percentage coefficient of variance (for inter and intraday run) for Ket and MDZ ranged between 8.208 ± 2.567 to 1.4 ± 1.6 and 6.7 ± 1.104 to 1.6 ± 2.0 , respectively. Peak shape, % CV, and % accuracy was taken into consideration to determine lower limits of quantitation (LLOQ) and lower limits of detection (LLOD) for Ket and MDZ. Representative chromatograms for both drugs for LLOQ extracted from plasma are shown in figure 3 (a, d). The LLOD was determined to be 10 ng/mL for Ket. This could probably be due to the interference from the plasma background. Lower concentration (ng/mL) value for the LLOD of Ket (with respect to MDZ) could also be because of the relatively lesser number of amine groups (shown in Supplementary figure 1(a,c)) that could get protonated to give relatively better S/N ratio [21, 22]. As a result, we have 0.5 ng/mL as LLOD for MDZ in comparison to 10 ng/mL LLOD for Ket.

It can be seen that this quantitation method could provide us the variance of 5–10% as per the recommendations provided in ICH guidelines. These values were extremely lower than previously reported method for quantitation of Ket and MDZ [20]. It has been reported that Ket and MDZ gets converted to DHNK (221.68 Da), NK (223.69 Da) and 1HMDZ (341.77 Da) metabolites in presence of CYP450 enzymes. The present LC- HRMS method used scanning mass range of 100 to 345 m/z. Hence, this method could also be explored for detection and quantitation of clinically relevant metabolites of Ket and MDZ.

3.3 Simultaneous Quantitation of NK, DHNK and 1HMDZ metabolites

LCMS runs of NK, DHNK and 1HMDZ (shown in supplementary figure 4) mix was done in presence and absence of Ket-MDZ mix. Figure 4 shows simultaneous detection of Ket, MDZ, NK, DHNK and 1HMDZ in single method. This indicates that present method could detect and quantitate, $n = 5$ analytes (two drug and three metabolites) in the presence of an internal standard CBZ. Two types of linearity equations were developed at low (shown in table 3 (a–c)) and high concentration ranges (supplementary table 1 (a–c)). At the low concentration range, NK ($y = 392.87x - 129630$, $r^2 = 0.9843$), DHNK ($y = 187.25x - 72783$, $r^2 = 0.9889$) and 1HMDZ ($y = 6921.5x - 1083609.44$, $r^2 = 0.9888$) was established for inter- and intra-day calibration runs. For the high concentration ranges (3000 to 25000 ng/mL), linearity equations were established for NK ($y = 1,082.07x - 2,212,700.13$, $r^2 = 0.99$), DHNK ($y = 459.37x - 927622$, $r^2 = 0.9906$), and 1HMDZ ($y = 4,902.96x - 1,325,900.20$, $r^2 = 0.97$). Average extraction recoveries were observed in acceptable range for NK, DHNK and 1HMDZ. The % accuracy and % CV for the all metabolites are shown in table 3 (a–c). Representative LCMS chromatogram for LLOQ is shown in figure 3 (b, c, and e). The %CV <10% was observed for both inter and intraday runs for calibration standards as per the recommendation of ICH guidelines. These values were observed to be complying with previously reported methods [17].

3.4 Method Validation for Quantitation of Ket, MDZ, NK, DHNK and 1HMDZ

Calibration curve was validated by quantitating random low, medium, and high concentrations of molecules (Ket, MDZ and their metabolites) spiked in human plasma using LCMS runs, shown in table 4 (a, b) and table 5 (a–c). Moreover, samples were subjected to inter-day study after a freeze (-20°C) and thaw cycle. Insignificant variations were observed as per the guidelines set for the validation of bioanalytical methods [17]. This indicates that the method for quantitation is robust and demonstrates considerable accuracy for simultaneous quantitation of Ket and MDZ and their metabolites (NK, DHNK and 1HMDZ) in clinical samples.

3.5 Clinical Application

The method was successfully applied to a critically ill patient on ECMO therapy. Plasma concentration-time profiles of Ket, MDZ, and their metabolites during the continuous infusion are shown in figure 5. The study protocol used an opportunistic blood sampling strategy that did not interfere with routine clinical care. As such, concentration fluctuations were anticipated throughout the course of ECMO due to sedation interruptions, surgical procedures, additional boluses to target sedation, and dose up-titrations to achieve adequate sedation and pain control. The assay was applicable and sensitive enough to discriminate among other drugs administered during the course of hospitalization. In real time, in an uncontrolled environment, this assay could be used to determine the best dosage when there are multiple factors influencing the treatment regimen of the patient. This includes studying the adsorption and sequestration of drugs into the ECMO circuitry [9, 23] or applying this assay to initiate population based pharmacokinetic studies.

Although the concentration-response relationship has not been investigated for both drugs during adult ECMO, concentrations observed were comparable to published reports. For

Ket, concentrations above 100–160 ng/mL were described to produce analgesia, whereas a value of 241 ng/mL invoked loss of consciousness [24, 25]. With MDZ, concentrations ranging from 400–600 ng/mL have been observed to produce deep sedation in the ICU [26]. The assay quantitated fluctuating drug levels well in the situation where sedation and analgesia changes occur within routine clinical care.

The parent compound and its metabolites may have important dosing implications. [14]. The present method was also able to quantify Ket metabolites (NK and DHNK) and MDZ metabolite (1HMDZ) with high accuracy. We observed a high metabolite concentration relative to the parent compounds analyzed presumably due to increased metabolism due to critical illness.

4. Conclusion

The present study reports a highly sensitive method for the simultaneous detection of Ket, MDZ and its metabolites (DHNK, NK and 1HDMZ) in human plasma using LC-MS in a patient on ECMO. The inter- and intra-day variation of the assay was found to be within the limits set by ICH guidelines. Unlike previous studies, this study shows high sensitivity towards the LLOD with high precision for Ket and MDZ molecules. This could be achieved due to the increased resolution of Ket and MDZ in the presence or absence of CBZ as internal standard. This assay was applied to study the concentration of Ket, MDZ and their metabolites over a period of 500 hours. In conclusion, this study provides a bioanalytical method to determine the concentration of Ket, MDZ, and its metabolites in human plasma and can also be applied for determination of appropriate dosing in ECMO patients in critical care units.

5. Limitations and Future Directions

Due to the limitations of employing therapeutic drug monitoring for sedatives and analgesics in a patient population requiring a rapid onset of effect, the current bioanalytical method will be difficult to use in some clinical settings. The present method was developed to complement clinical studies with the intention to develop understanding of the dose-exposure-response relationship in patients on ECMO using Ket and MDZ. Future explorations using modeling and simulation would be informative to derive appropriate dosing regimens for these patients requiring ECMO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MS	Mass spectrometry
LC or HPLC	High Performance Liquid Chromatography
SIM	Selection or Single Ion Monitoring
XIC	Extracted Ion Chromatogram
LLOD	Lower limit of detection
LLOQ	Lower limit of Quantitation
ECMO	Extra Corporeal Membrane Oxygenation
Ket	therapy Ketamine
MDZ	Midazolam
DHNK	Dehydronorketamine
NK	Norketamine
1HMDZ	1hydroxy midazolam
CBZ	Carbamazepine
IS	Internal Standard

Reference

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Highlights

- Simple, robust and highly sensitive, single ion monitoring (SIM) based LCMS method for the quantitation of Ketamine, Midazolam and their metabolites (n=5 molecules) in presence of carbamazepine as internal standard (IS).
- Both parent (Ket and MDZ) and metabolites (DHNK, NK and 1HMDZ) were detectable in nanogram concentrations in human plasma samples. The LCMS method was established and validated as per the norms set for bioanalytical methods by FDA and ICH guidelines.
- Our method was relatively able resolve both parent (Ket and MDZ) molecules in LCMS based single ion monitoring method.
- LCMS method was applied for quantitative mapping of (n=5) parent and metabolite molecules in a critically ill patient on Extra Corporeal Membrane Oxygenation (ECMO) therapy in IS. This makes it capable of being used in ECMO patients in clinics for deciding optimal dose for Ket and MDZ in real time situation of critical care units.

Figure 1:

The extracted ion chromatograms (XIC) while performing optimization of extraction protocol for quantitative estimation of Ket and MDZ at 100 ng/mL **a)** Blank plasma extracted in ACN 0.1% FA **b)** Ket extracted in ACN 0.1% FA **c)** Ket extracted in ACN, **d)** MDZ extracted in ACN, it shows splitting of peak **e)** MDZ extracted in ACN 0.1% FA, shows disappearance of peak split. The extraction of Ket and MDZ from plasma sample is optimum using ACN 0.1% FA.

Figure 2:
Representative chromatograms of Ket and MDZ (KM) with internal standard CBZ (KMC) and without the internal standard CBZ (KM) in plasma samples extracted in 0.1% FA ACN.

Figure 3 (a –c):

The XIC chromatograms of lower limit of quantitation (LLOQ) for Ket and its metabolites (DHNK and NK); (**d and e**) shows XIC chromatograms of LLOQs for MDZ and its metabolite 1HMDZ with their respective signal to noise ratios (S/N). All measurements are plotted in comparison to blank human plasma (BP) extracted in ACN 0.1%FA, with chromatograms extracted in respective and specific mass regions.

Figure 4:

Shows peaks for DHNK, NK and MDZ in presence (mix-2) and absence (mix-1) of Ket and MDZ. Detection of metabolites remains unaffected due presence or absence of parent molecules.

Figure 5:
Plasma concentration-time profiles of **A**) 1-hydroxymidazolam (1HMDZ) and midazolam (MDZ) and **B**) dehydronorketamine (DHNK), ketamine (Ket), and norketamine (NK) in a patient receiving a continuous infusion of both drugs.

Table 1:

The lower limit of detection (LLOD) and quantitation (LLOQ) for Ket, MDZ and their metabolites.

Analyte	RT (min)	LLOD (ng/mL)	LLOQ (ng/mL)
Ket	7.64	10	20
MDZ	7.83	0.5	10
NK	7.20	300	320
DHMK	7.24	410	470
1HMDZ	7.30	100	150

Table 2 (a, and b):

The coefficient of variance (% CV) and % accuracy for the calibration standards used for plotting calibration curves for Ket and MDZ.

Ket (a)				
	%CV		% Accuracy	
Conc. (ng/mL)	Inter Day	Intra Day	Inter Day	Intra Day
10	2.794	4.096	56.342	55.229
20	4.827	1.038	121.137	109.681
100	6.393	10.025	91.087	97.544
200	3.074	0.663	81.571	79.798
400	5.559	7.418	92.574	96.213
500	2.577	0.309	95.025	93.294
800	3.154	4.342	100.359	102.597
1000	6.324	8.685	99.898	104.365
1500	4.899	1.268	99.884	96.424
2000	3.161	4.514	100.963	98.706
MDZ (b)				
	%CV		% Accuracy	
Conc. (ng/mL)	Inter Day	Intra Day	Inter Day	Intra Day
10	11.991	4.486	92.184	81.691
20	5.967	7.528	94.668	91.634
100	2.090	2.941	102.676	92.016
200	4.432	2.216	100.068	94.167
400	6.900	1.725	105.545	97.175
500	5.759	1.152	104.997	98.153
800	8.296	1.037	103.032	92.238
1000	6.343	0.634	106.562	99.088
1500	3.034	0.202	102.060	97.812
2000	3.946	0.197	102.727	97.434

Table 3 (a-c):

The %CV and % accuracy for the calibration standards (low concentration range) for DHNK, NK and 1HMDZ.

DHNK (a)				
Conc. (ng/mL)	Intra Day		Inter Day	
	%CV	% Accuracy	%CV	% Accuracy
470	3.873	118.307	5.252	117.550
500	10.385	105.900	8.829	104.245
800	0.910	97.542	7.738	95.002
1000	8.629	95.783	5.535	93.639
1500	7.659	99.580	7.928	95.670
2000	1.876	103.686	0.297	103.863
NK (b)				
Conc. (ng/mL)	Intra Day		Inter Day	
	%CV	% Accuracy	%CV	% Accuracy
320	4.095	105.219	6.719	104.934
400	3.126	105.989	3.939	106.900
500	4.582	97.014	5.986	98.679
800	8.734	89.175	0.909	89.512
1000	3.145	89.245	3.247	90.644
1500	4.415	92.581	2.284	93.776
2000	4.479	107.848	1.690	106.750
1HMDZ (c)				
Conc. (ng/mL)	Intra Day		Inter Day	
	%CV	% Accuracy	%CV	% Accuracy
150	3.826	97.986	2.309	97.949
200	2.540	107.487	8.782	105.399
400	1.681	85.670	1.569	86.229
500	1.035	83.263	2.116	84.091
800	1.854	82.960	4.634	85.162
1000	1.579	87.011	0.021	96.691
1500	1.144	92.889	2.420	94.339
2000	0.557	108.023	3.605	105.516

Table 4 (a and b):

The %CV and %precision for random concentrations for Ket and MDZ used for the validation of calibration curve respectively.

Conc. (ng/mL)	Ket (a)			
	<i>Intra Day</i>		<i>Inter Day</i>	
	%CV	% Accuracy	%CV	% Accuracy
20	0.720	95.393	7.176	100.493
40	6.301	84.108	10.361	90.756
900	1.650	109.342	8.762	102.964
1600	0.299	97.941	9.903	107.094
Conc. (ng/mL)	MDZ (b)			
	<i>Intra Day</i>		<i>Inter Day</i>	
	%CV	% Accuracy	%CV	%Accuracy
30	2.739	106.250	8.998	99.894
40	0.369	104.041	7.966	110.252
900	9.907	87.560	4.457	84.885
1600	1.727	93.433	1.282	92.594

Table 5 (a–c):

The %CV and %precision for random concentration of DHNK, NK and IHMDZ used for the validation of calibration curve.

DHNK (a)				
Conc. (ng/mL)	<i>Intra Day</i>		<i>Inter Day</i>	
	%CV	% Accuracy	%CV	% Accuracy
470	4.125	103.319	7.775	103.677
600	5.466	83.827	3.072	84.250
900	6.083	100.040	2.257	100.962
9000	2.582	90.558	0.170	97.384
21000	1.071	95.428	2.097	94.174
NK (b)				
Conc. (ng/mL)	<i>Intra Day</i>		<i>Inter Day</i>	
	%CV	% Accuracy	%CV	% Accuracy
320	10.016	101.639	4.367	102.111
600	7.362	83.362	2.657	82.839
900	1.836	102.390	5.713	99.838
9000	3.605	92.787	0.146	91.508
21000	1.438	96.567	2.614	94.992
IHMDZ (c)				
Conc. (ng/mL)	<i>Intra Day</i>		<i>Inter Day</i>	
	%CV	% Accuracy	%CV	% Accuracy
150	3.799	101.547	3.777	102.016
500	1.332	91.941	0.564	92.194
1000	1.089	101.288	8.465	106.823
9000	1.557	96.110	1.065	96.958
21000	4.127	96.488	1.306	102.163