

Simultaneous HPLC–UV Determination of Ketamine, Xylazine, and Midazolam in Canine Plasma

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An isocratic reversed-phase high-performance liquid chromatography method with UV detection is developed and validated for the simultaneous determination of ketamine, xylazine, and midazolam in canine plasma. Analytes are extracted from alkalized samples into diethyl ether–methylene chloride (7:3, v:v) using single-step liquid–liquid extraction. Chromatographic separation is performed on a C₁₈ column using a mobile phase containing an acetonitrile–methanol–10 mM sodium heptanesulfonate buffer adjusted to pH 3, with glacial acetic acid (44:10:46, v:v) at a detection wavelength of 210 nm, with a total runtime of 10 min. The calibration is linear over the range of 78.125–5000 ng/mL for ketamine and 15.625–1000 ng/mL for xylazine and midazolam. The limits of detection are 17.8, 10.3, and 15.1 ng/mL for ketamine, xylazine, and midazolam, respectively. The extraction recoveries are 76.1% for ketamine, 91.0% for midazolam, and 78.2% for xylazine. The method is successfully used for clinical and pharmacokinetic studies of the three-drug fixed dose combination formulations.

Introduction

Ketamine is a dissociative anesthetic of the cyclohexylamine group used for chemical restraint and for the induction and maintenance of anesthesia in a number of species (1). Ketamine induces anaesthesia rapidly and causes minimal depression of the respiratory and cardiovascular systems, and it has a wide margin of safety (2). Unlike many anesthetics, ketamine usually stimulates cardiovascular function in normal animals, causing an increase in heart rate and mean arterial pressure. The use of ketamine as a sole anesthetic has been limited by muscle hypertonicity and myoclonus, violent recovery, and the occasional occurrence of convulsions (3). Xylazine is an α 2-adrenergic agonist sedative with analgesic properties that has a significant depression effect on heart rate and the respiratory system (4). Midazolam is a short-acting benzodiazepine, commonly used in intravenous anesthesia induction, short-term sedation, and oral hypnotic medication. Intravenous midazolam has been associated with respiratory depression and respiratory arrest, especially when used for sedation in noncritical care settings (5). The combination of ketamine–midazolam–xylazine has been successfully used in rabbits, pigs, parakeets, fowl, horses, dogs, deer, and tigers (6–13).

In order to improve the understanding of the interaction between ketamine, xylazine, and midazolam, a sound understanding of both the pharmacokinetics and pharmacodynamics of the drugs when coadministered to patients is required. Many methods have been developed to determine these three drugs, individually or in combination with other drugs or

metabolites. The methods for the analysis of ketamine include high-performance liquid chromatography (HPLC) (14–17) and LC–mass spectrometry (LC–MS) (18, 19). The techniques used for midazolam include HPLC (20–28), gas chromatography (GC) (29–34), GC–MS (35–37), LC–MS (38–42), and HPLC–MS–MS (43). The methods for the analysis of xylazine include GC (44), HPLC (45–49), GC–MS (50–52), and LC–MS (53).

Three methods have been reported for the simultaneous determination of ketamine and xylazine or midazolam (54–56). Only one of the published methods allows for the simultaneous determination of the three drugs using LC–MS (57), but ketamine and xylazine can't be separated, requiring MS equipment that may not be available in many laboratories.

In this paper, a simple, rapid, adequate sensitivity, and repeatable HPLC–UV method is described for the simultaneous determination of ketamine, xylazine, and midazolam from canine plasma. The simplicity of the proposed method is facilitated by using a single-step liquid–liquid extraction procedure, rather than using the expensive solid-phase extraction cartridges. Additionally, an application of the method is reported herein to determine the three drugs in canines after a single intravenous administration.

Experimental

Chemicals and reagents

Ketamine-HCl, midazolam-HCl, and xylazine-HCl were purchased from Sigma-Aldrich (St. Louis, MO). Diethyl ether, methylene chloride, NaOH, and glacial acetic acid were supplied by Sinopharm (Shanghai, China), acetonitrile and methanol were obtained from Tedia (Fairfield, CT), and sodium heptanesulfonate from Regis (Austin, Texas). All reagents used were of analytical grade except methanol and acetonitrile, which were of HPLC grade. Ultrapure water, prepared by a Milli-Q Reagent Water System (Millipore, MA), was used throughout.

Standard solutions and calibration standards

Ketamine, xylazine, and midazolam were dissolved in methanol to make the stock solutions (1.0 mg/mL). The stock solutions were stored at -20°C . The calibration range was selected by taking into account the concentrations expected in the plasma samples. The preparation of the calibration standard plasma samples (78.125, 156.25, 312.5, 625, 1250, 2500, 5000 ng/mL ketamine and 15.625, 31.25, 62.5, 125, 250, 500, 1000 ng/mL midazolam and xylazine) was accomplished by introducing

known amounts of stock solutions of three drugs to drug-free plasma.

Chromatographic conditions

A HPLC system manufactured by Waters (Milford, MA), which comprised of a 717 plus autosampler, a 600 controller, a 600 pump, and a 2487 Dual λ absorbance detector, measuring absorption at 210 nm. The integration software, Empower Pro 2, was used. Separation was achieved on a reversed-phase 5 μm C_{18} column (250×4.6 mm i.d.) (Cosmasil, Japan). The mobile phase consisted of an acetonitrile–methanol–10 mM sodium heptanesulfonate buffer adjusted to pH 3 with glacial acetic acid (44:10:46, v/v). The flow rate was 0.7 mL/min, which resulted in a backpressure of 1260 psi. The column temperature was adjusted to 30°C.

Animal experiment and sample collection

Seven Labrador dogs (four males and three females, weighing 26.79 ± 2.91 kg) participated in this study. The study protocol was approved by the Animal Ethics Committee of the Nanjing Police Dog Research Institute of Public Security Ministry. Their diets were prohibited for 12 h before the experiment, while water was taken freely. The anesthetics were applied intravenously to the dogs at the doses of 4.8 mg/kg for ketamine, 0.54 mg/kg for xylazine, and 0.12 mg/kg for midazolam. Samples of 2 mL blood were collected from the lateral saphenous vein at the following times: 0 (pre-dose), 5, 10, 20, 30, 40, and 50 min after administration. Blood samples were centrifuged in heparin vials at $2000 \times g$ for 15 min, and the separated plasma was immediately stored at -20°C prior to analysis.

Sample preparation

Aliquots (500 μL) of plasma were transferred into a screw-capped glass tube and alkalinized with 500 μL of 1 M sodium hydroxide. Then, 5 mL of diethyl ether–methylene chloride (7:3, v/v) was added, and the tubes were mixed using a vortex device for 5 min at 2000 rpm and centrifuged for 5 min at $4000 \times g$ at 4°C . The screw-capped tubes, containing the samples, were frozen for ~ 40 min at -20°C . The organic layer was transferred into conical glass centrifuge tubes and

concentrated (at 40°C) under a gentle stream of nitrogen. The residues were redissolved in 150 μL of the mobile phase, and 30 μL was injected into the HPLC system.

Results and Discussion

Method optimization

During the method development, the percentage of the mobile phase organic solvents was varied using different combinations of acetonitrile–methanol–water (28:10:62, 32:10:58, 36:10:54, 40:10:50, 44:10:46, 48:10:42, 52:10:38, 56:10:34, 60:10:30, v/v), but the separation of ketamine and xylazine was not satisfactory. When the 10 mM sodium heptanesulfonate was employed, good separation of these two drugs was achieved. Because ketamine and xylazine both contain a nitrogen atom with a lone pair of electrons, they will combine with H^+ based on the attraction between the positive and negative charges, changing ketamine and xylazine to the H^+ -conjugate. Subsequently, in the mobile phase, the H^+ -conjugates of ketamine and xylazine bind to the negative group of sodium heptanesulfonate and are converted to neutral molecules. Therefore, compared with the ionic forms of the two drugs, the neutral complexes result in an enhanced retention ability of the two analytes on the C_{18} column because the neutral complexes have weaker polarity than the ionized analytes, and compounds with a similar polarity to the column are retained or adsorbed effectively. In order to form the H^+ -conjugate, glacial acetic acid was added to the mobile phase to adjust the pH. Mobile phases of pH 5.5, 4.5, 3.5, and 3.0 were compared for their efficiency; when the pH of the mobile phase was adjusted to 3.0, high and narrow peaks for the three analytes were obtained. Here, the mobile phase composed of acetonitrile–methanol–10 mM sodium heptanesulfonate, adjusted to pH 3 with glacial acetic acid (44:10:46, v/v), was chosen to achieve good peak shape, satisfactory resolution, and a relatively short analysis time. The absorption maximum wavelengths of xylazine and midazolam were 225 and 220 nm, respectively, and ketamine absorbed strongly at 269 and 277 nm. Niedorf et al. (55) monitored ketamine and xylazine at 215 nm, while Adams et al. (56) assayed ketamine and midazolam at 210 nm. Herein, the detection wavelength was set at 210 nm, and proved to be suitable for the assay. The typical chromatograms of the experimental samples are shown in Figure 1.

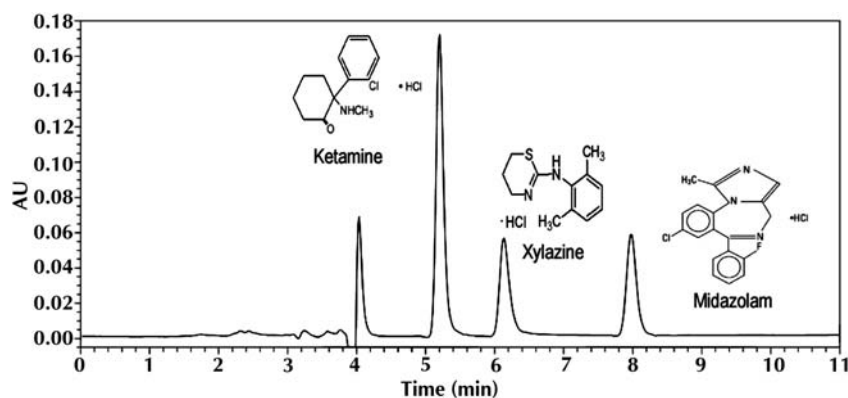


Figure 1. Typical chromatograms obtained with the described method. The plasma is from a dog 5 min after administration of the anesthetics.

Table ICalibration Curve Data for Ketamine, Xylazine, and Midazolam ($n = 6$)

Compound	Equation	Correlation coefficient	CV of slope (%)	CV of intercept (%)
Ketamine	$y = 173060 (\pm 8186)x + 19330 (\pm 2188)$	0.9997	4.7	11.3
Xylazine	$y = 355488 (\pm 10451)x - 1106.2 (\pm 87)$	0.9993	2.9	7.9
Midazolam	$y = 512677 (\pm 16406)x + 32.408 (\pm 3)$	0.9995	3.2	9.5

Table IIPrecision of the Determination of Ketamine, Xylazine, and Midazolam in Plasma ($n = 6$)

Analytes	Concentration (ng/mL)	Intra-day (RSD, %)	Inter-day (RSD, %)
Ketamine	156.25	1.8	3.3
	625	2.6	5.4
	2500	0.5	1.3
Xylazine	31.25	4.4	6.9
	125	2.8	4.1
	500	1.2	4.1
Midazolam	31.25	3.9	6.5
	125	2.6	4.6
	500	1.2	2.7

In the pilot study, the extraction technique was based on that reported by Niedorf et al. (55), which was used successfully for the extraction of ketamine and xylazine from canine plasma, but the method was time-consuming, and the samples were prone to emulsification after three vortexes. In order to avoid these two disadvantages, it was decided to use single-step liquid-liquid extraction. Several organic solvents, such as diethyl ether, diethyl ether-trichloromethane (3:1, v/v), and diethyl ether-methylene chloride (8:2, 7:3, 6:4, v/v), were tested. Diethyl ether was volatile, resulting in poor reproducibility. Diethyl ether-trichloromethane (3:1, v/v) could easily cause emulsification. As the concentration of methylene chloride in diethyl ether-methylene chloride increased, the recoveries of ketamine and midazolam also increased, and the recovery of xylazine was decreased. Finally, diethyl ether-methylene chloride (7:3, v/v) was selected, which provided acceptable recoveries for the three analytes (76.1% for ketamine, 78.2% for xylazine, and 91.0% for midazolam).

Method specificity

The degree of interference by endogenous plasma constituents with ketamine, xylazine, and midazolam was assessed by inspection of chromatograms derived from a processed blank plasma sample. Using the described method, separation is achieved within 9 min, the retention times of ketamine, xylazine, and midazolam were 5.2 min, 6.2 min, and 8 min, respectively. No interfering peaks were observed at the retention times of interest in plasma from untreated dogs. Atropine was the most commonly used pre-anesthetic medication in small animal immobilization in China and was injected into the prescribed chromatographic system (diluted with the mobile phase), eluted at 4.6 min, and did not show any interference.

Linearity

The linearity was investigated in the range of 78.125–5000 ng/mL for ketamine, and in the range of 15.625–1000 ng/mL for midazolam and xylazine. The calibration curves for the analytes

were done at seven levels of concentration (78.125, 156.25, 312.5, 625, 1250, 2500, and 5000 ng/mL for ketamine and 15.625, 31.25, 62.5, 125, 250, 500, and 1000 ng/mL for midazolam and xylazine). The linear correlation coefficient (R^2) for all calibration curves were greater than 0.999, indicating a good linearity (Table I). The R^2 of the presented method are excellent and consistent with the formerly published HPLC-UV methods (55, 56).

Limits of detection

The limits of detection (LOD) in the plasma were measured when the signal-to-noise ratio was better than 3. The LODs were 17.8, 10.3, and 15.1 ng/mL for ketamine, xylazine, and midazolam, respectively. The LODs of this method are comparable with the method described by Niedorf et al. (ketamine 19.5 ng/mL, xylazine 9.8 ng/mL) (55); however, they are higher than the method reported by Adams et al. (ketamine and midazolam < 5 ng/mL) (56).

Precision and accuracy

The precision was evaluated using quality control (QC) plasma samples spiked at three concentration levels (156.25, 625, and 2500 ng/mL for ketamine and 31.25, 125, and 500 ng/mL for xylazine and midazolam) using the optimized analytical method. Six replicates of QC samples at each concentration were analyzed on a same day to evaluate intra-day precision. For the evaluation of inter-day precision, six replicate QC samples at each concentration were analyzed on six consecutive days (one sample a day). The intra- and inter-day precisions (RSD) for the measured analytes were <4.4 and 6.9%, respectively, and are comparable with the results reported by Niedorf et al. (55) and Adams et al. (56). The technique displayed good intra- and inter-day repeatability for the three analytes (Table II).

The accuracy was evaluated by comparing the measured concentration with the true value of spiked controls. The accuracy results are summarized in Table III. The accuracies for quality control plasma samples ranged from 90.1% to 98.6% for the three drugs, and they are comparable with the results reported by Niedorf et al. (55) and Adams et al. (56).

Extraction recovery

The extraction recoveries for ketamine, xylazine, and midazolam were determined with three levels of concentration in spiked plasma. Six replicates of these samples were extracted and analyzed according to the method described herein. The recoveries were calculated by comparing the response of the quality control plasma samples with the response of the

identical standards prepared in the mobile phase which did not undergo sample pre-treatment. Despite the simple one-step extraction, the average recovery was above 76.1% for ketamine, 78.2% for xylazine, and 91.0% for midazolam.

Stability

The stability of the analytes in dog plasma was studied under different storage conditions: -20°C for 2 months, three freeze-and-thaw cycles, and post-preparation at 25°C and 4°C for 24 h. The stability results are summarized in Table IV. The analytes were stable in plasma samples when stored at -20°C

Table III

Method Accuracy for the Determination of Ketamine, Xylazine, and Midazolam in Plasma ($n = 6$)

Analytes	Spiked Concentration (ng/mL)	Measured \pm SD (ng/mL)	Method Accuracy (%)	RSD (%)
Ketamine	156.25	145.6 \pm 7.1	93.2	4.9
	625	598.8 \pm 26.9	95.8	4.5
	2500	2457.5 \pm 44.2	98.3	1.8
Xylazine	31.25	28.2 \pm 1.2	90.1	4.3
	125	121.6 \pm 6.3	97.3	5.2
	500	481.0 \pm 6.3	96.2	1.3
Midazolam	31.25	28.6 \pm 1.0	91.4	3.6
	125	121.9 \pm 5.7	97.5	4.7
	500	493.0 \pm 6.9	98.6	1.4

Table IV

Stability Results of Ketamine, Xylazine, and Midazolam ($n = 6$ at Each Concentration)

Storage condition	Nominal concentration ($\mu\text{g/mL}$)	Measured concentration ($\mu\text{g/mL}$)	Accuracy (%)	RSD (%)	
-20°C , 2 months	Ketamine	156.25	152.2 \pm 8.7	97.4	5.7
		625	596.9 \pm 30.7	95.5	5.1
		2500	2480 \pm 102.2	99.2	4.1
	Xylazine	31.25	29.8 \pm 1.9	95.4	6.3
		125	119.2 \pm 5.9	95.3	4.9
		500	481.7 \pm 19.3	96.3	4.0
	Midazolam	31.25	30.2 \pm 2.4	96.6	7.9
		125	122.6 \pm 8.7	98.1	7.1
		500	486.5 \pm 31.7	97.3	6.5
3 freeze-thaw cycles	Ketamine	156.25	152.3 \pm 4.3	97.5	2.8
		625	602.5 \pm 13.3	96.4	2.2
		2500	2465.1 \pm 46.8	98.6	1.9
	Xylazine	31.25	30.4 \pm 0.9	97.2	3.0
		125	122.5 \pm 3.6	98.0	2.9
		500	490.5 \pm 10.3	98.1	2.1
	Midazolam	31.25	30.0 \pm 0.51	96.1	1.7
		125	121.1 \pm 2.7	96.9	2.2
		500	486.2 \pm 11.7	97.2	2.4
25°C , 24 h	Ketamine	156.25	150.3 \pm 5.4	96.2	3.6
		625	600.6 \pm 28.2	96.1	4.7
		2500	2435.0 \pm 60.9	97.4	2.5
	Xylazine	31.25	29.8 \pm 1.2	95.4	4.1
		125	120.6 \pm 5.8	96.5	4.8
		500	483.5 \pm 17.9	96.7	3.7
	Midazolam	31.25	30.0 \pm 13.8	96.0	4.6
		125	119.9 \pm 5.5	95.9	4.6
		500	486.1 \pm 10.7	97.2	2.2
4°C , 24 h	Ketamine	156.25	151.3 \pm 5.3	96.8	3.5
		625	615.6 \pm 6.8	98.5	1.1
		2500	2452.5 \pm 12.3	98.1	0.5
	Xylazine	31.25	30.0 \pm 1.1	96.1	3.6
		125	121.8 \pm 5.8	97.4	4.8
		500	482.5 \pm 7.2	96.5	1.5
	Midazolam	31.25	30.6 \pm 0.8	97.9	2.6
		125	120.9 \pm 0.8	96.7	0.7
		500	486.1 \pm 8.3	97.2	1.7

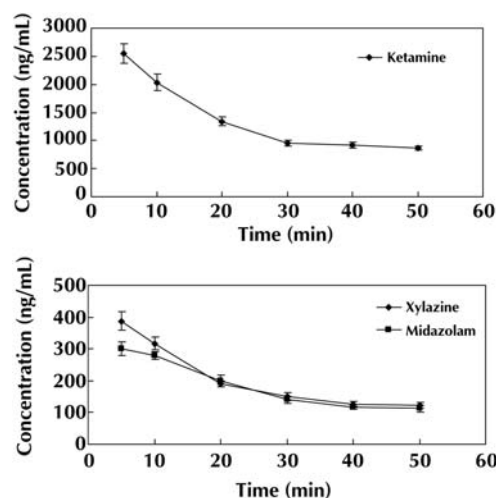


Figure 2. Ketamine, xylazine, and midazolam plasma concentrations measured in dogs after intravenous injection.

for 2 months and following three freeze–thaw cycles; the post-extraction solutions were also stable at 25°C and 4°C for 24 h.

Method application

The method was successfully applied for the monitoring of plasma levels of ketamine, xylazine, and midazolam in six dogs, after intravenous administration of a combination of 4.8 mg/kg for ketamine, 0.54 mg/kg for xylazine, and 0.12 mg/kg for midazolam. The plasma concentration-time curves are shown in Figure 2.

Conclusion

This is the first study dealing with HPLC–UV for the simultaneous determination of ketamine, xylazine, and midazolam from canine plasma. The simple process for the sample preparation and HPLC analyses with only a 10-min runtime enhanced the efficiency of the procedure. This is the first report concerning plasma concentrations of the three drugs in dogs after intravenous injection.

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