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Short Communication

Rapid high-performance liquid chromatographic method for the determination of ketamine and its metabolite dehydronorketamine in equine serum

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ABSTRACT

A simple, rapid and sensitive high-performance liquid chromatographic procedure has been developed for the determination of ketamine and dehydronorketamine in equine serum. Sample preparation consisted of mixing equal volumes of serum and acetonitrile–phosphoric acid (85%)–water (20:2:78, v/v/v), followed by ultrafiltration through a 10 000 molecular mass cut-off filter. Separation of these two analytes in the ultrafiltrate was accomplished on a reversed-phase phenyl column eluted with methanol–acetonitrile–phosphate buffer solution. Ketamine and dehydronorketamine were detected by a variable photometric UV-Vis detector set at 215 nm, and confirmed by a photodiode array detector operated in the 200–320 nm range. The limit of detection for ketamine was 5–15 ng/ml in equine serum. Additionally, the dehydronorketamine peak identity was tentatively confirmed by thermospray liquid chromatography–mass spectrometry.

INTRODUCTION

Ketamine (Fig. 1A) is used for the induction and maintenance of anesthesia and analgesia [1]. In humans and animals, ketamine is demethylated to form predominantly norketamine and dehydronorketamine (Fig. 1B) [1–3]. The determination of ketamine and its two main metabolites in biological fluids continues to be carried out by gas chromatography techniques with electron-capture [1,4–6] and mass spectrometric (GC–MS) [3,4] detection owing to their weak UV absorption and low effective clinical concentrations.

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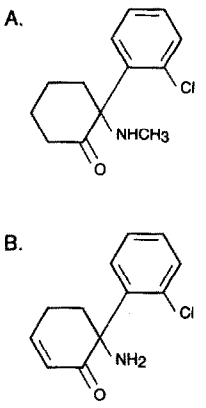

Fig. 1. Molecular structures of (A) ketamine and (B) dehydronorketamine.

Recently, several high-performance liquid chromatographic (HPLC) methods have been described which can determine ketamine in human plasma [7,8], ketamine and norketamine in rabbit serum [9], and racemic ketamine and norketamine together with their enantiomers in the plasma of horses [10] and in rat urine [11]. One of these methods [7] uses an on-line column-switching procedure with direct plasma injection without any pretreatment, but requires expensive columns and a column-switching valve system. The other HPLC procedure [9] for the determination of ketamine and norketamine in serum involves a multi-wavelength detection system and a peak deconvolution technique, which includes unresolved components upon chromatographic analysis and is therefore not suitable for routine analysis of biological fluids. These HPLC methods all involve time-consuming sample preparation, which complicates routine analysis. The majority require labor-intensive organic extraction, sometimes followed by derivatization [11].

The methodology described in this paper involves simultaneous determination of racemic ketamine and dehydronorketamine in equine serum with one-step sample preparation. Sample preparation was simplified by ultrafiltration of diluted serum (1:1) on a 10 000 molecular mass cut-off filter. Consistent recoveries of ketamine and the lack of volume transfers eliminate the need for an internal standard. These advantages allow investigators to study large sample sets in a reasonable time. Detection of ketamine and dehydronorketamine was achieved using photometric UV-Vis and photodiode array (PDA) detectors. The PDA detector was used to develop the assay, since ketamine and dehydronorketamine have no distinct absorption maximum, and to perform the confirmation of dehydronorketamine in equine serum. The photometric detector was used for quantitative analysis and improved sensitivity.

Our results also support the view that dehydronorketamine is a major metabolite in the serum of horses intravenously infused with ketamine. The tentative confirmation of dehydronorketamine present in equine serum was performed by thermospray mass spectrometry.

EXPERIMENTAL

Reagents and materials

The HPLC solvents were acetonitrile and methanol, highest purity solvent grade (Burdick & Jackson, Muskegon, MI, USA). HPLC-grade water was obtained from the Model 1000 Hydro Ultrapure water system from Hydro Services and Supplies (Research Triangle Park, NC, USA). Dehydronorketamine standard was not available. Ketamine hydrochloride standard was supplied by Fort Dodge Labs. (Crockville, MD, USA).

A 1 mg/ml (calculated as ketamine base) stock solution was prepared in acetonitrile–phosphoric acid (85%)–water (20:2:78, v/v/v). The working solution of 1 μg/ml was prepared daily from the stock solution. The microseparation system, Centricon-10, with a 10 000 molecular mass cut-off filter, was obtained from Amicon (Danvers, MA, USA).

The control equine serum was collected from horses not exposed to ketamine. It was used for blank analysis and was spiked with ketamine for assay validation. Equine serum samples were collected at various times (0–120 min) after infusion of ketamine. These samples were used for the dehydronorketamine confirmation. All samples were provided by NC State University, College of Veterinary Medicine (Raleigh, NC, USA).

Sample preparation procedure

A 0.5-ml aliquot of equine serum was diluted with an equal volume of aqueous solution containing acetonitrile–phosphoric acid (85%)–water (20:2:78, v/v/v). The sample was vortex-mixed for 10–15 s, placed in the Centricon-10 microseparation system and centrifuged for *ca.* 30 min at 3000 *g* with a 45° fixed-angle rotor. A 20–100 μl aliquot of colorless ultrafiltrate was injected into the HPLC system, and the column effluent was monitored by either a photometric or a PDA UV-Vis detector.

High-performance liquid chromatography

The HPLC equipment consisted of a Waters 820 workstation, two HPLC solvent-delivery systems (Models W600 and 590), a U6K injector, a 712 WISP automatic injector, a Model 990 plus PDA detector, a Model 481 variable-wavelength UV-Vis detector, and a column temperature control accessory set at 50°C. The HPLC separations were performed using a mobile phase of methanol–acetonitrile–phosphoric acid (85%)–0.01 *M* phosphate buffer (30:10:0.2:59.8, v/v/v/v). The phosphate buffer contained monosodium phosphate salt. The column was a 100 mm × 4.6 mm I.D. phenyl Spherisorb, 3 μm particle size, from Phenomenex (Rancho Palos Verdes, CA, USA). The column effluent was analyzed in the wavelength range 200–320 nm using the PDA detector or monitored by the UV-Vis variable detector set at 215 nm. Peak-area measurements were computed by the Waters 820 workstation. The recoveries of ketamine from equine serum were performed by analyzing five spiked samples at 50, 250, 1000, and 5000 ng/ml concentrations at 215 nm (Table I).

After comparison of the areas of ketamine in standard and equine serum samples, the amount of ketamine was determined by using LC/UV areas and calculated as follows: ng/ml = [ketamine (ng) × 2 × 1000]/[injection volume (μl)]. The multiplication by 2 in the equation accounts for the dilution of serum (1:1) with the solution for releasing protein-bound drug. Dehydronorketamine standard was not available, therefore the amounts of this compound were calculated as ketamine at 215 nm.

Thermospray mass spectrometry

Thermospray spectra were acquired using a mobile phase of methanol–0.1 *M* ammonium acetate (30:70, v/v), at a flow-rate of 1.2 ml/min. The column was a 100 mm × 4.6 mm I.D. phenyl Spherisorb, 3 μm particle size, from Phenomenex. The thermospray interface was operated at a source temperature of 300°C and a vaporizer temperature of 105°C. A Finnigan MAT 4800 quadruple mass spectrometer was operated in the pulse positive-ion/negative ion detection mode under full-scan condition for initial acquisition of the spectra for ketamine. For confirmation of dehydronorketamine in equine serum, the instrument was operated in the positive-ion mode, and ions were monitored under full scan from *m/z* 120 to 500. The thermospray spectrum of dehydronorketamine was obtained from the analyte collected from the equine serum under the HPLC conditions described in the previous section.

RESULTS AND DISCUSSION

Ketamine and its major metabolite in equine serum, dehydronorketamine, are weakly UV-absorbing compounds. Therefore a broad analytical window for these analytes was needed for the use of UV absorption at 215, 212.6, and even 200 nm (Fig. 2).

During investigation of stationary phases, it was found that a phenyl column could achieve a successful separation of these analytes from equine serum ultrafiltrate. Consequently, a 3-μm phenyl column was chosen, which also gave the necessary sensitivity for ketamine determination. Several elution systems were investigated to produce the optimal separation of these analytes, *e.g.* various amounts of acetonitrile, methanol, and phosphoric acid. The best results were obtained with the mobile phase described in Experimental.

Several solutions were tested for their ability to free protein-bound ketamine from equine serum. These solutions consisted of various proportions of acetonitrile, methanol, ethanol, and phosphoric acid. Based on the recovery of ketamine from equine serum, acetonitrile–phosphoric acid

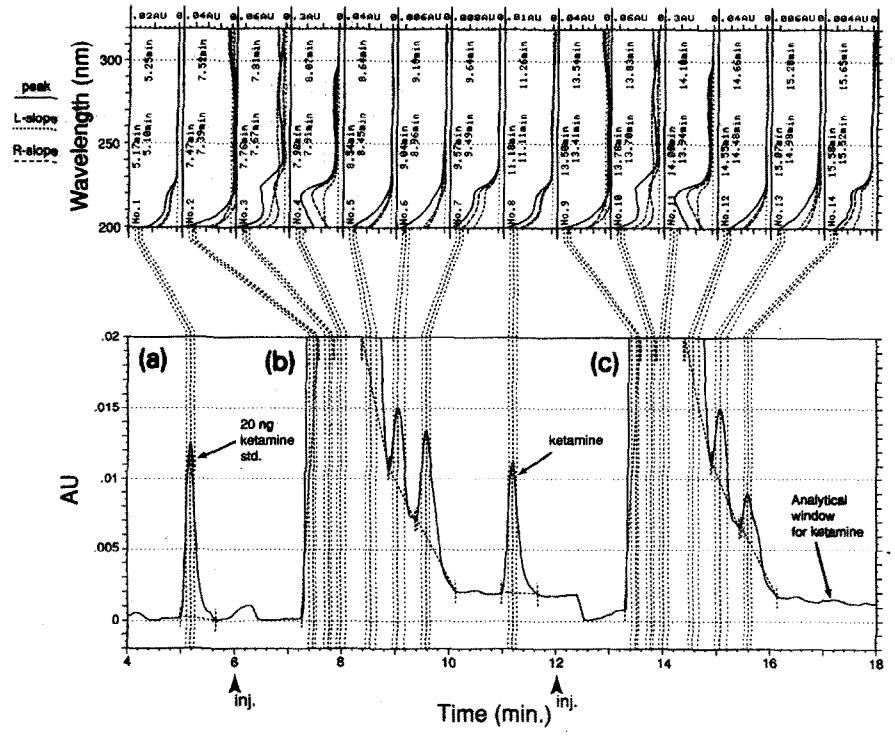

Fig. 2. Chromatograms of (a) 20 ng ketamine (0–6 min, injected at 0 min, not shown), (b) equine serum spiked with 1000 ng/ml ketamine (6–12 min, injected at 6 min), and (c) blank equine serum (12–18 min, injected at 12 min). Injection volumes were 20 μl. Photodiode array detection was used.

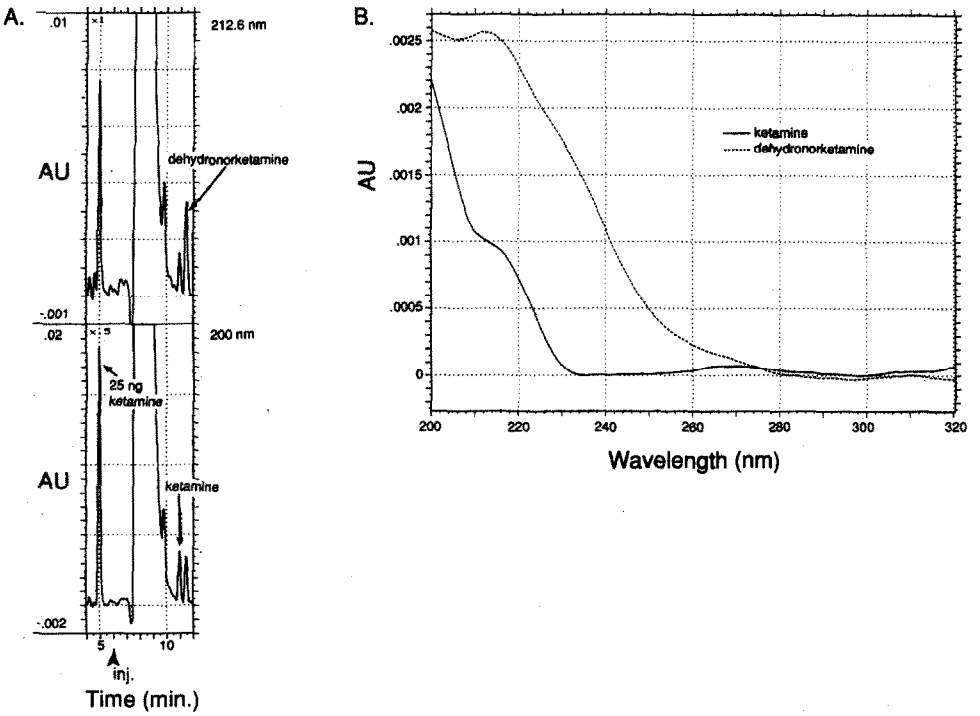

Fig. 3. (A) Typical chromatogram of serum obtained from a horse treated with ketamine and acquired at two wavelengths of 221.6 and 200 nm on the PDA detector. Injection volume was 20 μl. (B) UV spectral curves for ketamine and dehydronorketamine, peaks as seen in (A), generated on the PDA detector in 200–320 nm range.

(85%)–water (20:2:78, v/v/v) was deemed optimal for the extraction ultrafiltration step. The microseparation system with a 10 000 molecular mass cut-off filter was successful for the extraction and purification of ketamine from other serum components.

Fig. 2 shows LC–UV profiles of 20-ng ketamine standards (0–6 min run time, injected at 0 min, not shown), equine serum spiked with 1000 ng/ml ketamine (6–12 min run time, injected at 6 min), and blank equine serum (12–18 min run time, injected at 12 min). The chromatogram shows the maximum wavelength absorbance for all peaks in the 200–320 nm range (bottom) with their respective spectral curves (top). The curves for ketamine in standard (No. 1) and in the ultrafiltrate of equine serum (No. 8) are identical. The ketamine peaks were symmetric and well-resolved. There was an excellent analytical window for the ketamine peak on the blank equine serum chromatogram (12–18 min run time).

A typical chromatogram for ketamine and dehydronorketamine in equine serum obtained from a horse treated with ketamine and collected 15 min after administration of the drug is shown in Fig. 3A. This chromatogram was acquired with the PDA detector set at the maximum absorption wavelength for dehydronorketamine (212.6 nm) and ketamine (200 nm). This chromatogram also shows a 25-ng ketamine standard (0–6 min run time, injected at 0 min), and 20 μl equine serum dosed with ketamine (6–12 min run time, injected at 6 min). The retention time for ketamine in standard and equine serum was 5.06 and 5.08 min, respectively, whereas the retention time of dehydronorketamine was 5.57 min. Fig. 3B shows the LC–UV spectral curves (200–320 nm) for ketamine and dehydronorketamine acquired from the peaks of these analytes taken from the equine serum chromatogram. The curve for dehydronorketamine is substantially different from that for ketamine; this is primarily due to its additional chromophore.

Thermospray MS proved a specific and sensitive detection method for ketamine and its metabolite in equine serum. The thermospray spectrum for ketamine exhibited mainly an $[M + H]^+$ ion at m/z 238 (Fig. 4A). The negative-ion mode of operation is much less sensitive than the positive-ion mode. To confirm the presence and to tentatively identify the dehydronorketamine in equine serum, a concentrated sample collected under the described conditions was analyzed (*ca.* 200 ng analyte) in order to obtained a full-scan mass spectrum (Fig. 4B). The spectrum of dehydronorketamine showed only an $(M + NH_4 - H_2O)^+$ ion at m/z 221. The thermospray MS chromatogram (Fig. 4C) from an equine serum sample collected after intravenous infusion of ketamine verified the possible formation of dehydronorketamine and the ability to analyze ketamine. The diagnostic ions for dehydronorketamine at m/z 221 and ketamine at m/z 238 were at a maximum at their respective retention times (10 min for ketamine and 10.5 min for dehydronorketamine) on a phenyl 3-μm analytical column when a mobile phase of methanol–acetic acid–0.15 *M* ammonium acetate (30:1:69, v/v/v) was used. The detection of ketamine and dehydronorketamine separated from other equine serum compounds under the above conditions was performed using a UV-Vis detector as well. This analysis proved that ketamine was eluted earlier than its metabolite (data not shown).

Statistical data for ketamine determination in spiked equine serum are presented in Table I: they were obtained from the photometric detector at 215 nm. The percentage recovery was determined by the comparison of UV signal idensities from standard solutions (unextracted) and from extracted spiked equine serum, for the same concentration of ketamine. The mean recovery of ketamine from spiked equine serum in a 50–5000 ng/ml range was 106.3%. The UV PDA and photometric detection limits for ketamine were estimated to be 15 ng/ml and 5 ng/ml with an injection volume of 100 μl, based on 3:1 signal-to-noise ratio at 215 nm at 0.002 AUFS.

A study of the linearity of the photometric detector response was performed by injecting increasing amounts of ketamine standards ranging from 2 to 500 ng. The relationship between the peak area and the concentration of aqueous ketamine solution was linear over 5 μg/ml with a correlation coefficient of 0.9990 ($n = 8$).

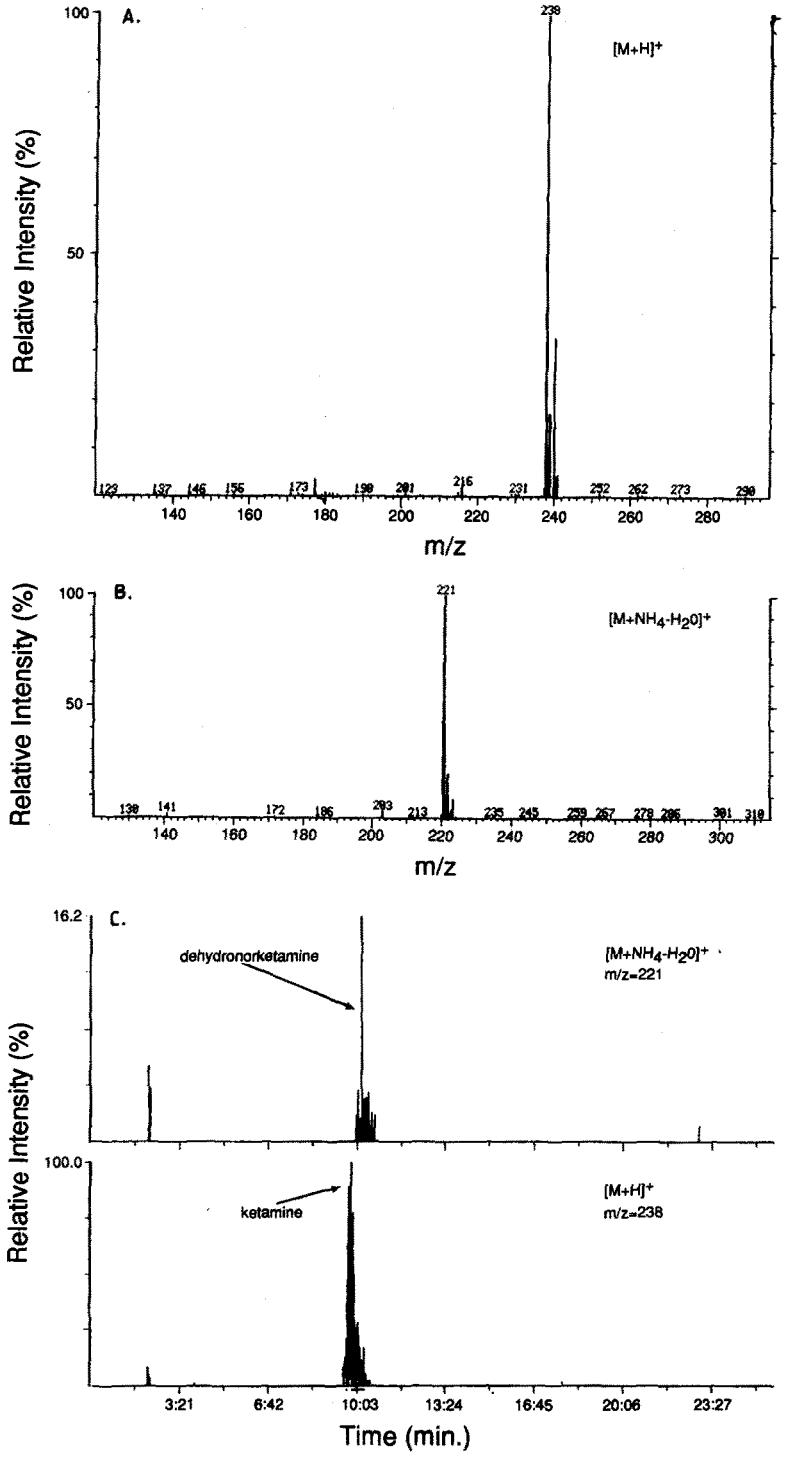

Fig. 4. (A) Thermospray MS spectrum of ketamine standard. (B) Thermospray MS spectrum of possible dehydronorketamine in dosed equine serum (15 min after intravenous infusion). (C) Thermospray MS chromatogram tentatively confirming the presence of dehydronorketamine and ketamine in equine serum collected 15 min after intravenous infusion. Injection volume, 100 μl.

TABLE I

STATISTICAL SUMMARY FOR KETAMINE RECOVERY FROM SPIKED EQUINE SERUM

Detection wavelength, 215 nm.

Amount spiked (ng/ml)	n	Amount recovered (ng/ml)		Coefficient of variation (%)	Recovery (%)
		Range	Mean ± S.D.		
5000	5	4992–5227	5055 ± 89.04	1.76	101.10
1000	5	1057–1140	1104 ± 43.34	3.92	110.40
250	5	238–262	249 ± 9.63	3.86	99.68
50	5	51–65	57 ± 5.10	8.94	114.00

CONCLUSION

An original HPLC method with UV detection has been developed to measure simultaneously ketamine and one of its metabolites, dehydronorketamine, in equine serum. The method provides a simple, sensitive and reliable analytical procedure for use in clinical studies. Owing to its ability to detect low concentrations, the assay is appropriate to analyze residues of ketamine and dehydronorketamine in equine serum. A norketamine was not found in serum obtained from horses given ketamine. A ketamine metabolite, dehydronorketamine, tentatively identified by UV–PDA and thermospray–MS detection, was found in serum from horses given ketamine by intravenous infusion. However, more work is needed to establish a definitive confirmation of this metabolite.

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