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Short communication

Simultaneous determination of ketamine and xylazine in canine plasma by liquid chromatography with ultraviolet absorbance detection

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic method for the simultaneous determination of ketamine and xylazine in canine plasma is described. Plasma samples (500 μl) are cleaned up via liquid–liquid extraction. The analytes and the internal standard clonidine are separated on a cyano (CN) column using a mobile phase containing acetonitrile–0.005 *M* phosphate buffer adjusted to pH 5.5 (3:2) at a detection wavelength of 215 nm. The method was validated according to specificity, sensitivity, accuracy and reproducibility and was used to determine the plasma concentrations of both compounds in dogs after intramuscular injection.
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Keywords: Ketamine; Xylazine

1. Introduction

More than 200 species including small and wild animals can successfully be anesthesized with a combined injection of the *N*-methyl-D-aspartate receptor antagonist ketamine and the α_2-adreno receptor agonist xylazine [1]. Especially in many wildlife species, better knowledge of the pharmacokinetic parameters of both compounds is desirable. Although several high-performance liquid chromatographic (HPLC) methods for the determination of one of both substances have been published [2–8], to our best knowledge, there is no report concerning their simultaneous determination, which is especially desirable in small species with regard to smaller plasma volumes required. To this aim, we present a HPLC method, which was established with canine plasma and validated according to the requirements published by Lindner and Wainer [9].

2. Materials and methods

2.1. Chemicals

Ketamine-HCl, xylazine-HCl and clonidine-HCl were purchased from Sigma (Taufkirchen, Germany). Diethyl ether, *n*-hexane, potassium dihydrogenphosphate, disodium hydrogenphosphate, HCl and NaOH were supplied by Merck (Darmstadt,

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Germany) and HPLC-grade acetonitrile by Lab-Scan (Dublin, Ireland). Distilled water was used throughout.

2.2. Solutions

Phosphate buffer (651.44 mg/ml KH_2PO_4 and 37.38 mg/ml Na_2HPO_4, pH 5.5, 0.005 *M*) was used for the mobile phase and for the stock solutions (1 mg/ml) of ketamine, xylazine and desipramine. These were stored at −20 °C and working solutions were made daily from the stock solutions by appropriate dilutions.

2.3. Apparatus and chromatographic technique

The chromatographic system consisted of an 816 autosampler (Spark, Emmen, The Netherlands), a 125 solvent module and a 166 detector (Beckman, Fullerton, CA, USA) measuring absorption at 215 nm. The software 32 Karat (Beckman) was used for instrument control and data acquisition. Separation was achieved on a reversed-phase 5 μm CN LiChrocart 250-4 column (Merck). The mobile phase consisted of acetonitrile–phosphate buffer (3:2). It was degassed by sonication and used recirculating at a flow-rate of 1.4 ml/min resulting in a system back pressure of about 125 bar. The column temperature was controlled with a SPH 99 HPLC oven (Spark) adjusted to 30 °C. The system was calibrated daily with freshly prepared standards. The analyte concentrations were determined via the peak heights of the analytes versus that of the internal standard.

2.4. Sample preparation

The procedure is based on a liquid–liquid back extraction technique described by Adams et al. [2] for ketamine and midazolam. It was modified for canine plasma and is shown in Fig. 1. Mixing steps were performed with a mixer (IKA vibrax VXR, Jahnke u. Kunkel, Staufen, Germany) at 1800 inversions per minute. Glass was used throughout due to the instability of plastics against diethyl ether.

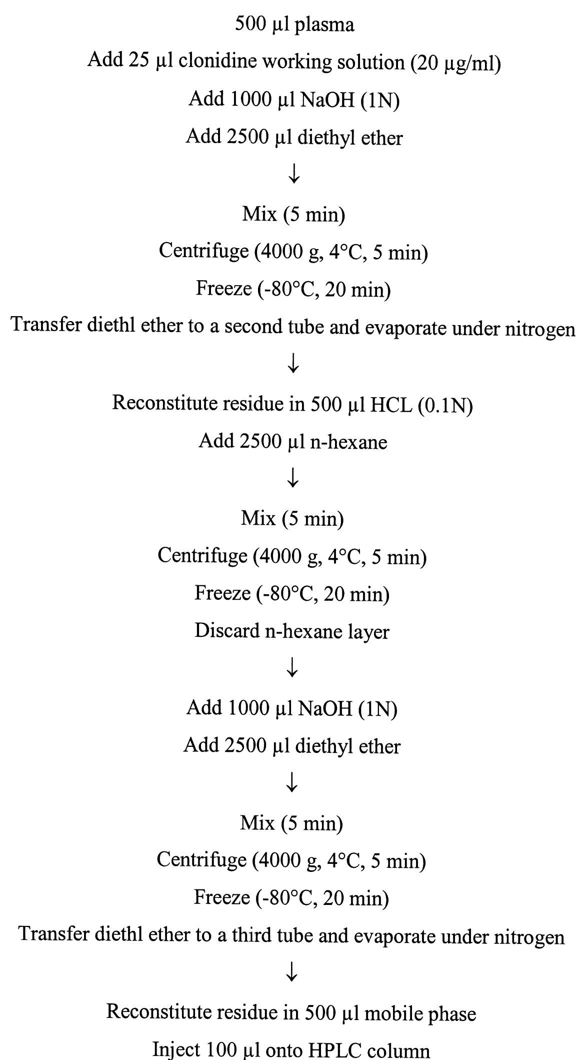

Fig. 1. Flow chart of the extraction technique.

2.5. Validation

2.5.1. Selectivity

Plasma aliquots of six healthy untreated dogs were extracted and chromatographed using the technique described in Sections 2.3 and 2.4. Chromatograms were examined for interferences at the retention times of interest. The chromatographic behavior of ketamine and xylazine metabolites was not examined.

2.5.2. Linearity of calibration

Calibration standards for ketamine and xylazine were prepared over the concentration range of 5000 to 19.5 ng/ml by adding appropriate dilutions of the stock solutions to drug-free plasma. The calibration curves were calculated by linear least-squares regression of measured versus added concentrations of the analytes.

2.5.3. Repeatability

The intra-day repeatability of the method was assessed by extracting and measuring five plasma samples of the same concentrations during a single analytical run. The inter-day repeatability was investigated by analyzing five aliquots of the same plasma samples at different days each. The aliquots were stored at −20 °C between the assays. Both assays were performed with high and low concentrated plasma samples (5000 or 156.25 ng/ml of both ketamine and xylazine).

2.5.4. Accuracy

The accuracy of the method was evaluated using eight drug-free plasma samples supplemented with different amounts of the analytes. The relationship between added and measured analyte concentrations was determined using a linear least-squares regression.

2.5.5. Stability

The stability of the analytes was assessed using five plasma standards (5000 ng/ml of both compounds) that were stored at −20 °C for 2 months. The measured concentrations were compared with those obtained from five freshly prepared plasma standards (5000 ng/ml of both compounds) and deviations were examined with the Mann–Whitney test. The stability of reconstituted plasma extracts was evaluated by repeated injection of six calibration samples from the same vials immediately after reconstitution and 24 h later. During this period the vials were stored at room temperature in the autosampler. The stability was calculated by comparison of the peak heights and deviations were examined with the Wilcoxon test.

2.5.6. Limits of detection and quantitation

A signal-to-noise ratio of 10 was defined as the limit of detection. It was determined for both ketamine and xylazine from the detector response after direct injection of calibration standards with decreasing analyte concentrations. Plasma standards were used in an analogous method to examine the limits of quantitation of both substances.

2.5.7. Absolute recoveries

The absolute recovery of ketamine and xylazine was evaluated in five independent experiments by means of equally concentrated calibration standards and plasma samples. The concentration used was 5000 ng/ml for both compounds and the recovery was calculated by comparison of the observed peak heights.

2.5.8. Application of the technique

The technique was used to measure plasma concentrations of ketamine and xylazine in laboratory dogs after intramuscular injection and in wolves after darting.

3. Results

Representative chromatograms obtained with the described method are shown in Fig. 2. Separation is achived within 16 min, ketamine, xylazine and clonidine are found at retention times of 4.5, 14.4 and 11.1 min with minimal variations. No interfering peaks can be observed at the retention times of interest in plasma from untreated dogs. The concentration versus detector response curves for both ketamine (r^2=0.9998) and xylazine (r^2=0.9999) are linear in the concentration range from 5000 ng/ml to the minimum detectable amount of 19.5 ng/ml for ketamine and 9.8 ng/ml for xylazine. The limit of quantitation was 78.1 ng/ml for ketamine and 19.5 ng/ml for xylazine. Calibration curves obtained in plasma from untreated dogs are linear in the concentration range from 5000 ng/ml to the specified limits of quantitation (ketamine: r^2=0.9998, xylazine: r^2=0.9997). The accuracy of the method was proved by excellent agreement between added versus measured concentrations of ketamine and

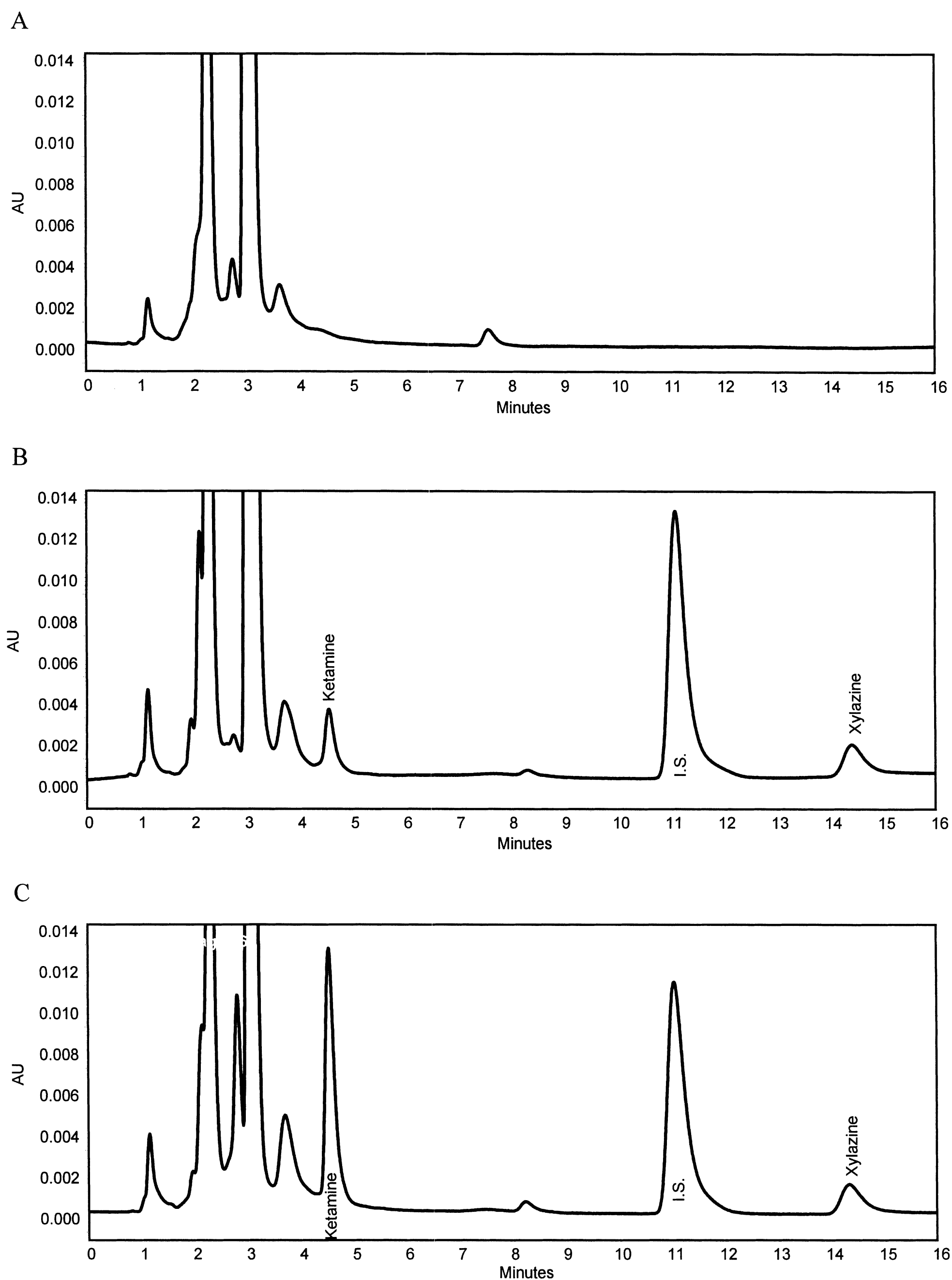

Fig. 2. Representative chromatograms obtained with the described method. (A) Drug-free plasma. (B) Drug-free plasma with ketamine (156.25 ng/ml) xylazine (156.25 ng/ml) and clonidine (I.S. 2000 ng/ml) added. (C) Plasma from a dog 50 min after administration of the anesthetics containing 1225 ng/ml ketamine and 178 ng/ml xylazine.

Table 1
Intra- and inter-day repeatability (mean±SD) of ketamine and xylazine plasma assays

Concentration added (ng/ml)	n	Concentration measured (ng/ml)	RSD (%)
Intra-day repeatability			
Ketamine			
156.25	5	156±3	2.2
5000	5	5012±135	2.7
Xylazine			
156.25	5	146±3	1.9
5000	5	4943±101	2.0
Inter-day repeatability			
Ketamine			
156.25	5	147±11	7.6
5000	5	4970±282	5.7
Xylazine			
156.25	5	188±15	7.1
5000	5	4936±112	2.3

xylazine. Least-squares regression lines between both concentrations were linear (ketamine: r^2= 0.9949, xylazine: r^2=0.9994) and yielded slopes of 1.019 for ketamine and 0.9863 for xylazine, which is close to the ideal value of 1. Table 1 shows the intra- and inter-day repeatability of ketamine and xylazine plasma assays. The method is characterized by good repeatability with low relative standard deviations (RSDs) at intra-day assays and anticipated greater variability at inter-day assays. The absolute recovery (mean±SD) calculated from five plasma assays was 96.2±3.8% for ketamine, 94.4±1.9% for xylazine and 94.6±3.4% for the internal standard clonidine. The stability assay showed no significant difference for xylazine and 3.95% lower values (P=0.0317) for ketamine after 2 months of storage at −20 °C as compared with freshly prepared plasma samples. There was no statistically significant decrease in the measured peak heights of ketamine, xylazine and the internal standard clonidine after 24 h of storage at room temperature. The technique was used to determine pharmacokinetic parameters in dogs. Fig. 3 shows an example of the observed plasma concentrations after intramuscular injection.

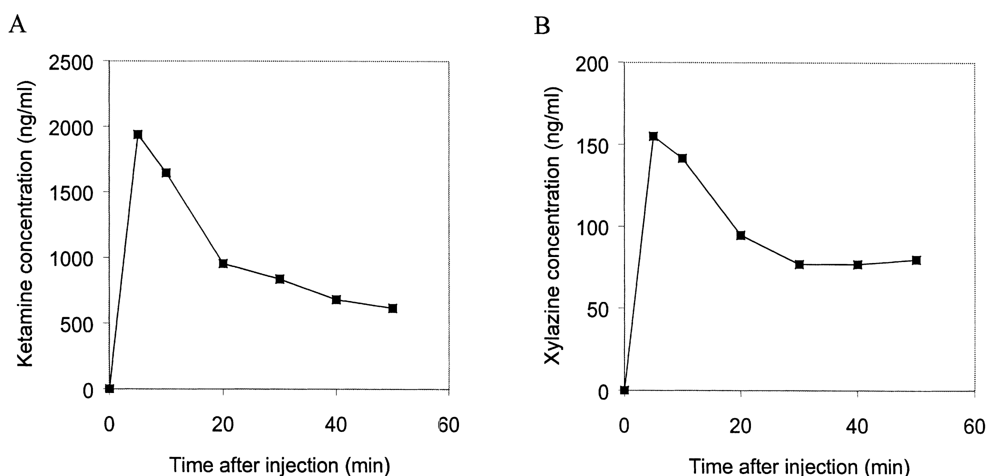

Fig. 3. Ketamine (A) and xylazine (B) plasma concentrations measured in a dog after intramuscular injection.

4. Discussion

The method described above allows the simultaneous determination of ketamine and xylazine with HPLC standard equipment in a simple and effective manner. The extraction technique is primarily based on the method described by Adams et al. [2], which was also used successfully by Gross et al. [6] in a slightly modified way for the extraction of ketamine and bupivacaine from human plasma. It was chosen after unsatisfactory preliminary results regarding selectivity and recovery obtained by solid-phase extraction at high pH with ODS cartridges (data not shown). In contrast to this, the liquid extraction procedure results in very clean samples and reproducible measurements. Recovery rates of about 95% for ketamine, xylazine and the internal standard are excellent and consistent with results from other authors using liquid–liquid extraction for one of the substances [2,3,5,8]. A modification of the original method [2], which used direct back-extraction with HCl from diethyl ether, lies in the evaporation of the organic phase as previously proposed by Bolze and Boulieu [5]. The residue is reconstituted in HCl and extracted with *n*-hexane, which results in equally good clean-up characteristics with superior recovery as compared to the original method. Adams et al. [2] directly injected HCl onto the column, which in our system generated unreproducible drifting baselines. Other authors [5,6] suggest the evaporation of the aqueous layer, which additionally eliminates the danger of an injection of partially remaining organic phase. In order to avoid the time-consuming evaporation of aqueous solutions we decided to principally follow Yanagihara et al. [8] and reextract the compounds in a third step, which resembles the first step and eliminated the problem of drifting baselines. The cyano (CN) column was chosen in agreement with Gross et al. [6] and the detection wavelength of 215 nm at the absorption maximum of ketamine as described by other authors [7,8] in order to account for the lower absorption coefficient of this compound as compared with xylazine (λ_{max}=225 nm).

Gross et al. [6] used desipramine as internal standard that was found at a retention time of 17 min in our system. As compared to this substance the calculation of the analyte concentrations relative to clonidine resulted in distinctly smaller within-day variations in six independent experiments (data not shown). Furthermore the run times are not prolonged with clonidine in contrast to desipramine. Along with the good accuracy and repeatability of the method clonidine is proven to be a suitable internal standard. Both analytes were found to be stable under storage conditions and in reconstituted plasma extracts. The deviations of ketamine concentrations after 2 months of storage at −20 °C as compared to freshly prepared samples lie well within the specified range of inter-day variations.

In conclusion, the described method shows good specificity, sensitivity, reproducibility, accuracy and a limit of quantitation sufficient for drug monitoring after systemic administration. It is thus suitable for the simultaneous determination of ketamine and xylazine using plasma samples of only 500 μl volume.

References

[1] H. Wiesner, Tierärztl. Prax. 26 (1990) 2.

[2] H.A. Adams, B. Weber, B. Bachmann, M. Gúerin, G. Hempelmann, Anaesthesist 41 (1992) 619.

[3] A. Akbari, B.J. Gordon, P.B. Bush, J.N. Moore, J. Chromatogr. 427 (1988) 207.

[4] M. Alviniere, P.L. Toutain, J. Chromatogr. 222 (1981) 308.

[5] S. Bolze, R. Boulieu, Clin. Chem. 44 (1998) 560.

[6] A.S. Gross, A. Nicolay, A. Eschalier, J. Chromatogr. B 728 (1999) 107.

[7] S.S. Seay, D.P. Aucoin, K.L. Tyczkowska, J. Chromatogr. 620 (1993) 281.

[8] Y. Yanagihara, M. Ohtani, S. Kariya, K. Uchino, T. Aoyama, Y. Yamamura, T. Iga, J. Chromatogr. B 746 (2000) 227.

[9] W. Lindner, I.W. Wainer, J. Chromatogr. B 707 (1998) 1.