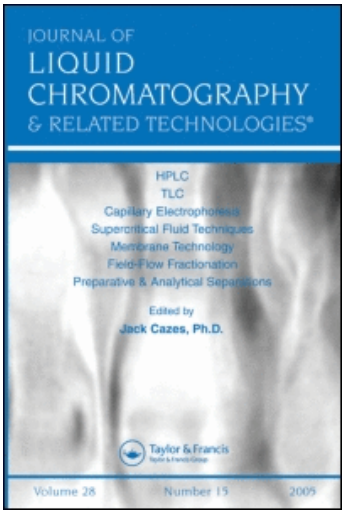

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LIQUID CHROMATOGRAPHIC ASSAY OF XYLAZINE IN SHEEP AND CATTLE PLASMA

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ABSTRACT

A high-performance liquid chromatographic method for the determination of xylazine in sheep and cattle plasma at levels as low as 3 ng/ml has been developed. Xylazine was extracted from plasma under alkaline conditions with diethyl ether and, then, reextracted into phosphoric acid. Following addition of octane-1-sulfonate, xylazine was extracted as ion pair into chloroform and analyzed on a reversed-phase C_{18}, 5 μm, column. Overall mean recovery of the method was found to be 99 ± 2.6%.

INTRODUCTION

Xylazine hydrochloride (2-(2,6-dimethylphenylamino)-4H-5,6-dihydro-1,3-thiazine hydrochloride) is extensively used in veterinary practice for its potent sedative, analgesic and myorelaxant properties (1-11). Since xylazine is administered in small doses, sensitive methods are required to investigate the pharmacokinetics of the drug in domestic and wild animals.

Xylazine has been analyzed in plasma by various chromatographic methods. Among these, high-performance liquid chromatography (HPLC) (12-13) seems to be superior to thin-layer (14) and gas chromatography (15-16) in terms of simplicity and efficiency. Nevertheless, these HPLC methods either lack the desirable sensitivity (12) or suffer from interferences of indigenous compounds (13).

This report describes a sensitive, accurate and precise HPLC method for the analysis of xylazine in sheep and cattle plasma. Sample preparation is based on direct extraction at alkaline conditions followed by ion-pair formation and reextraction at acidic conditions. Using this analytical scheme, interfering peaks are not observed even when plasma contains 3 ng/ml xylazine.

EXPERIMENTAL

Instrumentation

HPLC was carried out on a Gilson (Villiers-le-Bel, France) system consisting of a Model 802 manometric module, a Model 302 piston pump, a Model HM/HPLC dual-beam variable-wavelength UV-vis spectrophotometer set at 220 nm, and a Model N1 variable-span recorder. A HPLC-Technology (Macclesfield, UK) Model TC 831 column oven, set at 35 °C, permitted temperature regulation. Injections were made on a Hichrom, 25 cm X 4.6 mm I.D., stainless-steel column prepacked with Nucleosil 120, C_{18} 5-µm, through a Rheodyne 7125 sample injector equipped with a 100-µl loop.

HPLC Procedure

The mobile phase used was a mixture of 450 ml of acetonitrile and 550 ml of 0.02 N phosphoric acid solution. The mobile phase was degassed using helium and delivered at a rate of 1 ml/min. Under these conditions the retention time of xylazine was 5.0 min. Recordings were made at a chart speed of 0.5 cm/min and a detector sensitivity of 0.020 a.u.f.s.

Chemicals

Xylazine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Octane-1-sulfonic acid sodium salt and HPLC grade acetonitrile were obtained from Merck-Schuchardt (Munchen, FRG). All other chemicals used were of analytical reagent grade. Deionized water was distilled before use.

Stock solutions of xylazine hydrochloride were prepared in methanol (ca. 1 mg/ml) and stored at -25 °C. Aliquots of these stock solutions were diluted with mobile phase immediately before use, to give working solutions containing xylazine hydrochloride in the range 0.1-10 µg/ml.

Extraction Procedure

A 2-ml sample was transferred to a 15-ml centrifuge tube, mixed with 0.5 ml of 0.1 N sodium hydroxide, and extracted with 8 ml of diethyl ether. After centrifugation for 1 min at 1000g, a 7.5 ml aliquot of the upper organic phase was transferred into another centrifuge tube to be vortexed with 3 ml of 0.02 N phosphoric acid and centrifuged. A 2.5-ml volume of the bottom acidic

layer separated was mixed with 0.3 ml of 0.5 M aqueous octane-1-sulfonate solution, pH 2, and extracted with 5 ml of chloroform. Following extraction, tube content was centrifuged and a 4.5-ml volume from the bottom chloroform layer was taken to be further dried under a stream of nitrogen at 35 °C. The residue remaining was dissolved in 100 µl of mobile phase, and a 50-µl aliquot was used for HPLC analysis.

Calibration curves were constructed by plotting peak heights versus concentration from 50-µl injections of each of the prepared working solutions. The concentration of xylazine in samples was calculated by reference to calibration curve and multiplication by appropriate dilution factor.

RESULTS AND DISCUSSION

The extraction of xylazine from plasma samples was carried out at alkaline conditions. At these conditions the ionization of xylazine, a molecule with distinct basic character, is suppressed and, thus, partitioning of the unionized form into diethyl ether can be easily effected.

Further cleanup could be performed by submitting the extract to partition with aqueous phosphoric acid. This procedure, although simple and rapid resulted, however, in appearance of interfering peaks in the chromatograms recorded. Interferences have also been observed by Akbari et al. (13) in a pertinent experiment on horse blood, while Alvinerie and Toutain (12) did not noticed any interference using chloroform as extracting solvent. Since these interferences are associated with the alkalinity of

the sample, Akbari et al. (13) applied a salting-out extraction process throw the use of sodium sulfate, to overcome the problem. This process resulted, however, in reduction but not in elimination of interferences. This is the reason why authors suggested alteration of the mobile-phase pH in cases where interferences are encountered. Furthermore, the salting-out process suffers from certain drawbacks; the amount of sodium sulfate, its contact time, and the total extraction time were all reported (13) to have an adverse effect on the cleanup and recovery obtained.

Considering the above, attempts were made to further purify the aqueous phosphoric acid extract by partitioning it with chloroform. As the partition coefficient of xylazine prevent its extraction from acidic solutions into organic solvents, xylazine molecules had to be converted, prior to extraction, to ion pairs with octane-1-sulfonate anions. This procedure resulted in effective carryover of xylazine ion pairs into chloroform. The extraction was most efficient when the concentration of octane-1-sulfonate in its aqueous solution was higher than 0.4 M and the pH value of the solution had been adjusted at 2.

The effectiveness of the cleanup procedure permitted chromatographic analysis of plasma samples under isocratic conditions. Using a Nucleosil C_{18} 5-μm stationary phase and aqueous acetonitrile containing dilute phosphoric acid as eluent, the chromatograms recorded (Fig. 1) were free of interfering extraneous peaks. Xylazine was eluted in 5.0 min and no changes in retention time was noted with continual column use.

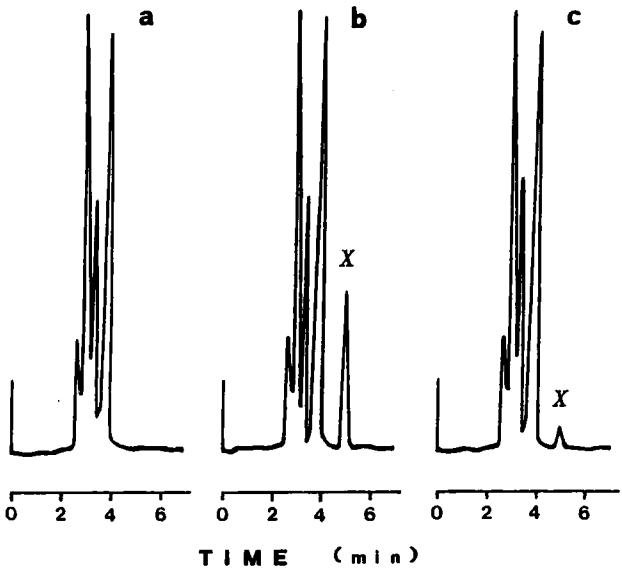

FIGURE 1. Typical chromatograms of a blank plasma sample (a), a sample containing 25 ng/ml (b), and a sample containing 3 ng/ml (c) of xylazine (X). Mobile phase, acetonitrile–0.02 N phosphoric acid solution (45:55, v/v); column, Nucleosil 120, C_{18} 5-μm, 25 x 0.46 cm; temperature, 35 °C; flow rate, 1 ml/min; detection wavelength, 220 nm; detector sensitivity, 0.020 a.u.f.s.; chart speed, 0.5 cm/min; injection volume, 50 μl.

Under the mentioned conditions, determinations of xylazine in plasma at levels down to 3 ng/ml (peak to noise ratio : 3) could be readily effected. This limit could be further lowered by using larger sample size, due to the absence of any interfering peaks in sample chromatograms.

Regression analysis of the data obtained by running a series of xylazine working solutions showed the response to be linear in the range studied [0.020 a.u.f.s., y=-0.06+1.78x, correlation co-

TABLE 1

Presicion and Accuracy Data for the Determination of Xylazine in Plasma (n = 5)

Concn. added (ng/ml plasma)	Mean concn. found (ng/ml)	Std dev	Rel. std dev (%)	Recovery (%) (mean ± SD)
5	5.1	0.2	3.5	103.0 ± 3.7
25	24.5	0.4	1.6	97.9 ± 1.5
50	49.5	0.5	1.0	99.0 ± 1.0
100	97.0	0.8	0.8	97.0 ± 0.8
200	195.7	2.4	1.2	97.9 ± 1.2
500	495.2	4.7	0.9	99.0 ± 1.0
			Overall mean recovery ± SD	99.0 ± 2.6

efficient (r) = 0.9999, where y represents peak height in mm and x the quantity of xylazine injected in ng].

The precision and the accuracy of the proposed method were studied by spiking plasma samples at six fortification levels with standard xylazine hydrochloride and analyzing five replicates. The results are tabulated in Table 1. The overall mean recovery of xylazine was 99 ± 2.6%

In conclusion, the results of the present study show that the proposed HPLC method, although more complicated than the existing ones, does not suffer from interferences and, thus, is an efficient and reliable means of quantitating xylazine in sheep and cattle plasma. Minor modifications should made the method suitable for residue studies in milk and tissues as well. Moreover the analytical scheme on which this method is based might also be useful for extracting basic drugs, in general, from biological materials.

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