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Note

Determination of xylazine in blood components using high-performance liquid chromatography

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Xylazine (Fig. 1) has been widely used in equine veterinary medicine as a potent non-narcotic sedative exhibiting analgesic and muscle relaxant properties [1–11]. Xylazine 2-(2,6-dimethylphenylamino)-4H-5,6-dihydro-1,3-thiazine, is also widely used in food-animal, wildlife and companion-animal veterinary medicine for production of similar effects [9, 11–13].

Xylazine (molecular mass 256.8) is readily soluble in water and methanol. It is less soluble in diethyl ether and chloroform. Previous xylazine assay methods in biological fluids included spectrophotometry [14,15], thin-layer chromatography [16], gas chromatography [16,17] and high-performance liquid chromatography (HPLC) [18].

Detection of xylazine in cells, however, requires an extremely clean extraction process and a clear chromatogram with a minimum of interference peaks. The present method was developed to satisfy this requirement. This report describes a rapid, specific, sensitive and inexpensive HPLC technique for the determination of xylazine in blood components.

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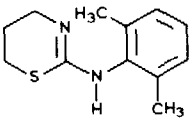

Fig. 1. Chemical structure of xylazine.

EXPERIMENTAL

Samples and chemicals

Xylazine hydrochloride was obtained from Haver-Lockhart (Bavet Division Miles Labs., Shawnee, KS, U.S.A.). Analytical-grade reagents were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

A stock solution of 100 μg/ml xylazine hydrochloride in methanol was prepared and stored at room temperature. HPLC standard solutions were prepared daily.

Composite blood samples from several horses were collected intravenously (i.v.) from the jugular vein and fractionated with an IBM 2997 blood cell processor (International Business Machines, Cranbury, NJ, U.S.A.) using the continuous-flow centrifugation technique [19]. The hemapheresis products (concentrated white blood cells, plasma, packed red blood cells and whole blood) were collected from the extracorporeal blood circuit of a blood cell separator. A sodium citrate solution (4%) was mixed with the blood as part of the hemapheresis process to prevent clotting of its components. The packed cell volume of concentrated white blood cells and concentrated red blood cells was determined and adjusted to 65–70% by the addition or removal of plasma. All samples were immediately stored at $-70\,^{\circ}$C prior to analysis.

Sample extraction

To break the cellular membrane, 1 ml of thawed cell suspension was mixed with 1 ml of deionized water and sonicated for 1 h. Sodium sulfate (1.5 g) was added to the cell solution and the sample was vortexed for 10 s and allowed to stand for an additional 3 min. A 10-ml volume of diethyl ether was added, capped, and the mixture was rigorously vortexed for 10 s. After centrifugation (600 *g*) for 5 min, the supernatant was decanted to a clean test tube and dried under a stream of nitrogen at approximately 37 °C. The residue was dissolved in 100 μl of acetonitrile for HPLC analysis.

For extraction of xylazine from plasma, 1 g of sodium sulfate was added to 1 ml of horse plasma mixed on a vortex mixer for 5 s. The plasma was allowed to stand for 3 min and xylazine was extracted with diethyl ether as previously described.

Chromatographic conditions

The isocratic chromatography was conducted using a Waters (Milford, MA, U.S.A.) Model 6000A solvent delivery pump. The separation was performed on a μBondapak C_{18} (30 cm $\times$ 2.9 mm I.D.) column at ambient temperature. The system was equipped with a fixed-wavelength absorbance detector (254 nm). The degassed mobile phase, consisting of acetonitrile–$10^{-3}M$ aqueous hydrochloric acid–methanol (65:30:5), was pumped through the column at a flow-rate of 1 ml/min.

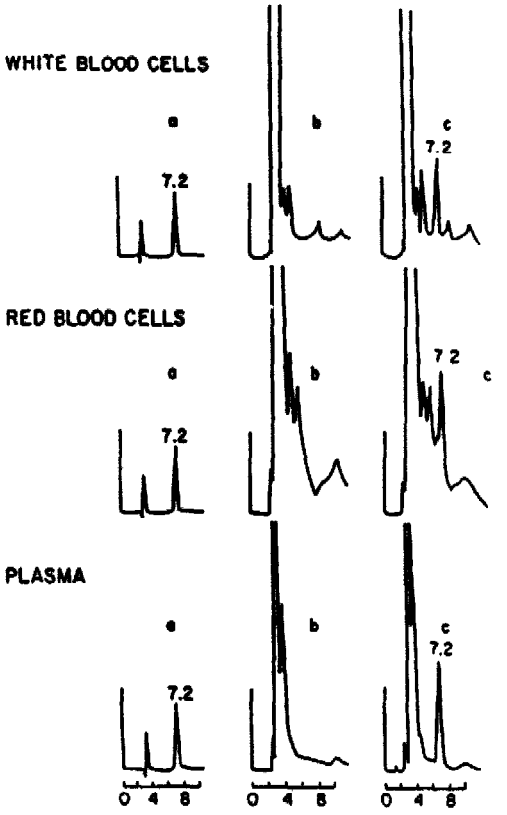

Fig. 2. Chromatograms of (a) standard xylazine, (b) control samples and (c) samples of hemapheresis products in a horse 4 min after a single i.v. injection of xylazine (0.44 mg/kg.)

Analyses

The chromatographic column was preconditioned. Several injections of the standard solution of xylazine (1 μg/ml) were made prior to sample analysis. A calibration curve was obtained by injecting 50-μl xylazine standards (1000, 700, 500, 200, 50 and 20 ng/ml) in the mobile phase and measuring the peak heights. Samples were injected in acetonitrile to minimize background interferences.

In order to monitor column performance, retention time, the recorder response, etc., a standard solution was analyzed after every fifth sample. A reagent blank was analyzed with each set of analyses. Xylazine levels were quantified by comparing retention time and peak height of components in the sample with the analytical standards.

RESULTS AND DISCUSSION

Several extraction methods and mobile phases were evaluated. The extraction procedure and chromatographic conditions described in this report gave optimum

TABLE I

RECOVERY OF XYLAZINE FROM FORTIFIED HORSE BLOOD COMPONENTS

Mean of three replications per sample ± one standard deviation. WBC = White blood cells, RBC = red blood cells.

Sample No.	Concentration (ng/ml)	Recovery (%)		
		Plasma	WBC	RBC
1	800	93.6 ± 3.3	95.9 ± 2.6	96.8 ± 2.6
2	400	91.3 ± 2.5	96.1 ± 2.5	99.5 ± 3.3
3	200	91.6 ± 2.6	97.9 ± 3.8	97.5 ± 2.5
4	100	95.0 ± 4.0	97.9 ± 2.8	98.9 ± 2.5
5	50	95.1 ± 4.1	96.6 ± 2.3	99.8 ± 2.6
6	20	94.8 ± 3.8	98.3 ± 4.5	97.8 ± 3.1
7	10	95.5 ± 4.7	99.4 ± 3.8	99.5 ± 3.1
8	5	93.4 ± 4.1	99.4 ± 4.5	99.5 ± 4 1
Mean ± overall S.D.*		93.8 ± 3.7	97.7 ± 3.5	98.7 ± 3.0

*Overall S.D. was computed as:

$$\text{S.D.} = \left(\frac{1}{8} \sum_{i=1}^{8} \text{S.D.}_i^2 \right)^{1/2}$$

separation with a short retention time. Although xylazine is more soluble in polar solvents, use of diethyl ether as the extraction solvent resulted in quantitative recovery with minimal coextractives. At low xylazine concentrations, diethyl ether extraction from plasma yielded an average extraction efficiency of 93.8 ± 3.7% with a minimum of interferences. Extraction under alkaline pH conditions (normally used for alkaline extraction) did not improve xylazine recovery but did increase analytical interferences. Reducing the amount of sodium sulfate used in the extraction step results in an increase in coextractives. Nevertheless, a larger amount of sodium sulfate causes the solidification of the mixture and resulting poor recovery. The sodium sulfate contact time during extraction has a direct effect on the clean-up and recovery obtained. If the mixture is left for longer than 3 min the sodium sulfate solidifies with resulting reduced recovery. Finally, the diethyl ether extraction period is also critical since prolonged extraction periods result in increased coextractives. If interferences are encountered, the retention time of xylazine can be shifted to a suitable location of the chromatogram by altering the pH of the mobile phase.

Chromatograms obtained from cells and plasma extractions are shown in Fig. 2. Under given chromatographic conditions, the retention time of xylazine was 7.2 min. A detection limit of 5 ng/ml is shown in Fig. 2 (a concentration with the signal-to-noise ratio of 3:1 was considered the limit of detectability).

Recovery of xylazine from white blood cells, red blood cells and plasma was 97.7 ± 3.5, 98.7 ± 3.0 and 93.8 ± 3.7%, respectively (Table I). Detector response

was found to be linear over the range of 50–1000 ng/ml. Extraction efficiencies of 97–100% were routinely obtained with clean extract. The limit of detection can be lowered by increasing the volume of sample from 1 to 2–5 ml. It should be noted, however, that increased sample size results in increased coextractive levels.

The extraction procedure described in this report allows quantitative determination of xylazine in various blood components. The previously reported methods suffer from coextractives interference, high detection limits or both. The method was used successfully to determine xylazine in horse and cattle blood components.

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