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Note

Rapid extraction and determination of xylazine in greyhound urine using high-performance liquid chromatography

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Xylazine, 5,6-dihydro-2-(2,6-xylidino)-4H-1,3-thiazine, is a widely used veterinary drug. The compound's sedative, analgesic and muscle relaxant properties are due to its action on the autonomic and the central nervous system. Xylazine shares certain pharmacological properties with a number of structurally related drugs, for example phenothiazines. Although it is intended as a veterinary drug, its metabolism in racing greyhounds is not well documented. A highly potent drug, it is used more extensively to sedate much larger animals. The pharmacokinetics of xylazine in the plasma of horses, cattle, sheep and dogs, by both intravenous and intramuscular routes, is reported [1]. In dogs, the onset of sedative action is between 10 and 15 min after injection and the period of analgesia is reported to be relatively short (15–30 min) [2]. No literature is available concerning metabolic pathways in racing greyhounds or relating to unchanged amounts of xylazine after administration.

Analytical methods reported for the determination of the xylazine in biological fluids include spectrophotometry and thin-layer chromatography (TLC) [3], high-performance liquid chromatography (HPLC) [4,5] and gas chromatography (GC) [6–8]. These methods require the drug to be isolated from the biological material using liquid–liquid extraction, reversed-phase solid-phase extraction with, for example, C_8 or C_{18} cartridges, or non-selective solid-phase extraction with, for example, XAD resins. Also, most of the literature is concerned with serum or plasma rather than urine extractions. The advantages

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of solid-phase extractions are well documented: in general they are fast, efficient and avoid emulsion formation which often occurs in solvent extractions.

No literature is available concerning the selective solid-phase extraction or determination of parent xylazine in the urine of racing greyhounds. This paper provides a highly efficient, rapid extraction method and an analysis procedure which can detect parent xylazine in the urine of racing greyhounds up to 8 h after dosing. The method will be applied for pharmacokinetic studies in racing greyhounds.

EXPERIMENTAL

Reagents

Xylazine (Rompun®) and diazepam were obtained from Bayer U.K. (Newbury, U.K.) and Roche Products (Welwyn Garden City, U.K.), respectively.

High-performance liquid chromatography

The chromatographic system consisted of a continuous-flow Kratos Spectroflow 400 pump (Analytical Instruments, Cambridge, U.K.) which was used to deliver solvent at a flow-rate of 2 ml/min. The eluent was monitored at 225 nm with a Pye Unicam PU4025 variable-wavelength ultraviolet detector. The column was 25 cm × 4.6 mm I.D. pre-packed with Hypersil 5-μm octadecylsilane C_{18} (HPLC Technology, Macclesfield, U.K.) and fitted with a Rheodyne 7125 injection system incorporating a 20-μl loop. The system was operated at room temperature (20°C).

Separation was achieved with an eluent of 1 g of tetramethyl ammonium hydroxide dissolved in deionised water (250 ml)–acetonitrile–methanol (250:150:10, v/v), giving a retention time of 6.5 min for xylazine and 9.4 min for diazepam used as an internal standard. All solvents used were of HPLC grade (Rathburn Chemicals, Walkerburn, U.K.).

Greyhound samples

Urine samples were collected at timed intervals from four greyhounds following a single intramuscular injection of 0.05 ml (Rompun, 2% solution) per kilogram body weight of a racing greyhound and were stored at −20°C before analysis. Each greyhound was dosed three times allowing sufficient time between dosings for total excretion to have occurred.

Spiked urine samples in the range 10–1000 ng/ml were prepared by adding aliquots of a standard solution of xylazine in methanol (1 mg/ml) to blank greyhound urine.

Extraction procedure

Bond-Elut® columns containing cyanopropyl (CN) packing material (100 mg) with a capacity of 1 ml were positioned in a Vac-Elut® system. Vacuum

pressure was adjusted to 50 kPa and each column was conditioned with methanol (2×1 ml) followed by deionised water (pH 7, 1×1 ml). Without allowing the column to dry out, the urine sample (1 ml), mixed with deionised water (pH 7, 0.5 ml) and diazepam (100 μl of a 15 μg/ml solution in methanol) as an internal standard, was added to the column. The urine was drawn through and the column was dried under vacuum for 3 min.

Methanol (50 ml) was added to 36% (w/w) hydrochloric acid (3 ml) to give methanolic hydrochloric acid. The drug was then eluted with methanolic hydrochloric acid–acetonitrile (50:50, v/v, 2×0.25 ml).

The eluent was then directly analysed by HPLC.

The positive identification of xylazine was based on comparison with standards, absorbance maxima (225 nm) and retention time.

Recovery

The percentage xylazine recovery was determined from spiked samples, using the procedure given, by comparing the peak area obtained after injection of an extract from a spiked urine sample of known concentration with that produced by the same concentration of drug in methanol. Each measurement was taken as the average of two determinations.

Quantitation

The quantitation of parent xylazine from actual samples was carried out using the ratio of xylazine peak areas to diazepam standard peak areas. A series of ratios, xylazine standard peak area divided by diazepam standard peak area, were calculated, varying the xylazine concentration. Straight-line graphs were obtained relating the ratio to xylazine concentration, and from this parent xylazine concentrations were calculated.

RESULTS AND DISCUSSION

The HPLC system described here was suitable for the analysis of xylazine form the urine of racing greyhounds. The extracts were extremely clean and the recoveries from spiked urine samples were 98.4±3.0%. No interference from endogenous compounds was noted (Fig. 1).

The linear relationship calculated between peak-area ratio (R) and the concentration of xylazine (x) in urine between 10 and 1000 ng/ml was $R=95.20x+0.3$ ($r=0.997$) at 0.02 a.u.f.s. Standard curves constructed on three different days showed good reproducibility over the concentration range used, with a coefficient of variation of 3.16%. The accuracy of the assay over three days was 90.34±4.6%, and the detection limit was 10 ng/ml.

Three of the four greyhounds showed a peak excretion time of 2 h and very similar excretion patterns. The fourth greyhound showed a peak excretion after 3 h and a somewhat erratic excretion pattern. This occurred in all three dosing

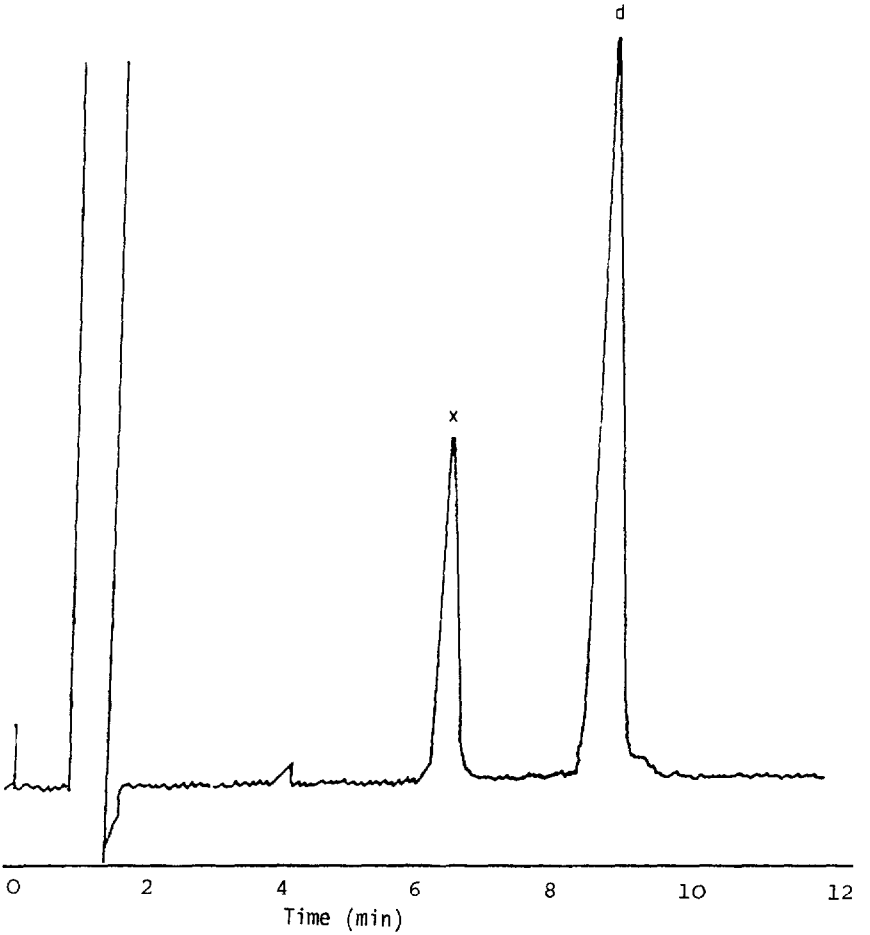

Fig. 1. Extract from greyhound urine (greyhound No. 3, 2-h sample). Mobile phase: 1 g tetramethyl ammonium hydroxide in deionised water (250 ml)–acetonitrile–methanol (250:150:10). Flow-rate: 2.0 ml/min. Detection wavelength: 225 nm. Samples: x = xylazine; d = diazepam.

trials for that particular greyhound. Unfortunately, samples could not be obtained from the greyhounds before 2 h since they were sedated. This observation differs from that of Newkirk and Miles [2].

Using the average excretion values between the dogs, an excretion curve for unchanged xylazine was produced (Fig. 2). An average peak excretion value of 0.29 μg/ml parent xylazine would be expected 2 h after dosing. A total of 2.7% of the parent dose was excreted within 8 h and no xylazine was detected after this time.

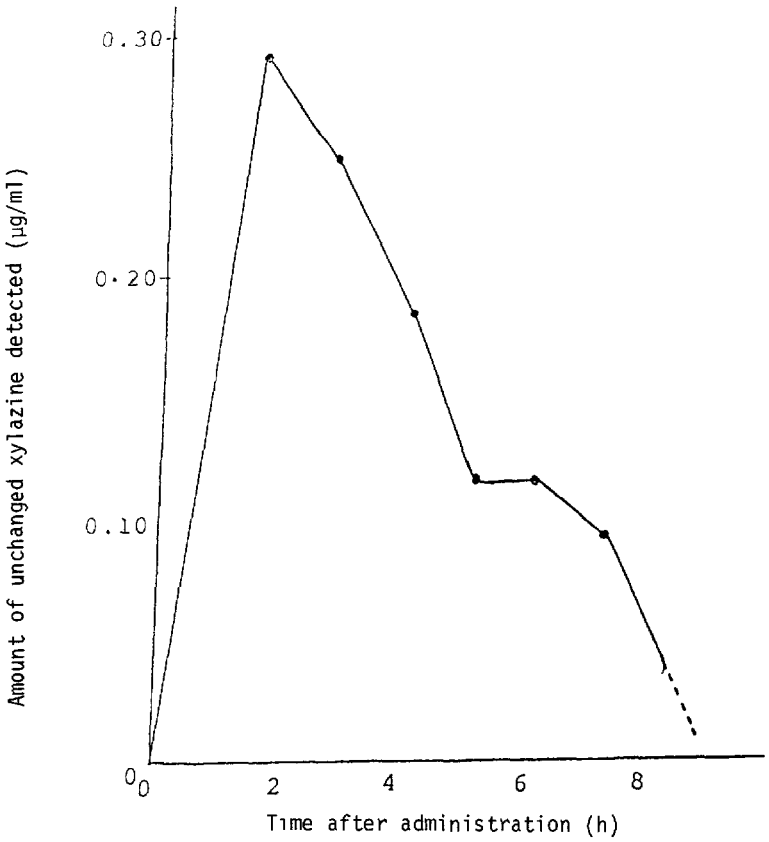

Fig. 2. Mean excretion pattern ($n=4$) of unchanged xylazine in greyhound urine after single-dose administration.

CONCLUSION

The detection of parent xylazine up to 8 h after the intramuscular injection of a single therapeutic dose shows that this analytical procedure is suitable for the detection of the misuse of this drug in greyhound racing. Since any greyhound seen to deteriorate markedly on previous performances is sampled immediately post race, parent xylazine from an effective dose would be detected.

The method described demonstrates quantitative recovery of the drug using the cyanopropyl columns. It is rapid (up to ten samples can be extracted simultaneously) and has minimal sample volume and solvent requirements.

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