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Determination of alclofenac in equine plasma and urine by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method to measure plasma and urinary alclofenac levels in equine biofluids is described. Isolation of the drug from plasma is achieved using liquid–liquid extraction with diethyl ether. Reversed-phase C_{18} solid phase extraction is used for the extraction of free and conjugated alclofenac from urine. The reproducibility and accuracy of the method were well within acceptable limits over the concentration ranges 0–10 and 0–20 μg/ml, respectively, for plasma and urine. Starting with 2 ml of plasma, a concentration of 0.1 μg/ml could easily be measured; the limit of quantification in urine (0.5 ml) was 1 μg/ml. Hydrolysis of urine with strong alkali resulted in the decomposition of alclofenac. A pharmacokinetic profile of alclofenac in the horse is shown.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used in equine medicine and have been found as illicit substances in body fluids of horses at racecourses or jumping events [1]. The detection of these doping substances in horse body fluids is facilitated by a knowledge of how such drugs are metabolized and eliminated in this species.

Nowadays, most racing laboratories are confronted with the detection in equine body fluids of drugs developed for use in humans. Alclofenac (4-allyloxy-3-chlorophenylacetic acid) is a human NSAID with analgesic and antipyretic actions [2]. Although a number of papers have appeared on the isolation and quantification of alclofenac and its metabolites, most reported data have related to the use of the radiolabelled compounds [3,4]. Published methods for the determination of alclofenac in plasma and urine involved trimethylsilylation [5] or methylation [6] with subsequent gas chromatographic (GC) analysis.

This paper reports a high-performance liquid chromatographic (HPLC) method developed for the determination of free alclofenac in horse plasma and free and conjugated alclofenac in urine with UV detection after separation on a reversed-phase column.

EXPERIMENTAL

Experimental animals

Three tablets of Mervan (Continental Pharma, Brussels, Belgium), corresponding to a total amount of 3 g of alclofenac, were administered by stomach tube to a standard-bred mare. Heparinized blood samples and urine were collected during a 96-h period after drug administration.

Reagents

The reference substances alclofenac and diclo-

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fenac were obtained through Continental Pharma and Ciba-Geigy (Basle, Switzerland). Diethyl ether, methanol and acetic acid (analytical grade) were obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade) was from Alltech (Deerfield, IL, USA). Aqueous HPLC solvent was prepared using water obtained with Milli-Q water purification system from Millipore (Brussels, Belgium). The enzymic preparation *Suc Helix pomatia* (SHP) containing 100 000 Fishman units/ml of β-glucuronidase and 1 000 000 Roy units/ml of arylsulphatase was obtained from IBF (Villeneuve, France). Adsorbex C_{18} solid phase extraction (SPE) columns were from Merck. Ammonia buffer (pH 9.5) was prepared by the addition of ammonia to a saturated ammonium chloride solution.

Equipment

The HPLC system was composed of a Model SP 8800 solvent-delivery pump (TSP, San Jose, CA, USA) and a Model SP 8880 autosampler (TSP). The detector was a Focus forward optical scanning detector (TSP) set at 220 nm. Chromatographic data (peak heights) were generated through the Labnet (TSP) communication system with a PS2/2386 computer (IBM). The column was a Nucleosil $5C_{18}$, 100 mm × 3 mm I.D., 5 μm (Chrompack, Antwerp, Belgium) with an appropriate pre-column. The loop volume was 20 μl. Chromatography was carried out at room temperature.

For the determination of alclofenac in urine, the mobile phase comprised 45% acetonitrile and 55% water–acetic acid (99:1, v/v). The flow-rate was 0.5 ml/min. Plasma alclofenac concentrations were determined using an essentially isocratic method with acetonitrile and water–acetic acid (99:1), followed by flushing of the column after the elution of the compounds of interest. The solvent gradient programme was as follows: initial acetonitrile was 48% for 8 min (flow-rate 0.5 ml/min); it was gradually increased to 90% from 8.1 to 9.9 min (flow-rate 1 ml/min). The equilibration time was 10 min (flow-rate 0.5 ml/min).

Analytical procedures

Plasma. The concentration of alclofenac in plasma was measured by a modification of the HPLC method for mofebutazone [7]. Plasma samples were prepared in duplicate by pipetting 2.0 ml into a 15-ml screw-capped tube, followed by the addition of 50 μl of internal standard solution (diclofenac in methanol, 50 μg/ml), 250 μl of 1 *M* HCl and 5 ml of diethyl ether. Extraction was performed on a rolling mixer for 15 min. After centrifugation (10 min), the organic layer was transferred to a clean screw-capped tube and evaporated under nitrogen at 36°C. The extraction step was repeated and, after evaporation of the combined organic extracts, the residue was redissolved in 200 μl of mobile phase and briefly vortex-mixed, and 20 μl were injected.

Urine without hydrolysis. The SPE cartridges, used with a vacuum manifold, were preconditioned by washing with methanol (5 ml) followed by phosphate buffer (0.1 *M*, pH 6.0, 5 ml). To 0.5 ml of urine, 2 ml of phosphate buffer (pH 6.0) were added, and the pH was adjusted to 6. The sample was centrifuged (5 min) and passed through the activated cartridge at a flow-rate of 2 ml/min. Thereafter the cartridges were rinsed with 2 ml of phosphate buffer (pH 6), and elution was performed with 1 ml of methanol–water (80:20). The injection volume was 20 μl.

Stability of alclofenac undere alkaline conditions. Urine (0.5 ml) spiked with alclofenac to a final concentration of 2.5 μg/ml or 10 μg/ml (n = 5) was made alkaline with, respectively, 50 μl of 1 *M* NaOH or 50 μl of ammonia buffer (pH 9.5). The solution was placed on a rolling mixer for 30 min, then the internal standard solution was added and the pH was brought to 6.0. Extraction was performed as described above.

Alclofenac was also determined in spiked urine at 4 μg/ml or 10 μg/ml (n = 5) without hydrolysis or after heating at 56°C for 3 h (pH 9.5), respectively.

Influence of time and temperature on alkaline hydrolysis. Urine samples were subjected to alkaline hydrolysis (pH 9.5) at 56°C for 0.5, 1, 2, 3 or 4 h, or 42°C overnight.

Enzymic hydrolysis of urine. Urine samples (0.5

ml) were buffered with 100 μl of 1 *M* sodium acetate buffer (pH 5.2) and 10 μl of SHP were added. Hydrolysis was performed at 56°C for 2.5 h. After cooling, the hydrolysate was acidified with hydrochloric acid and subsequently analysed as described above.

Quantitative determination. Standard curves were obtained by subjecting spiked plasma and urine to the appropriate extraction method in quadruplicate at each concentration. The prepared alclofenac concentrations were 10, 5, 2.5, 1, 0.5, 0.25 and 0.10 μg/ml for plasma and 20, 10, 8, 4, 2 and 1 μg/ml for urine.

The precision of the assay was measured at two different concentrations: 0.5 and 2.5 μg/ml in plasma, and 2 and 8 μg/ml in urine.

Plasma samples taken after the administration of aclofenac were analysed in duplicate, while urine samples were processed by two different procedures (unhydrolysed and alkaline hydrolysis at pH 9.5), and each sample was analysed in duplicate for each method.

Extraction recovery. The recovery of alclofenac was estimated from the changes in peak-height ratios when the drug was added to the plasma (urine) and the internal standard was added to the final extract, compared with the peak-height ratios when both the drug and the internal standard were dissolved in the mobile phase.

Statistical analysis

The significance of differences between mean values was determined by *t*-tests for paired data.

RESULTS AND DISCUSSION

For the extraction of alclofenac from biological fluids before GC analysis, liquid–liquid extraction methods were used [5,6]. However, when a conventional extraction procedure with diethyl ether was used in combination with HPLC and UV detection at 220 nm, serious analytical and sensitivity problems were encountered, particularly for horse urine. As there is a trend, in racing laboratories also, towards fully automated systems based on liquid–solid extraction via disposable columns, this rapid preparation technique was used here to assess alclofenac in equine urine.

Under the described conditions, the peaks corresponding to alclofenac and diclofenac were well resolved, sharp and symmetrical. No endogenous compound extracted at the same time interfered with these peaks. In order to avoid interferences from late-eluting substances, a solvent programme was used in the HPLC plasma assay. The total analysis time was somewhat reduced by using a slightly higher percentage of acetonitrile. The retention times for alclofenac and diclofenac were 2.9 and 5.7 min, respectively. Urine treated with alkali did not contain late-eluting components, so alclofenac was assayed by an isocratic HPLC method. The retention times for alclofenac and diclofenac were 3.4 and 7.3 min, respectively.

Linear calibration graphs were obtained in the ranges 0–10 μg/ml and 0–20 μg/ml for plasma and urine, respectively. The respective correlation coefficients were 0.9999 and 0.9960. At a signal-to-noise ratio of 3, the limit of quantitation of alclofenac in equine plasma and urine was 0.1 μg/ml and 1 μg/ml, respectively. Recovery measurements on spiked plasma and urine revealed a significant difference between liquid–liquid and solid-phase extraction. Generally, the separation of NSAIDs from a biological fluid using solvent extraction is relatively difficult and not precise, because of the hydrophilic character of these drugs [8,9]. However, the use of a double extraction step in the plasma assay as described here resulted in fairly good recoveries and reproducibility (Table I). Although the average recovery was nearly quantitative using SPE for urine, the precision was inferior to that for the plasma assay.

For both urine and plasma assays, no interferences were found with either veterinary NSAIDs, including flunixin, naproxen, phenylbutazone and oxyphenbutazone, or the human drugs piroxicam, flurbiprofen, ketoprofen, ibuprofen and indomethacin.

It has been established that, in order to improve the detection of NSAIDs from equine urine, alkaline hydrolysis at room temperature is required [10,11]. However, when urine containing alclofenac was treated with 1 *M* NaOH considerable decomposition of the drug occurred.

TABLE I

EXTRACTION RECOVERY AND PRECISION OF THE ALCLOFENAC ASSAY

Extraction recovery (n = 5)		Precision (n = 5)		
Concentration added (μg/ml)	Recovery (%)	Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)
Plasma		*Plasma*		
0.5	76.4 ± 3.8	0.5	0.47 ± 0.01	2.5
2.5	77.0 ± 2.0	2.5	2.48 ± 0.03	1.3
Urine		*Urine*		
4	97.2 ± 6.2	2	1.70 ± 0.11	6.6
10	94.5 ± 5.0	8	7.86 ± 0.46	5.8

Analogous analytical problems associated with the lability of the NSAID indomethacin on alkali treatment have been reported elsewhere [12,13]. However, the use of ammonia buffer (pH 9.5) for the hydrolysis of conjugates did not result in a significant decrease of the recovery of alclofenac from urine (Table II). As alkaline hydrolysis will be influenced by reaction time and temperature, alclofenac administration urine samples were hydrolysed (n = 5) at pH 9.5 at 42°C overnight, and for different time periods at 56°C. Generally higher alclofenac amounts were found after hydrolysis at 56°C than after hydrolysis at 42°C. The concentration of alclofenac reached a maximum after 3 h at 56°C. The stability of alclofenac at pH 9.5 during 3 h at 56°C was therefore also evaluated, using two different concentrations (4 μg/ml and 10 μg/ml). Five-fold determinations did not reveal significant differences in alclofenac concentrations without hydrolysis and after alkaline treatment (pH 9.5) for 3 h at 56°C (Table II).

Several NSAIDs, including indomethacin [12] and fenclofenac [14], undergo phase II metabolism and are excreted as glucuronides in the horse. However, when an alclofenac administration urine was hydrolysed with SHP (five determinations), the concentration of alclofenac was not significantly different from the value found in unhydrolysed urine. Therefore, glucuronidation of alclofenac does not seem to occur in the horse.

TABLE II

STABILITY OF ALCLOFENAC IN URINE ON ALKALI TREATMENT

Concentration expected (μg/ml)	Concentration found (mean ± S.D., n = 5) (μg/ml)		
	Without treatment	pH 9.5, 25°C, 30 min	pH 9.5, 56°C, 3 h
2.5	2.58 ± 0.17	2.49 ± 0.19	–
10	11.10 ± 0.07	11.15 ± 1.47	–
4	3.91 ± 0.12	–	4.09 ± 0.21
10	10.30 ± 0.16	–	10.77 ± 0.30

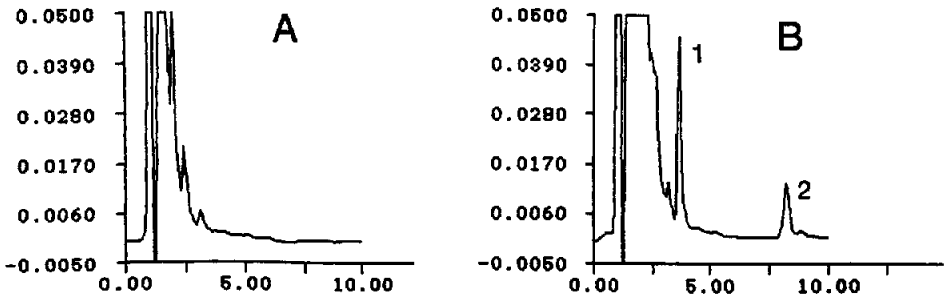

Fig. 1. Chromatograms of alclofenac in equine urine (unhydrolysed) before (A) and 9 h after (B) an oral administration of 3 g of alclofenac. Peaks: 1 = alclofenac; 2 = diclofenac.

Another reason for the failure to detect alclofenac glucuronides in horse urine could be the resistance of the acylglucuronide towards the glucuronidase enzyme.

Chromatograms of urine samples collected before and 9 h after the administration of alclofenac to the horse are shown in Fig. 1. The highest concentration of alclofenac (24.5 μg/ml free and 55.6 μg/ml total alclofenac, assuming a 100% hydrolysis efficiency) was obtained in the 24-h sample (Fig. 2). The last sample in which alclofenac could be detected was collected 48 h after administration (1 μg/ml). The total amount of alclofenac excreted in the urine after 12 h is very low, and corresponds to 1.7% of the dose. Fig. 3 illustrates a plasma concentration *versus* time profile for alclofenac in the horse following a 3-g oral dose. Plasma levels reached a maximum of 42.1

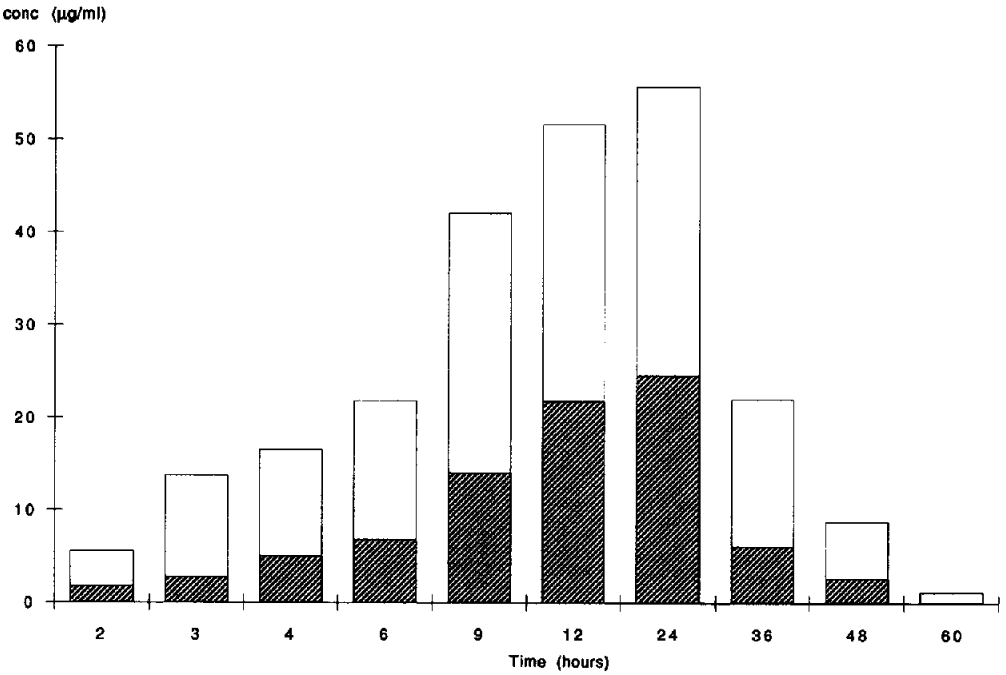

Fig. 2. Time variation of the urine concentration of alclofenac in the horse. Shaded areas indicate free and clear areas conjugated alclofenac.

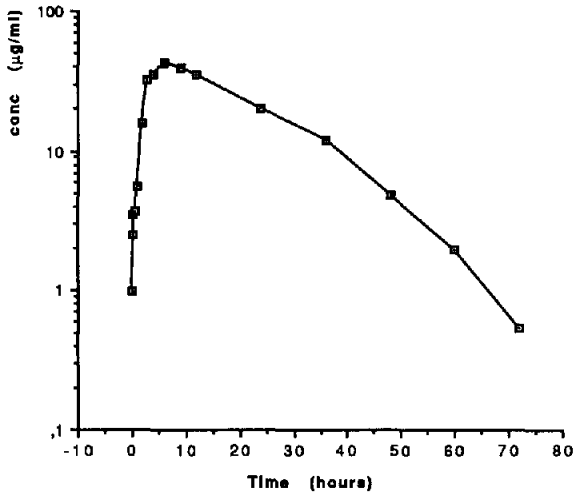

Fig. 3. Plasma concentration–time profile for alclofenac in the horse.

µg/ml after 6 h. The drug was still detectable in plasma 72 h after administration, thus ensuring that the detection limit of the method does not represent a drawback in pharmacokinetic studies on alclofenac in the horse.

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