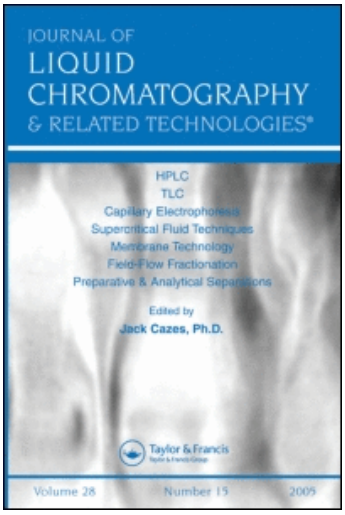

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LIQUID CHROMATOGRAPHIC DETERMINATION OF MECLOFENAMIC ACID IN EQUINE PLASMA

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ABSTRACT

Meclofenamic acid is extracted to dichloromethane together with the internal standard diclofenac sodium. After evaporation of the organic solvent the residue is dissolved in the chromatographic eluent and analyzed by liquid chromatography. The acids are separated on a column packed with Spherisorb ODS with methanol - phosphate buffer as the eluent and detected at 280 nm. A possible metabolite of meclofenamic acid was also detected in the chromatograms. The detection limit for meclofenamic acid in plasma was 0.361 µmol/L (0.107 µg/ml) for 1.5 ml sample size. The conditions of quantitative extraction of meclofenamic acid and diclofenac to dichloromethane are given.

INTRODUCTION

Meclofenamic acid is a potent non-steroidal anti--inflammatory agent belonging to the fenamate group [1]. The metabolism of the drug in man has been studied with tritium-labeled drug in combination with fluoro-

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metry, gas chromatography, and thin-layer chromatography [2,3]. The drug has been approved in some countries for the treatment of horses and analysis of meclofenamic acid in equine plasma has been performed with fluorometric assay [4,5].

Meclofenamic acid was included in a study of the effects of drugs on performance in the horse [6]. Quantitative analysis of the acid in equine plasma was required to correlate possible effects of the drug with the plasma concentrations. Our method involves liquid chromatographic isolation of meclofenamic acid after an initial extraction and concentration of the substance together with the internal standard, diclofenac sodium. Fluorometric detection of the chromatographic eluent was tested but photometric detection at 280 nm gave better sensitivity.

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Constametric I pump (Milton Roy Company, Riviera Beach, Fla., U.S.A.), a sample injection valve with a 20 µl loop (Rheodyne, Berkeley, CA, U.S.A.), and an ultraviolet detector with variable wavelength, LDC Spectro Monitor III.

The chromatographic columns were of 316 stainless steel, 100 mm x 3.0 mm, equipped with zero volume Swagelok unions and column end fittings. Two-µm stainless steel frits from Altex were used. The columns were packed with LiChrosorb RP-18, 5 µm (Merck, Darmstadt, G.F.R.) or Spherisorb ODS, 10 µm (Phase Separations Ltd., Queensferry, United Kingdom).

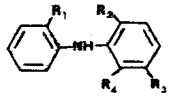
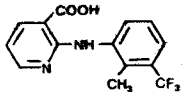
A Shimadzu spectrophotometer, UV-210 A, was used for the photometric measurements in the batch extraction experiments and the determination of pH was performed with a Metrohm 620 pH-meter.

Chemicals and Reagents

Methanol and dichloromethane were of analytical-reagent grade quality and obtained from Merck. Meclofenamic acid (mol. wt. = 296.2) and mefenamic acid were kindly supplied by Parke, Davis & Co. (Pontypool, United Kingdom), diclofenac sodium by Ciba-Geigy AG (Basle, Switzerland) and flunixin meglumine by Schering Corporation (Kenilworth, NJ, U.S.A.). Their structures are given in Table 1. Tetrabutylammonium hydrogensulfate (TBA) was obtained from Hässle AB (Mölndal, Sweden). The phosphate buffers used in the batch extraction and chromatographic experiments were prepared from sodium dihydrogen phosphate and disodium hydrogen

TABLE 1.

Structure of the Acids

Formula	Name	R_1	R_2	R_3	R_4
	Mefenamic acid	COOH	H	CH_3	CH_3
	Meclofenamic acid	COOH	Cl	CH_3	Cl
	Diclofenac sodium	CH_2COONa	Cl	H	Cl
	Flunixin				

phosphate. Citrate buffer, prepared from citric acid and sodium hydroxide, was used in the preparation of plasma samples. All buffers had an ionic strength of 0.1 and were prepared with ion-exchanged and distilled water.

Determination of Distribution Ratio

Meclofenamic acid was dissolved in 0.1 M sodium hydroxide as the acid has a limited solubility at lower pH. The solubility of meclofenamic acid at pH 7 is ca. 91 µmol/L (27 µg/ml) [3]. Diclofenac sodium was dissolved in 0.05 M disodium hydrogen phosphate. Buffer solutions with known concentrations of the acids were prepared. The excess of sodium hydroxide, used to dissolve the meclofenamic acid, was neutral-

ized with phosphoric acid. The buffer solutions containing the drugs were mechanically shaken with equal volumes of dichloromethane at 25°C in a water bath for 30 min. After phase separation, the absorbance of the aqueous phase was measured photometrically and the concentration of the acid was calculated using 278 nm, ε = 6570 for meclofenamic acid and 276 nm, ε = 10600 for diclofenac. The concentration of the acid in the organic phase was then calculated from the initial concentration. The pH of the aqueous phase was also measured after phase separation.

Chromatographic Technique

The eluents were prepared by mixing methanol and phosphate buffer pH 6.1. The solution was allowed to stand overnight and was treated in an ultrasonic bath for some minutes before use. TBA, when used, was dissolved in the phosphate buffer.

The columns were packed at 39 MPa with methanol as the driving liquid. LiChrosorb RP-18 was suspended in dichloromethane (0.1 g/ml) and the column was packed downwards [cf. 7]. Spherisorb ODS was suspended in methanol and the column was packed by the upwards slurry packing technique [8].

The void volume of the column, V_m, was determined by injecting sulfathiazole, which was not retained in

the system. This value of V_m was used to calculate the capacity ratio, k'.

Sampling

Blood samples were collected in vacuum tubes, Venoject®, containing sodium heparin. Plasma was prepared and stored frozen at -20°C until analyzed.

Pooled equine plasma from four horses that had not received any drug was used as blank plasma.

Sample Preparation

Meclofenamic acid was dissolved in 0.025 M disodium phosphate with the addition of 0.1 M sodium hydroxide. The solution was diluted with 0.025 M disodium phosphate to obtain aqueous samples containing 1.6-81 µmol/L (0.48-24 µg/ml) of meclofenamic acid. Plasma samples were spiked with 0.68-16 µmol/L (0.20-4.8 µg/ml) (50 µl/ml plasma).

Analytical Method

Plasma (1.50 ml) was mixed with 2.00 ml of citrate buffer pH 4.6, containing 0.53 µmol/L (0.15 µg/ml) of diclofenac sodium as internal standard, 5.00 ml of dichloromethane was added and the tube was rotated for 30 minutes. After centrifugation, ca. 3 ml of the organic phase was transferred to another tube and evaporated. The residue was dissolved in

200 µl of the chromatographic eluent and 20 µl was injected onto the liquid chromatographic column.

The chromatographic isolation was performed on a column containing Spherisorb ODS (10 µm) with 40 % methanol in phosphate buffer pH 6.1 as the eluent. The analysis time was 14 minutes at a flow-rate of 0.8 ml/min. UV-detection was performed at 280 nm.

All calculations of the drug concentrations were based on peak height measurements.

RESULTS AND DISCUSSION

Choice of Liquid Chromatographic System

Flunixin, diclofenac sodium, and mefenamic acid, all belonging to the group of anti-inflammatory drugs, were included in the chromatographic study as conceivable standards together with meclofenamic acid. At first LiChrosorb RP-18 was used as support with mixtures of methanol - buffer pH 6.1 as eluents. Meclofenamic acid and mefenamic acid did not separate from each other on this support. Flunixin was eluted too close to the front and consequently diclofenac was chosen as the internal standard. Figure 1A shows how the retention is influenced by the concentration of methanol in the eluent. Extracts from equine plasma

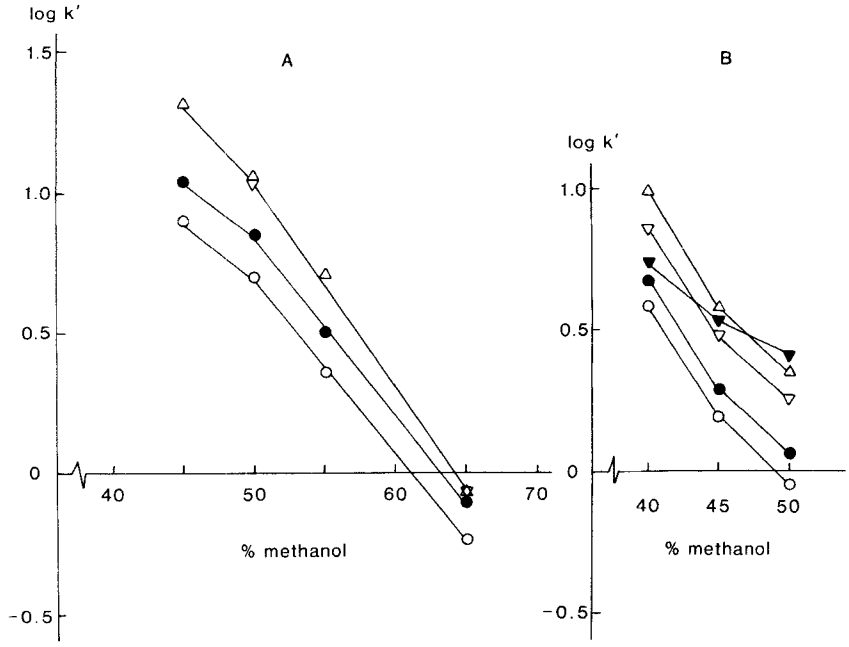


FIGURE 1. Influence of the Methanol Content in the Eluent on the Capacity Ratios.
Support: A = LiChrosorb RP-18; B = Spherisorb ODS.
Eluent: Methanol in phosphate buffer pH 6.1.
Δ = meclofenamic acid; ∇ = mefenamic acid;
● = diclofenac; o = flunixin; ▼ = interfering peak.

showed, however, a peak that interfered with diclofenac in the chromatogram. A separation could not be achieved by changing the pH or the methanol concentration of the eluent.

However, the separation was possible after the addition of TBA to an eluent containing 66 % methanol. In this system the interfering peak was eluted in the front and the acids emerged later, migrating as ion-

-pair with TBA. One disadvantage of the use of TBA was the poor column stability and another problem was the low solubility of meclofenamic acid in the eluent containing TBA.

Small differences in the selectivity between the acids were obtained by exchanging LiChrosorb RP-18 for Spherisorb ODS as the support (Figure 1B). About 10 % less methanol was needed in the eluent to get the same retention of meclofenamic acid on this support compared with LiChrosorb RP-18. Figure 1B shows the influence of methanol on the retention. The retention of the interfering peak was much less influenced than was the retention of the drugs by the concentration of methanol. A chromatogram of a standard solution of the acids is given in Figure 2.

During the investigation, it was observed that plasma extracts from only one horse contained the interfering peak in the chromatogram and that this peak might be related to some plastic caps in the storage of plasma samples. The caps were exchanged for further work. Spherisorb ODS was used for the plasma studies with 40 % methanol in phosphate buffer pH 6.1 as the eluent.

Extraction

Both meclofenamic acid and diclofenac are protolytes in aqueous solutions and can be transferred in

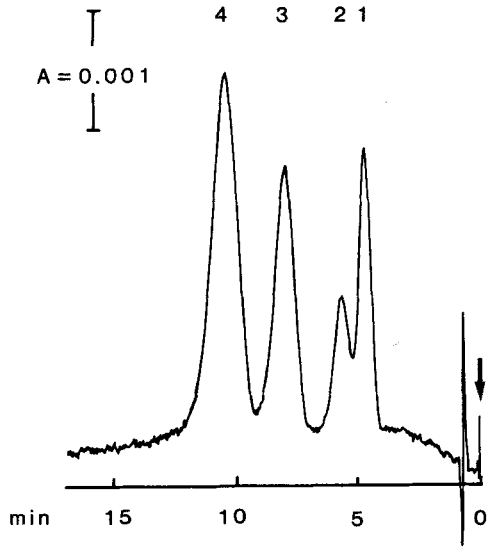


FIGURE 2. Chromatogram of Some Acidic Drugs.
Support: Spherisorb ODS, 10 µm.
Eluent: 40 % methanol in phosphate buffer pH 6.1 (0.8 ml/min, 3500 kPa).
Peaks: 1 = flunixin (25 ng); 2 = diclofenac (18 ng); 3 = mefenamic acid (50 ng); 4 = meclofenamic acid (152 ng).

uncharged forms into organic solvents. The degree of extraction will depend both on the nature of the organic solvent and the pH of the aqueous solution [9]. The extraction of an acid, HX, can be expressed by the distribution ratio, D_{HX}

$$D_{HX} = \frac{C_{HXorg}}{C_{HXaq}} = \frac{[HX]_{org}}{[HX]+[X^-]} = \frac{K_D \cdot a_{H^+}}{K'_{HX} + a_{H^+}} \qquad (1)$$

where

$$K_D = \frac{[HX]_{org}}{[HX]} = \text{distribution constant of HX} \quad (2)$$

$$K'_{HX} = \frac{a_{H^+} \cdot [X^-]}{[HX]} = \text{apparent dissociation constant of HX} \quad (3)$$

If the constants are known the distribution ratio can be estimated at any pH and the degree of extraction, P %, to the organic solvent calculated by use of

The Formula $$P\% = 100 \cdot \left(1 + \frac{V_{aq}}{V_{org} \cdot D_{HX}}\right)^{-1} \quad (4)$$

where V_{org} and V_{aq} are the volumes of the organic solvent and the aqueous solution, respectively.

The distribution ratios of meclofenamic acid and diclofenac were determined between dichloromethane as organic solvent and phosphate buffers of pH 7.5-9.5. Inversion of eq. (1) gives

$$\frac{1}{D_{HX}} = \frac{1}{K_D} + \frac{K'_{HX}}{K_D \cdot a_{H^+}} \quad (5)$$

Plots according to eq. (5) gave linear relationships and K'_{HX}/K_D was calculated from the slope. The intercept was too small to enable a calculation of K_D. The results are given in Table 2 together with the experimental conditions.

In our analytical procedure the extraction is performed at a pH of about 5.2, which for both meclofena-

TABLE 2.
Partition Coefficients
Aqueous solution: Phosphate buffer (μ = 0.1).
Organic solvent: Dichloromethane.

Acid	$C_{HXaq} \cdot 10^4$	$C_{HXorg} \cdot 10^4$	pH	$\log (K'_{HX}/K_D)$	pK'_{HX}
Meclofenamic acid	0.25-0.97	0.53-1.3	8.4-9.4	-9.11	4[a]
Diclofenac	1.4 -3.7	0.92-3.2	7.5-8.5	-7.84	4.0[b]

C_{HX} denotes the total concentration of the acid in mol/L. [a] Ref. [1], [b] Ref. [10].

mic acid and diclofenac gives a theoretical recovery of 99.8 % (eq. 4). The acids are concentrated about 4.5 times by the extraction procedure.

Buffer solutions of meclofenamic acid, 1.6-81 μmol/L (0.48-24 μg/ml), analyzed according to the method, showed a deviation from linearity at higher concentrations of meclofenamic acid. The obtained standard curve was compared with standards of meclofenamic acid - diclofenac dissolved in the eluent and not taken through the extraction step, Figure 3. The non-linearity might be due to the low solubility of meclofenamic acid in acidic aqueous solutions. The standard curve was considered linear up to 17 μmol/L (5 μg/ml). The same degree of linearity has previously been reported for the fluorometric analysis of meclofenamic acid [5].

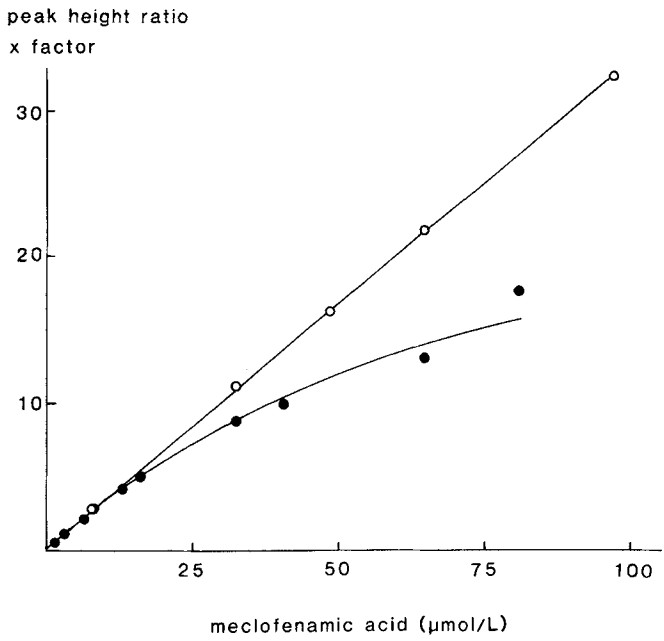


FIGURE 3. Standard Curve of Meclofenamic Acid. ● = meclofenamic acid dissolved in 0.025 M phosphate buffer pH 9.2 and extracted according to the Analytical Method; o = meclofenamic acid directly injected onto the column. Internal standard: Diclofenac. Chromatographic conditions as in Figure 2.

Determination of Meclofenamic Acid in Equine Plasma

The standard curve in Figure 4 is prepared from spiked equine plasma in the range of 0.68-16 µmol/L (0.20-4.8 µg/ml). An absolute recovery of 95 % was obtained. The recovery was not increased by doubling the extraction time.

Figure 5 shows chromatograms of two plasma samples taken before and 45 min after an oral dose of meclofe-

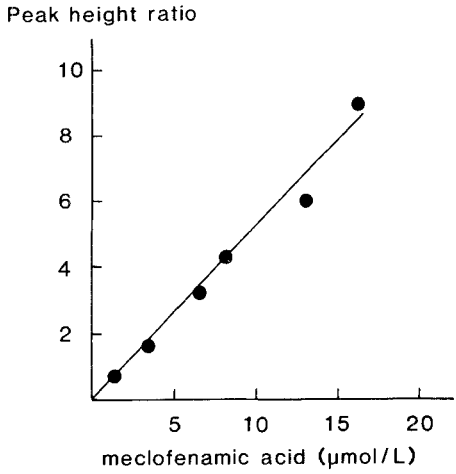


FIGURE 4. Standard Curve for the Determination of Meclofenamic Acid in Equine Plasma. Analyses performed according to the Analytical Method with diclofenac (0.53 μmol/L) as the internal standard. Chromatographic conditions as in Figure 2.

namic acid. From the chromatogram the presence of a metabolite of meclofenamic acid is strongly evident. It can be isolated and quantified with the present liquid chromatographic method after its structure elucidation. This is an improvement compared with the fluorometric assay [4,5] where probably the metabolite is codetermined with the parent drug after extraction from acidified solutions with carbon tetrachloride [3].

The simple work-up procedure, with a single extraction of the plasma sample to dichloromethane followed by evaporation of the organic solvent and redissolving in a small volume of eluent, will result in rather

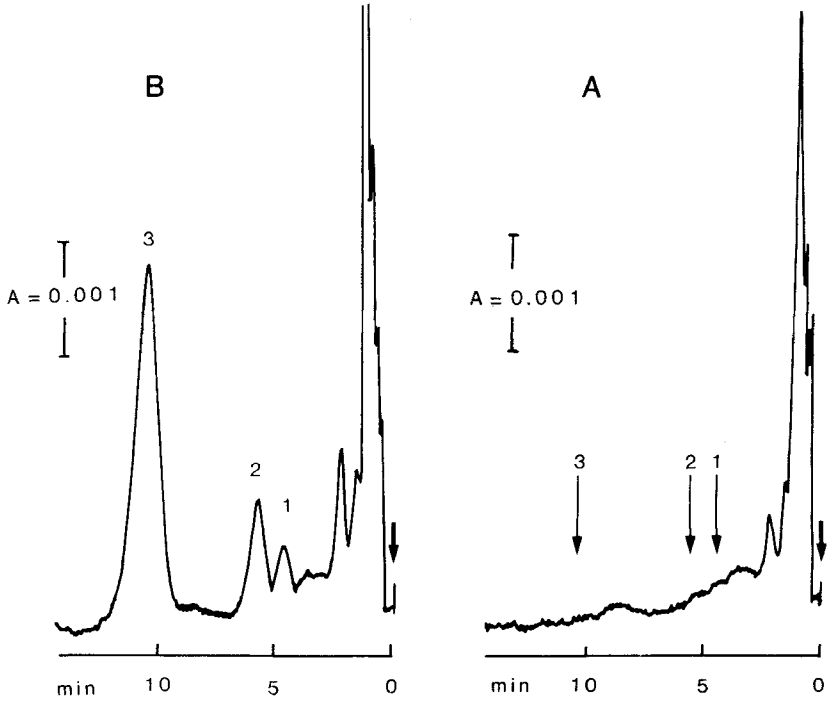


FIGURE 5. Chromatograms from Equine Plasma. Conditions as in Figure 2. Peaks: 1 = possible metabolite; 2 = diclofenac (internal standard); 3 = meclofenamic acid; A = blank plasma; B = plasma sample 45 minutes after oral administration of meclofenamic acid, 2.2 mg/kg body weight. Calculated concentration of meclofenamic acid, 7.4 µmol/L (2.2 µg/ml).

dirty samples to be injected onto the liquid chromatographic column. This causes no problem provided the column is changed after the injection of 200 plasma samples.

The recovery and precision of the analytical method, summarized in Table 3, were calculated from repeated analyses of spiked equine plasma at the con-

TABLE 3.
Recovery and Precision of the Method.
Data obtained with internal standard, diclofenac sodium 0.47 µmol/L (0.15 µg/ml). The relative standard deviation, s_r %, is calculated from repeated analyses of spiked plasma samples.

Drug added µmol/L	µg/ml	Drug found %	s_r %	n
6.50	1.92	102.3	4.95	9
1.30	0.384	96.3	12.6	5

centration levels of 6.50 µmol/L and 1.30 µmol/L. The relative standard deviation, 4.95 % and 12.6 %, respectively, can probably be reduced by the injection of a larger volume onto the column to increase the signal to noise ratio. With the present injection of a 20 µl sample, the detection limit in equine plasma, defined as the concentration which gives a signal twice the base-line noise, was 0.361 µmol/L (0.107 µg/ml) for meclofenamic acid.

Figure 6 shows the plasma concentration of meclofenamic acid obtained from one horse after oral administration of meclofenamic acid, 2.2 mg/kg body weight. With our method, meclofenamic acid can be determined in equine plasma up to 12 hours after a single oral dose. The simple sample treatment, high column stability,

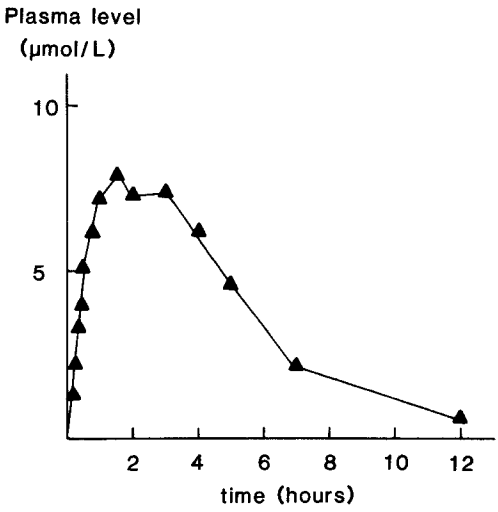


FIGURE 6. Plasma Levels of Meclofenamic Acid after Oral Administration. Administered dose: 2.2 mg/kg body weight.

rapid liquid chromatographic separation of meclofenamic acid, and the possibility of metabolite determination constitute a powerful tool for pharmacokinetic measurements (work in progress).

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