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GAS CHROMATOGRAPHIC ANALYSIS OF FLUNIXIN IN EQUINE URINE AFTER EXTRACTIVE METHYLATION

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SUMMARY

A quantitative method for the analysis of flunixin, 2-(2-methyl-3-trifluoromethylanilino) nicotinic acid, in equine urine by gas chromatography with nitrogen–phosphorus detection has been developed. Flunixin and the internal standard, mefenamic acid, N-(2,3-xylyl) anthranilic acid, were analysed after extractive methylation of the carboxylic acid group using methyl iodide. The extraction and alkylation conditions of flunixin and mefenamic acid have been studied. The detection limit of the method was 0.25 μmol/l flunixin in urine (74 ng/ml). Flunixin was found to be conjugated to 96.5% in equine urine, and the conjugate was spontaneously hydrolysed to free flunixin. This approach can also be used to confirm the presence of flunixin or mefenamic acid in horse urine in the doping control of racehorses.

INTRODUCTION

We began working with flunixin some years ago because the drug was found as a positive dope in our doping control analysis. The drug was confirmed with gas chromatography–mass spectrometry (GC–MS), which involved derivatization of the polar acidic group to obtain good chromatographic behaviour. We also analysed flunixin in horse urine after treatment of horses with the drug [1], but obtained low reproducibility.

The analysis of flunixin in horse plasma by a simple liquid chromatographic method has been described [2,3], but to analyse the substance in urine a more extensive clean-up of the sample from endogenous compounds was necessary. Flunixin has also been analysed in equine urine by gas chromatography (GC) with electron-capture detection after silylation with N,O-bis(trimethyl-

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silyl)acetamide [4], but a non-linear standard curve was obtained. A GC screening procedure for non-steroidal anti-inflammatory drugs in horse urine, including flunixin, using on-column methylation with trimethylanilinium hydroxide as the methylating reagent, has also been reported [5].

No basic data for the extraction of flunixin were found in the literature, and we decided to investigate the extraction and alkylation conditions for flunixin as a model substance of the large group of anti-inflammatory drugs with similar structures. Mefenamic acid, which is structurally related to flunixin, was used as the internal standard.

This paper describes the analytical method that has been developed to determine free and total flunixin in authentic urine samples.

EXPERIMENTAL

Apparatus

GC with nitrogen–phosphorus detection (NPD) was carried out with a Perkin-Elmer 8310 instrument equipped with an AS 8300 autosampler. The separations were performed on a fused-silica capillary column (25 m×0.32 mm I.D.) coated with a 0.25-μm film of 5% methylphenyl silicone (Quadrex, New Haven, CT, U.S.A.). Helium was used as carrier gas at a flow-rate of 3.2 ml/min. Splitless injections were made at a column temperature of 80°C. The split valve was closed 30 s prior to sample injection and was opened 90 s after injection. After 2 min at 80°C the column temperature was increased to 200°C at 30°C/min and held there for 10 min. The temperature of the injector was 280°C and the detector temperature was 330°C.

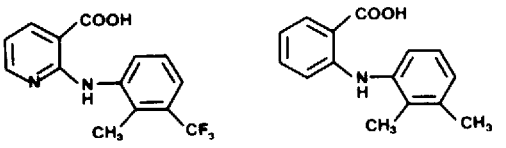
GC with flame ionization detection (FID) was carried out with a Shimadzu GC-7A instrument equipped with a glass column (1.5 m×2.0 mm I.D.) packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh. Nitrogen was used as carrier gas at a flow-rate of 55 ml/min. The temperature of the column was 200°C when the alkylation rate of flunixin was studied. The alkylation rate of mefenamic acid was studied with a column temperature maintained at 200°C for 8 min and then programmed at a rate of 8°C/min to 220°C and held there for 4 min. The temperature of the injector was 280°C. The initial studies of the recovery of flunixin from urine were also performed with this instrument.

In the batch extraction experiments the photometric measurements were performed with a Shimadzu spectrophotometer, UV-210 A, and the pH measurements with a Metrohm 620 pH meter. A Metrohm 605 pH meter was used to measure the pH in the determination of acid dissociation constant.

A Buchler vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.) was used in the preparation of samples.

Chemicals

Flunixin meglumine was kindly supplied by Schering (Kenilworth, NJ, U.S.A.) and mefenamic acid by Parke, Davis & Co. (Pontypool, U.K.). The structures of the compounds are given in Fig. 1. Hexacosan, puriss, and tetracosane, purum, were obtained from Fluka (Buchs, Switzerland).



Flunixin **Mefenamic Acid**

Fig. 1. Structures of flunixin and mefenamic acid.

Tetrabutylammonium (TBA) hydroxide was prepared from a solution of TBA iodide (E. Merck, Darmstadt, F.R.G.). The iodide was exchanged for hydroxide by shaking with silver oxide [6]. The solution was tested for the absence of iodide with silver nitrate, then it was extracted ten times with equal volumes of dichloromethane. The concentration of TBA in the aqueous solution was determined by the picric acid method [7].

Carbonate, phosphate and citric buffers were prepared with an ionic strength of 0.1.

Determination of acid dissociation constant

The acid dissociation constant was determined photometrically [8]. Four solutions of pH 5.73–6.29, phosphate buffer pH 9.28 and citric buffer pH 3.8 were prepared with a concentration of flunixin of $4.78 \cdot 10^{-4}$ *M*. The absorbance of the solutions was measured at 282 nm.

Determination of partition coefficients

The distribution studies were performed in centrifuge tubes using equal volumes of organic and aqueous phases. The tubes were shaken for 30 min in a waterbath thermostatted at 25.0 ± 0.1 °C, then centrifuged. The phases were separated, and the concentration of flunixin in the aqueous phase was determined by photometric measurements at 282 nm using the molar absorbance, $\epsilon = 1.369 \cdot 10^4$. The concentration of flunixin in the organic phase was then calculated from the initial concentration ($3.5–5.4 \cdot 10^{-4}$ *M*) [9].

In the ion-pair extraction study, flunixin-TBA was first formed in the organic phase [10]. This was done by equilibrating an aqueous solution of flunixin meglumine and TBA hydroxide in carbonate buffer (pH 10.2) with dichloromethane.

The distribution of mefenamic acid as an acid and as an ion pair with TBA was determined in a similar way. The photometric measurements were performed at 284 nm using $\epsilon = 1.027 \cdot 10^4$.

Evaluation of alkylation conditions

The conditions for extractive alkylation were chosen according to ref. 11, but TBA was used instead of tetrahexylammonium (THeA). Fluxinin meglumine ($2.0 \cdot 10^{-3}$ *M*) was first shaken at pH 3 with an equal volume of dichloromethane to obtain flunixin as an acid in the dichloromethane phase. The dichloromethane contained tetracosan ($3.0 \cdot 10^{-3}$ *M*) as a non-reacting internal standard. To 5.0 ml of the dichloromethane solution was then added 2.0 ml of TBA hydroxide

(0.0025 *M*) in carbonate buffer (pH 10.2) and 157 µl of methyl iodide, i.e. 0.5 *M* in the dichloromethane solution. The mixture was shaken at 50 °C; 20-µl aliquots of the organic phase were withdrawn at 5, 10, 15, 20, 30, 45, 60, 120 and 180 min and mixed with 1.0 ml of dichloromethane and 1.0 ml of phosphate buffer (pH 3) in order to quench the reaction. Each resulting organic phase was injected (2 µl) into the chromatograph (GC–FID).

The experiment was repeated with mefenamic acid ($4.1 \cdot 10^{-3}$ *M*). Hexacosan ($3.0 \cdot 10^{-3}$ *M*) in dichloromethane was used as the non-reacting internal standard.

Synthesis of methyl flunixin

Flunixin meglumine (250 mg) was dissolved in 100 ml of carbonate buffer (pH 10.3), and 2 ml of TBA (0.1 *M*) were added. The solution was divided into twelve portions and each was shaken with 20 ml of methyl iodide (0.5 *M*) in dichloromethane for 30 min at 50 °C. The tubes were centrifuged and the phases were separated. The organic phases were first shaken with phosphate buffer (pH 3) and sodium hydroxide (0.2 *M*) and were then pooled and evaporated. The residue, methyl flunixin, was recrystallized in diethyl ether and dissolved in hexane. The hexane solution was filtered through a 0.2-µm filter and evaporated. The purity of the remaining crystals was checked by GC.

Analysis of free flunixin

To 1.0 ml of urine were added 9.0 ml of citric buffer (pH 3.3), 100 µl of the internal standard, mefenamic acid (50 µg/ml) dissolved in 0.1 *M* sodium hydroxide, and 10.0 ml of dichloromethane–methanol (98:2). The tube was agitated for 15 min at room temperature and centrifuged for 10 min at 1000 *g*. The organic phase was transferred to a new tube containing 2.0 ml of carbonate buffer (pH 10.2). The tube was agitated for 15 min at room temperature and centrifuged for 10 min at 1000 *g*, and the organic phase was discarded. To the aqueous phase were added 100 µl of TBA hydroxide (0.05 *M*) in sodium hydroxide (0.1 *M*) and 5.0 ml of methyl iodide (0.5 *M*) in dichloromethane. The tube was shaken for 30 min in a water-bath at 50 °C, cooled to room temperature and centrifuged for 10 min at 1000 *g*. The organic phase was transferred to another tube and evaporated in vacuum at 50 °C. The residue was dissolved in 200 µl toluene and 1 µl was subjected to GC–NPD system.

Analysis of free and conjugated flunixin

To 3.2 ml of urine were added 1.6 ml of sodium hydroxide (0.5 *M*) to hydrolyse conjugated flunixin. The solution was shaken for 20 min at room temperature and then centrifuged for 10 min at 1000 *g* [4]. To 0.2 ml of the mixture were added 1.8 ml of citric buffer containing phosphoric acid to give pH 3.5, and 100 µl of mefenamic acid (50 µg/ml) dissolved in 0.1 *M* sodium hydroxide. The sample was extracted with 5.0 ml of dichloromethane–methanol (98:2), and the organic phase treated as above.

Drug administration

A therapeutic dose of flunixin meglumine, corresponding to 1.1 mg/kg flunixin, was given intramuscularly to one standard-bred horse, a gelding. Urine samples

were spontaneously voided and the total amount was collected until 48 h after the administration of the drug. One urine sample was also collected before treatment. The urine samples were kept at −20°C, and all were analysed five days after drug administration.

Quantitative analysis

The concentrations of flunixin in the samples were determined by comparison of the detector response for known concentrations of flunixin added to the urine relative to the internal standard. In GC–FID peak-height measurements were performed whereas in GC–NPD peak areas were measured. The linearity of the methods was tested by analysing known concentrations of flunixin added to buffer solutions (pH 7.4) and to horse urine.

When the urine samples from the horse treated with flunixin meglumine were analysed, the median value of four standards analysed in the same set was calculated and correlated to the standard curve. The standards were prepared in urine to a concentration of 10 and 50 μg/ml for the analysis of free and total concentration of flunixin, respectively. The standards were stored at −20°C.

Stability of conjugated flunixin in urine

Portions of the urine samples (those collected before administration of flunixin meglumine and between 8 and 18.5 h afterwards) were kept for two weeks at room temperature (20–23°C). Analysis of these samples for free and total concentration of flunixin was made in duplicate on days 1, 7 and 14. On days 7 and 14 the urine and standard (10 μg/ml) were diluted ten times with phosphate buffer (pH 7.4) before being analysed for free flunixin.

RESULTS AND DISCUSSION

Extraction

The apparent acid dissociation constant, K'_a, of flunixin was determined photometrically. The mean values of four determinations at different pH gave $pK'_a = 5.82 \pm 0.02$.

A study was made of the extraction of flunixin ($3.5–5.4 \cdot 10^{-4}$ M) as an acid using four organic solvents, dichloromethane, dichloromethane–methanol (98:2), dichloromethane–2-propanol (85:15) and diethyl ether. A calculation of the degree of extraction (P_{org}) according to

$$P_{org} = 100 \cdot \frac{1}{1 + V_{aq}/(D_A \times V_{org})}$$

using the obtained distribution ratio, D_A, and a phase volume ratio (V_{aq}/V_{org}) = 1, gave 94% extraction of flunixin from a buffer solution of pH 3.3–3.5 to dichloromethane [9]. (For an acid $D_A = K_D(1 + K'_a \times a_{H^+}{}^{-1})^{-1}$ where K_D is the distribution constant of the uncharged acid between organic and aqueous phase. For an ion pair $D_A = K_{ex} \times [TBA]$ and the equilibrium is given by the extraction constant, K_{ex}, and the concentration of the counter ion.) Both dichloromethane–2-propanol (85:15) and diethyl ether gave higher extraction, although the amount of coextracted endogenous substances increased as well. Mefenamic acid, the internal standard, was extracted to 99% from a buffer solution

of pH 3.3–3.5 to dichloromethane. Dichloromethane–methanol (98:2) was finally selected. This solvent gives about the same extraction degree as dichloromethane but the presence of a small amount of alcohol may help to prevent adsorption losses. On back-extraction of the acids into a buffer solution of pH 10.2 the flunixin remaining in the organic phase was less than 1%.

The extraction constants of flunixin and mefenamic acid as ion pairs with TBA were determined with dichloromethane as the organic phase and carbonate buffer, pH 10.2 and 10.8, respectively, as the aqueous phase. At these pH values the partition of the acids in acidic form is negligible. The distribution, ion-pair and dissociation constants are summarized in Table I. The constants were evaluated graphically [9].

The difference in the distribution constant for mefenamic acid and flunixin was 3 log units and was larger than expected from structural differences between the acids [9,12]. Flunixin has weaker intramolecular hydrogen bonds than mefenamic acid, which makes flunixin more hydrophilic. This was also reflected by the dissociation constants: flunixin was found to be a weaker acid, $pK'_a = 5.82$, than mefenamic acid, $pK'_a = 4.2$ [13]. The distribution properties of mefenamic acid are too marked for it to be the ideal internal standard for the analysis of flunixin from the extraction point of view. The internal standard was chosen because of the similarity in alkylation rates for flunixin and mefenamic acid, and because methyl mefenamate had a suitable GC retention time.

The difference between the ion-pair extraction constants was smaller, 1 log unit (Table I). The found dissociation constant for the ion pair, TBA-mefenamic acid ($\log K_{diss} = -2.4$), was surprisingly large. Previously, 20–600 times smaller dissociation constants have been found in dichloromethane with TBA as the counter ion ($\log K_{diss} = -3.5$ to -5.2) [9].

Dilution of the urine with buffer or ethanol before extraction of flunixin increases the relative recovery in the analytical procedure. This aspect was studied with flunixin added to urine to a concentration of 50 μg/ml. Aliquots of the urine

TABLE I

DISTRIBUTION, ION-PAIR EXTRACTION AND DISSOCIATION CONSTANTS

Acid	Aqueous phase	Organic phase	$\log K_D$*	$\log K_{ex}$	$\log K_{diss}$
Flunixin	Buffer, pH 6.88–7.70	Dichloromethane	1.22	–	–
Mefenamic acid	Buffer, pH 8.58–9.43	Dichloromethane	4.5	–	–
Flunixin	Buffer, pH 6.61–7.57	Dichloromethane–methanol (98:2)	1.24	–	–
Mefenamic acid	Buffer, pH 8.83–9.48	Dichloromethane–methanol (98:2)	4.6	–	–
Flunixin	Buffer, pH 7.64–8.54	Dichloromethane–2-propanol (85:15)	2.04	–	–
Flunixin	Buffer, pH 7.70–8.55	Diethyl ether	2.66	–	–
Flunixin	TBA, pH 10.2	Dichloromethane	–	4.97	−4.7
Mefenamic acid	TBA, pH 10.2	Dichloromethane	–	5.97	−2.4

*Calculated with $pK'_a = 5.82$ for flunixin and $pK'_a = 4.2$ for mefenamic acid [13].

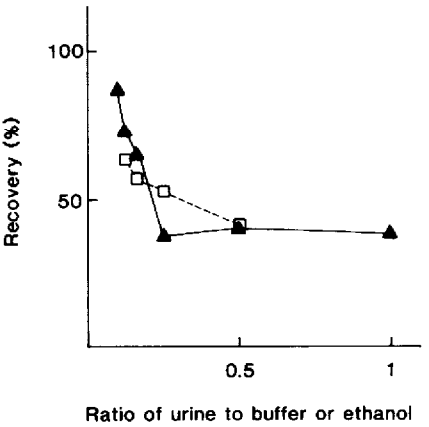


Fig. 2. Extraction and alkylation of diluted urine samples. (▲) Dilution with buffer; (□) dilution with ethanol.

(1.0 ml) were then diluted with phosphate buffer (pH 3.5) or ethanol. The samples were extracted with dichloromethane–2-propanol (85:15); equal volumes of aqueous and organic phase were used. The results are given in Fig. 2. The relative recovery increased from 38 to 87% when the urine samples were diluted ten-fold with buffer solution before extraction. Dilution with buffer was preferred, as ethanol gave 10% lower recoveries at higher dilutions. It is important to dilute the urine samples after hydrolysis, because when flunixin was added to urine and the sample hydrolysed, the relative recovery, without dilution prior to extraction, was only 28%. We have no explanation for these findings, but the recovery seemed to decrease when the urine was viscous and/or contained a lot of endogenous material.

Extractive alkylation

The alkylation reaction studied in carbonate buffer (pH 10.2)–dichloromethane was complete within 30 min for both flunixin and mefenamic acid (Fig. 3). The methylation of the acids was rate-limited, as both flunixin and mefenamic acid are extracted to 99% as ion pairs with TBA during the alkylation procedure. The carboxylic acid group in the molecules was methylated, and the structures have been verified by MS.

Initial studies showed that the amount of methyl flunixin formed was higher if the alkylation was performed with carbonate buffer (pH 10.2) as the aqueous phase instead of sodium hydroxide (0.2 *M*).

Recovery and precision

Linear standard curves were obtained (Fig. 4A and B). The relative recovery of free and total flunixin in urine was 97 and 115%, respectively, comparing standard curves of flunixin in phosphate buffer and urine. The higher recovery of total flunixin obtained from urine might reflect degradation of flunixin in the buffer solution under alkaline conditions. The time for alkaline treatment in both buffer and urine was limited to 30 min, as Chay et al. [4] have shown that no

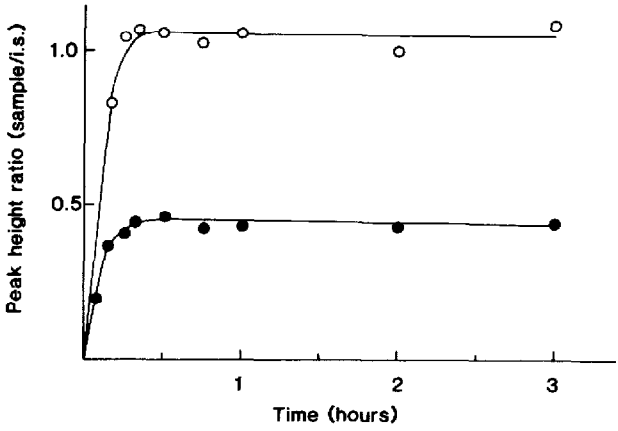


Fig. 3. Time course for methylation of flunixin and mefenamic acid. Aqueous phase, 2.0 ml of TBA (0.0025 *M*) in carbonate buffer (pH 10.2); organic phase, 5.0 ml of methyl iodide (0.5 *M*) in dichloromethane with internal standard, tetracosan or hexacosan. (○) Flunixin ($2.0 \cdot 10^{-3} M$); (●) mefenamic acid ($4.1 \cdot 10^{-3} M$).

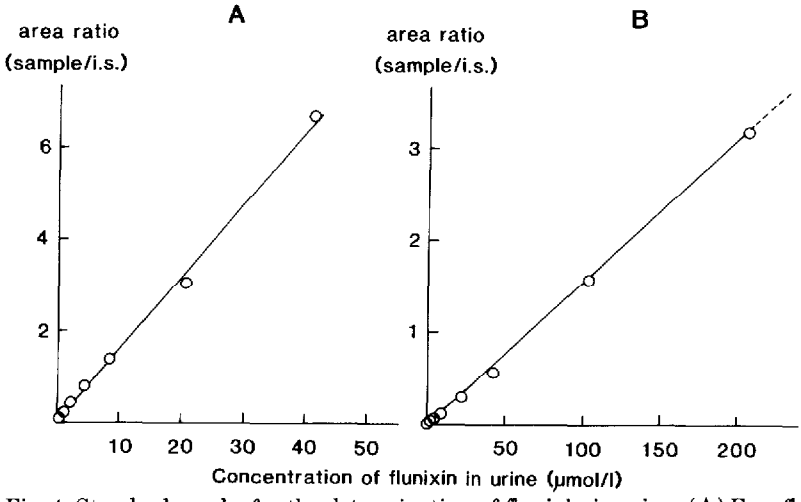


Fig. 4. Standard graphs for the determination of flunixin in urine. (A) Free flunixin; (B) total flunixin. The regression data for the calibration curves are $y = 0.156x + 0.058$ ($r = 0.9988$; $n = 7$; 0.25–41.4 μM) for free flunixin and $y = 0.0155x - 0.016$ ($r = 0.9998$; $n = 8$; 0.83–207 μM) for total flunixin. (The standard graph for total flunixin could be expanded to 500 μM.)

increase in total flunixin occurred after this time. The internal standard cannot compensate for degradation of flunixin, but urine standards and samples were treated the same.

The relative standard deviation (R.S.D.) for free flunixin was 1.7% at the 33.7 μM (10 μg/ml) level ($n = 8$) and 11.2% at 0.83 μM (0.25 μg/ml) ($n = 9$). For total flunixin the R.S.D. was 5.2% at the 168.5 μM (50 μg/ml) level ($n = 8$) and 20.1% at 0.83 μM (0.25 μg/ml) ($n = 10$). This can be compared with the R.S.D. of 7.7% obtained when ten samples containing 243 μM free flunixin in urine (72 μg/ml) were analysed using our previous method [1]. In this method we started

with 3 ml of urine and made the extraction with diethyl ether without any dilution of the urine with buffer. The GC analysis was made on a packed column with FID, and the internal standard was added prior to injection of the sample. The detection limit in urine with our previous method was 8.4 μM (2.5 μg/ml) with GC–FID. This can be compared with 0.25 μM free flunixin (74 ng/ml), which is the detection limit for the present method with GC–NPD, measured at twice the background noise.

The absolute recovery of flunixin was only 39% for free flunixin (10 μg/ml) and 42% for total flunixin (50 μg/ml). This was calculated by comparing the area obtained on injection of a known concentration of methyl flunixin dissolved in toluene with the area obtained when urine samples of flunixin were analysed taken aliquots in the analytical procedure.

The low absolute recovery obtained indicates that losses of the substance took place. The alkylated products may be rearranged during evaporation of the solvent or substances may be lost due to adsorption. The extraction and alkylation processes could be studied in isolation, although in higher concentrations. The losses, however, seem to be the same for flunixin and mefenamic acid as the area ratios were more reproducible than the areas under the methyl flunixin peaks. In this regard, mefenamic acid was an excellent internal standard in the analyses of flunixin.

Analysis of flunixin in urine after drug administration

Chromatograms of urine samples collected before and 18–27 h after the administration of flunixin meglumine to the horse are shown in Figs. 5 and 6. In all the chromatograms, there were peaks resulting from endogenous compounds, and there was also one peak (after 4.1 min) produced by tributylamine. Tributylamine was formed from TBA on injection of the sample. The corresponding deg-

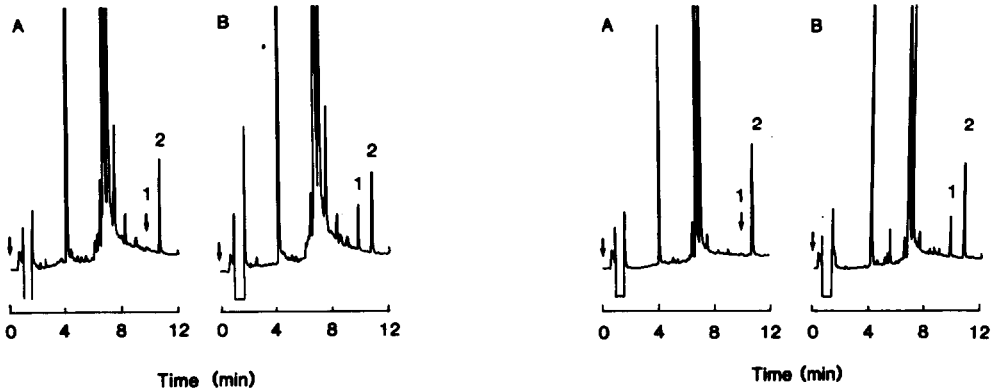


Fig. 5. Gas chromatograms of flunixin in samples of equine urine before (A) and 18–27 h after (B) an intramuscular injection of flunixin meglumine (1.1 mg flunixin per kg body weight) to a horse. Urine concentration of free flunixin = 2.7 μM (0.81 μg/ml). Peaks: 1 = methyl flunixin; 2 = methyl mefenamate.

Fig. 6. Gas chromatograms of flunixin in equine urine after base hydrolysis of the urine. Urine samples as in Fig. 5. Urine concentration of total (free + conjugated) flunixin = 28.7 μM (8.5 μg/ml). Peaks: 1 = methyl flunixin; 2 = methyl mefenamate.

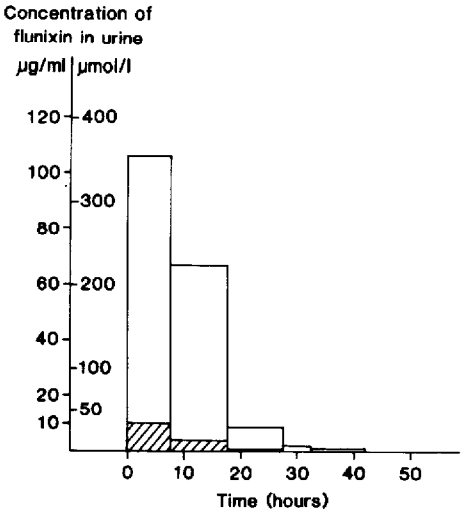


Fig. 7. Time variation of concentration of flunixin found in the urine. Shaded areas indicate free flunixin and clear areas total (free+conjugated) flunixin.

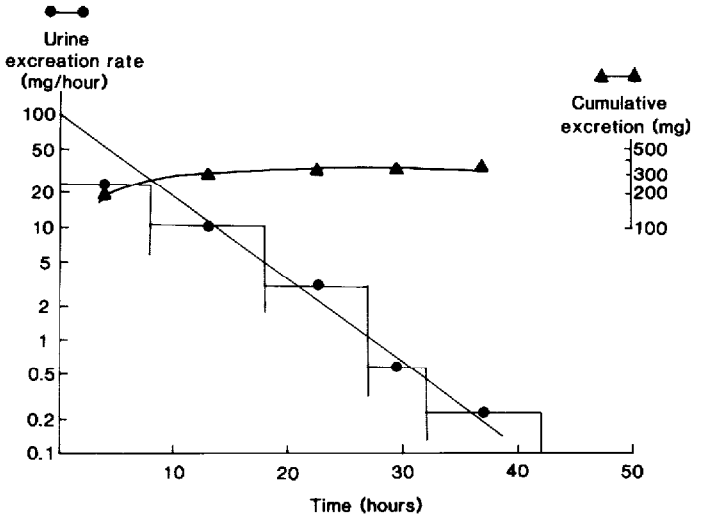


Fig. 8. Elimination of flunixin from the horse. (●) Urinary excretion rate of total (free+conjugated) flunixin; (▲) cumulative urinary flunixin excretion.

radation of THeA to trihexylamine has earlier been reported in the analysis of barbituric acids [14].

The highest concentration of flunixin (9.9 µg/ml free flunixin and 106 µg/ml total flunixin in urine) was obtained in the first urine sample collected. The concentrations decreased thereafter as shown in Fig. 7. The last sample in which flunixin could be detected was collected 32–42 h after administration (0.24 µg/ml free flunixin and 0.89 µg/ml total flunixin in urine). Flunixin was found to be conjugated to 96.5%. A common metabolic pathway for non-steroidal anti-inflammatory drugs in humans is glucuronide conjugation [15].

The total amount of flunixin excreted in the urine corresponds to 64% of the

dose. From the plot of the total amount of flunixin excreted versus time (Fig. 8), the elimination half-life of flunixin was calculated by slope analysis [16] to be 4.2 h. This is in accordance with the earlier study by Chay et al. [4].

Stability of flunixin and conjugated flunixin in horse urine

The urine sample collected at 8–18 h was kept at room temperature for 14 days. The total concentration of flunixin in this sample was 67 μg/ml and was the same on days 1 and 14, but the concentration of free flunixin was found to increase over the period. The analysis gave 94% flunixin conjugation on day 1, 63% on day 7 and 9% on day 14. This means that there was no degradation of flunixin itself, but conjugated flunixin was spontaneously hydrolysed to give the parent drug.

Doping control analysis

The degree of hydrolysis of conjugated flunixin in samples taken at horse-racing tracks cannot be controlled during transport. The samples are normally kept in a refrigerator for one to two nights and then transported at ambient temperature for one to two days, occasionally longer, before the analyses can be performed. This is often no problem in doping control analysis as the question here is only to confirm the presence of the drug without reporting concentration values.

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