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Disposition of human drug preparations in the horse IV. Orally administered fenoprofen

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Abstract

Plasma and urinary concentrations of the non-steroidal anti-inflammatory drug fenoprofen were determined by a high-performance liquid chromatographic procedure following oral administration of a dose of 3 g to fed and fasted horses.

In plasma, fenoprofen was present in detectable concentrations for 6-12 h. Free access to hay significantly reduced the peak plasma concentration and bioavailability of fenoprofen, and large interindividual differences in absorption and elimination pattern occurred. In fasted horses, fenoprofen was rapidly absorbed with a mean half-life of 0.10 h. Maximum concentrations were found 0.63 ± 0.21 h after dosing. The elimination half-life was 0.9 h.

As early as 1 h after dosage, fenoprofen could be detected in hydrolysed and unhydrolysed urine, and remained detectable up to 48 h. The maximum excretion rate and peak concentration occurred 2 h after administration, irrespective of the feeding schedule. In fed horses, a second maximum occured after 9 h. The percentage of the dose excreted as unchanged fenoprofen in 12 h was $13.0 \pm 6.8\%$. A recovery of $21.9 \pm 7.4\%$ and $42.2 \pm 7.0\%$ of the dose was obtained after enzymatic and alkaline hydrolysis, respectively. At least three hydroxylated metabolites were detected in hydrolysed urine.

Keywords: Disposition; Doping analysis; Fenoprofen; Horse

1. Introduction

Several drugs developed for human use have been reported by the Association of Official Racing Chemists as illicit substances in the bodily fluids of horses at racecourses or jumping courts. The detection of such substances in equine biofluids is facilitated by the knowledge of how such drugs are metabolised and eliminated in this species. In previous works, the disposition of indomethacin after the administration of a human suppository preparation [1], the excretion and metabolism of fencamfamine [2] and the pharmacokinetics of alclofenac [3] in horses have been described. In the present study, the disposition of the orally administered human nonsteroidal anti-inflammatory drug (NSAID) fenoprofen in the horse is reported. The bioavailability was studied both under fasting and hay feeding conditions.

2. Material and methods

2.1. Experimental animals

Ten standard-bred mares (375-450 kg, mean 425 ± 31 kg) received five tablets FEPRON® (Dista Products Division, Eli Lilly Benelux,

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Brussels, Belgium) corresponding to a total dose of 3 g fenoprofen, mixed with a small amount of oats. The horses of one group ($n = 5$) were fasted from the afternoon preceding the day of the experiment; the second group ($n = 5$) was offered hay before dosing. All horses were allowed water ad libitum during the experiment and pelleted food was offered 8 h after the administration of fenoprofen.

The bioavailibility study was not carried out as a cross-over study in the same individuals. However, a group of five animals is a sufficient population for pharmacokinetic work.

Heparinized blood samples (10 ml) were collected by direct venipuncture of the right jugular vein. Sampling times were 0 (control), 5, 10, 15, 20, 30, 40, 50, 60 min and 2, 3, 4, 5, 6, 9 and 12 h after the administration. Blood samples were centrifuged at 1700 g for 10 min and plasma was removed and stored at 4 °C until analysis. Plasma samples were analysed in duplicate. A balloon-tipped catheter was placed in the bladder of each mare. The total volume in the bladder was collected over a 12 h period after drug administration with the same sampling times as for blood from the 1 h sample onwards. After this 12 h period, aliquots of urine were taken every 12 h until 72 h after dosing. Urinary pH and volume were measured and aliquots analysed in duplicate. Whenever necessary, dilutions were made with blank urine.

2.2. Reagents

The reference substances fenoprofen and prenazone (internal standard) were obtained from Eli-Lilly (Brussels, Belgium) and Boehringer Ingelheim (Brussels, Belgium), respectively. HPLC grade acetonitrile was from BDH (Poole, UK). Aqueous HPLC solvent was prepared using water obtained with the MILLI-Q water purification system from Millipore (Brussels, Belgium). Acetone, diethyl ether, hydrochloric acid, sodium hydroxide and sodium bicarbonate, all ACS grade, were purchased from Merck (Overijse, Belgium). Iodomethane and tetrahexylammonium hydrogen sulphate (THAHS) were obtained from UCB (Drogenbos, Belgium) and Fluka (Buchs, Switzerland), respectively. The enzyme preparation Suc Helix Pomatia (SHP) containing 100 000 Fishman units per ml of β-glucuronidase and 1 000 000 Roy units per ml of arylsulphatase was obtained from IBF (Villeneuve, France).

2.3. Equipment

The HPLC system (TSP, Fremont, CA, USA) consisted of a SP 8800 pump, Model SP 8880 autosampler and a Focus forward optical scanning detector set at 230 nm. Data (peak heights) were generated through the LABNET communication system with a PS 2/386 computer (IBM).

A 100 × 3 mm i.d. reversed-phase column packed with octadecyl silica (5 μm Nucleosil, Chrompack, Antwerpen, Belgium) attached to an appropriate guard column was used. The loop volume was 20 μl. A gradient elution with acetonitrile and water–acetic acid (99:1) was performed for the assay of plasma fenoprofen. The solvent program was as follows: initial acetonitrile 50% for 7 min (flow rate 0.5 ml min^{-1}); the column was flushed with 80% acetonitrile from 7.6 to 11 min (flow rate 0.5 ml min^{-1}). Equilibration time was 8 min. For the determination of fenoprofen in urine, flushing of the column was omitted.

GC/MS was carried out on a Hewlett-Packard (Palo Alto, CA, USA) 5993 instrument equipped with a J & W (Folsom, CA, USA) fused silica column (30 m × 0.245 mm i.d., DB-5MS). Injections were made with an all-glass moving needle; the column was directly coupled to the MS source. GC conditions were: injector temperature, 270 °C; oven temperature programme from 150 to 300 °C at a rate of 10 °C min^{-1}; transfer line temperature 270 °C. Helium was used as carrier gas (0.8 ml min^{-1}). Electron impact (EI) spectra were recorded at 70 eV.

2.4. Drug assay

The concentration of fenoprofen in plasma and urine was determined by a recently published method [4]. Briefly, plasma samples (2 ml) were treated with 250 μl of 1.0 mol l^{-1} hydrochloric acid, 50 μl IS added, the resulting mixture briefly vortexed, and fenoprofen extacted twice with 5 ml diethyl ether (15 min). The combined organic layers were evaporated under nitrogen at 36 °C and the residue redissolved in 200 μl acetonitrile–1% acetic acid (50:50 V/V). The limit of quantification was 0.05 μg ml^{-1}. Urine samples (0.5 ml) were acidified with 250 μl 1.0 mol l^{-1} hydrochloric acid

and 50 μl IS added. Extraction was performed with 5 ml diethyl ether (15 min). The organic layer was washed by vortexing with 1 ml of a freshly prepared $NaHCO_3$ solution (1%). The diethyl ether layer was separated and subsequently treated as described for plasma. The limit of quantification was 0.2 μg ml^{-1}.

2.5. Enzymatic hydrolysis

Urine (0.5 ml) was buffered with 0.1 ml of 1 mol l^{-1} sodium acetate buffer (pH 5.2) and 20 μl of SHP added. Hydrolysis was performed for 2.5 h at 56 °C. After cooling, the hydrolysate was analysed as described above.

2.6. Stability of fenoprofen at pH 5.2

Urine (0.5 ml) spiked with fenoprofen (final concentration 8 μg ml^{-1}) was analysed ($n = 5$) with and without treatment with sodium acetate buffer as described above.

2.7. Alkaline hydrolysis

Urine (0.5 ml) was treated with 50 μl of 1 mol l^{-1} sodium hydroxide for 30 min at room temperature. The hydrolysate was then acidified with 300 μl of 1 mol l^{-1} hydrochloric acid and subsequently analysed.

2.8. Stability of fenoprofen under alkaline conditions

Urine (0.5 ml) spiked with fenoprofen (final concentration 8 μg ml^{-1}) was analysed without treatment ($n = 5$), and after alkaline hydrolysis ($n = 5$) with 1 mol l^{-1} NaOH and saturated ammonium buffer (pH 9.5), respectively.

2.9. Influence of time and temperature on alkaline hydrolysis

Administration urine from horse # 1, 3 h after dosing ($n = 5$), was subjected to alkaline hydrolysis at room temperature (30 min) and at 42 °C (30 min and 2 h).

2.10. GC/MS confirmation and detection of metabolites

Blank urine (1 ml) and urine 4 h after administration was hydrolysed with NaOH. Extractive methylation was performed using the method of Lisi et al. [5] by adding 150 μl of 0.2 mol l^{-1} THAHS and 5 ml of 0.5 mol l^{-1} CH_3I in toluene. Mixing was carried out at room temperature for 20 min, followed by a clean-up step using SM-7 resin. The residue was dissolved in 200 μl ethyl acetate and 1 μl injected for GC/MS analysis.

2.11. Plasma protein binding in vitro

The binding of fenoprofen to horse plasma protein was determined by ultrafiltration. The plasma was obtained from a fresh pooled heparinised blood sample. Solutions of fenoprofen (10 μg ml^{-1} and 5 μg ml^{-1}) were prepared with plasma. Quadruplicate samples (1 ml) were centrifuged in the micropartition apparatus (model MPS-1, Amicon) at 1500*g* for 20 min. Fenoprofen concentrations were measured by the HPLC method.

2.12. Pharmacokinetic analysis

The time at which peak concentration oc-

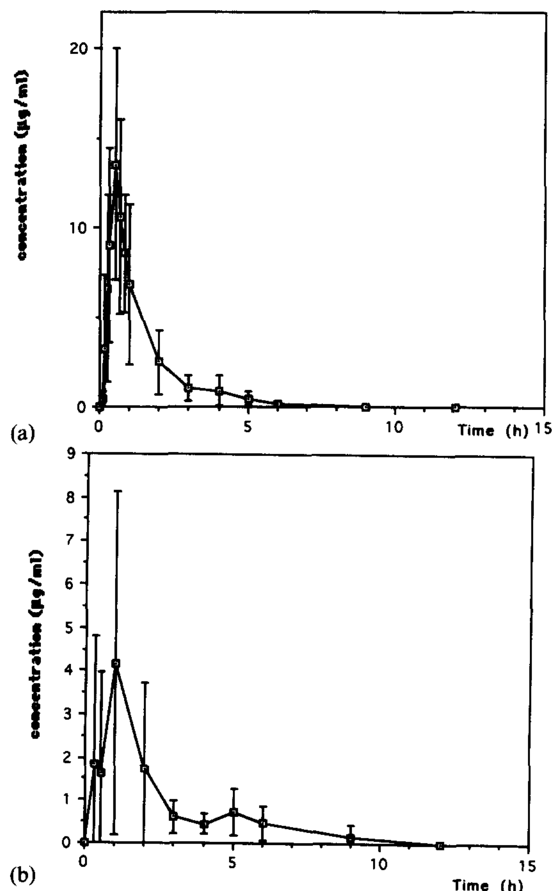

Fig. 1. Mean plasma concentration–time curve in (a) fasted horses and (b) fed horses, following 3 g fenoprofen ($n = 5$).

curred (t_{max}) and the peak concentration itself (C_{max}) were determined from the graphs. Plasma fenoprofen concentration–time curves were analysed by use of an iterative non-linear regression programme [6]. This PK non-lin programme allows calculations of other pharmacokinetic variables including AUC, $t^{1/2}_{abs.}$ and $t^{1/2}_{el.}$ The arithmetic mean $\pm$ SD was calculated for all values with the exception of $t^{1/2}$, for which the harmonic mean was considered more appropriate.

Differences between values were compared using Student's t-test for unpaired data, and were considered to be significant at the 95% probability level.

3. Results and discussion

Studies in animals and humans have shown that fenoprofen, a phenylpropionic acid derivative, has anti-inflammatory, analgesic and antipyretic properties. Comparative studies in man [7] suggest that daily doses of 2.4 g fenoprofen provide relief approximately equivalent to that of 3.9 g aspirin. Considerable amounts of fenoprofen were detected in bile, indicating the possible occurrence of an enterohepatic circulation [8]. After oral administration of 250 mg fenoprofen in man, preliminary plateaus between 30 and 60 min after ingestion have been observed [9]. A lag time of 10–15 min before the start of the absorption process or until the plasma fenoprofen concentrations could be detected by the GC method was also observed. In the present study, however, fenoprofen could already be detected in plasma after 5 min. The mean plasma fenoprofen concentration–time curve is illustrated in Fig. 1. Absorption occurred very rapidly when the horses had no access to feed at the time of dosing. The maximum mean concentration of 13.1 ± 6.3 μg ml^{-1} was achieved in 0.63 ± 0.21 h. After 0.5–1 h, plasma fenoprofen concentrations fell rapidly and exponentionally with a harmonic mean terminal half-life of 0.90 h, comparable to that found in humans [9].

Significant differences in absorption as reflected by C_{max} and $t^{1/2}_{abs.}$ (Table 1) occurred following the administration of fenoprofen to horses with free access to hay before and after dosing. Intersubject variability in the absorption pattern was noticed: a broad absorption plateau, more than one concentration peak, and a normal pattern. The bioavailability of fenoprofen as shown by the AUC seemed to be lower in horses fed with hay. The plasma concentrations at all sampling times up to 2 h were lower in fed than in fasted horses. Drug absorption from the gastrointestinal tract may be affected by the feeding schedule. Moss [10] showed that the absorption pattern of phenylbutazone following oral administration to horses varies, and this variation has been confirmed in other studies [11–13]. Similar variations are also found after administering fenoprofen to horses fed with hay. A significant reduction in C_{max} and the occurrence of a second concentration peak was observed in fed horses (Fig. 1). Such a decrease and multiple concentration peaks were also noticed when flunixin was administered to horses having free access to feed [14]. One of the reasons for the difference in the absorption pattern between fed and fasted horses could be the binding of fenoprofen to hay. Maitho et al. [15] speculated that phenylbutazone was bound to hay and that this greatly limited the absorption for a time. It was also postulated that the release of the drug in the caecum and the colon as a consequence of fermentative digestion of the

Table 1
Pharmacokinetic parameters after administration of 3 g fenoprofen to fasted and fed horses

Horses	Fasted						Fed					
	#1	#2	#3	#4	#5	mean	#6	#7	#8	#9	#10	mean
AUC (μg h ml^{-1})	23	15	17	13	17	17 ± 4	11	6	7	11	6	9 ± 2 [a]
C_{max}(μg ml^{-1})	22.8	12.0	13.7	12.0	8.0	13.1 ± 6.3	6.25	1.09	1.59	5.79	0.86	3.11 ± 2.66 [a]
T_{max}(h)	0.5	0.5	1	0.5	0.67	0.63 ± 0.21	0.33	1	6	1	0.33	1.73 ± 2.40
$t^{1/2}_{abs.}$(h)	0.082	0.16	0.075	0.10	0.49	0.10	1.38	2.12	2.52	0.74	2.33	1.47 [b]
$t^{1/2}_{el.}$(h)	0.68	1.35	1.09	0.83	0.97	0.90						

[a] $p \leq 0.005$.
[b] $p \leq 0.001$.

Table 2
Stability of fenoprofen in urine during hydrolysis, and influence of time and temperature on the recovery of the hydrolysis

A. Spiked horse urine (8 µg ml⁻¹)	
Without treatment ($n = 5$)	7.86 ± 0.19
NaOH, 30 min, room temp. ($n = 5$)	7.77 ± 0.08
NH_4^+ buffer, 30 min, room temp. ($n = 5$)	7.57 ± 0.27
NH_4^+ buffer, 3 h, 56 °C ($n = 5$)	7.20 ± 0.08
Buffer pH 5.2, 2 h, 56 °C ($n = 5$)	7.39 ± 0.17
B. Administration urine (conc. found, µg ml⁻¹)	
NaOH, 30 min, room temp. ($n = 5$)	8.91 ± 0.42
NaOH, 30 min, 42 °C ($n = 5$)	8.59 ± 0.43
NaOH, 2 h, 42 °C ($n = 5$)	8.53 ± 0.49

food might explain the delayed plasma concentration peak when horses had free access to hay and feed. In addition, the differences in absorption pattern could be ascribed to the pH in the stomach, which is influenced by the feeding schedule. The pH in the equine stomach is more acid after a period of food deprivation [16], which could also contribute to the higher fenoprofen peak concentration obtained in fasted horses.

Plasma protein binding was calculated to be $89.0 \pm 0.7\%$ and $92.2 \pm 1.4\%$ for fenoprofen concentrations of 10 and 5 µg ml^{-1} respectively. These values are lower than those found for the in vitro binding of fenoprofen to human serum albumin, indicating that 99% was protein bound [8].

Formation of acyl glucuronides is a major metabolic pathway for several NSAIDs in humans. In the horse, however, phase II metabolism of carboxylic acids additionally includes glycine and taurine conjugation [17]. These conjugates can be hydrolysed by alkali. However, in previous works on NSAIDs in the horse it was shown that indomethacin decomposed during alkaline hydrolysis (1) and that mild alkaline conditions (pH 9.5) were required to hydrolyse alclofenac conjugates (3). When fenoprofen spiked horse urine was subjected to alkaline conditions at room temperature, no statistically meaningful differences in concentration were found compared to untreated spiked urine (Table 2). From the analysis of an administration sample, it appeared that alkaline hydrolysis at room temperature for 30 min is sufficient to hydrolyse the alkali labile conjugates.

Following oral administration of Fepron®, the parent and conjugated drug could already be detected in the urine of all horses 1 h after dosage. With a limit of quantification of 0.2 µg ml^{-1}, fenoprofen remained detectable in alkaline hydrolysed urine up to 48 h for most horses (Fig. 2). Generally, the detection time was 24–36 h in enzymatically hydrolysed urine and 12–24 h in unhydrolysed urine. Maximum excretion rates of parent and total (free + conjugated) fenoprofen in fasted horses both occurred 2 h after dosage. The range was 294–709 mg h^{-1} (alkaline hydrolysis). In horses fed hay, the maximum excretion rates were obtained 3 h after administration and were not significantly different from the values obtained in fasted horses. However for three out of five fed horses a second maximum occurred after 9 h. For all feed deprived horses, fenoprofen urinary peak concentrations, both conjugated and unconjugated, were obtained after 2 h. The maximum concentration range after alkaline hydrolysis was 1346–2680 µg ml^{-1}. For horses with free access to hay, urinary fenoprofen peaked at 2 h and a second maximum was observed after 9 h.

The percentage of the dose excreted as fenoprofen in the first 2 h (in 10 horses) was $13.0 \pm 6.8\%$. Part of the conjugated fenoprofen could be hydrolysed by β-glucuronidase arylsulphatase, increasing the recovery to $21.9 \pm 7.4\%$. Alkaline hydrolysis resulted in the detection of $42.2 \pm 7.0\%$ of the administered dose. The large difference in recovery between *helix pomatia* and alkaline hydrolysis could be due to the activity of the glucuronidase enzyme. Acyl migration in ester glucuronides results in the formation of a mixture of isomers and partial resistance to hydrolysis by β-glucuronidase. This phenomenon has been described for some NSAIDs in humans [18].

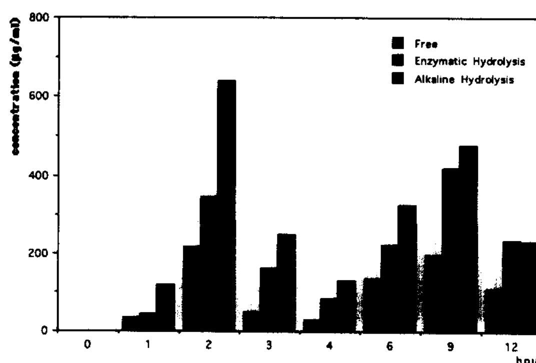

Fig. 2. Urinary fenoprofen concentrations in horse #6 (fed).

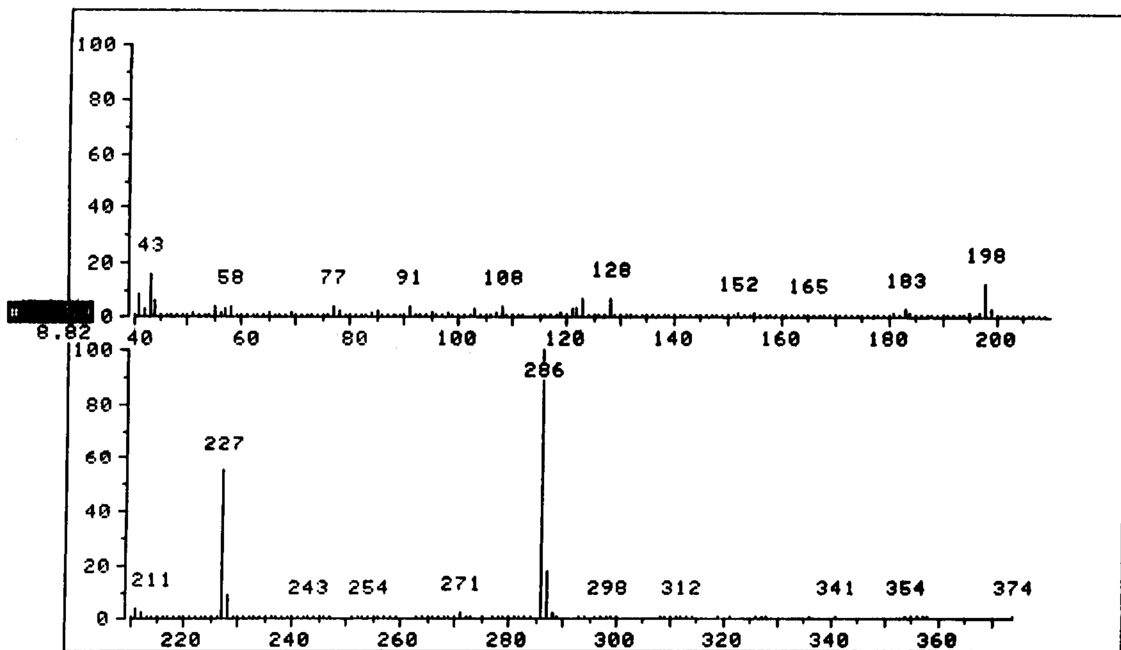

Fig. 3. Mass spectrum of a monohydroxylated metabolite in a methylated urine extract obtained 4 h after administration of fenoprofen.

In humans, fenoprofen is extensively metabolised to 4′-hydroxyfenoprofen and its conjugates [9]. GC/MS analysis of a methylated urine extract obtained 4 h after the administration of fenoprofen revealed the presence of at least three hydroxylated metabolites. As indicated by the molecular ion of 286 u, the major metabolite appeared to be mono-hydroxylated fenoprofen (Fig. 3). From the presence of the ion 198 u it is concluded that the hydroxy group is located on the phenyl moeity of the molecule. Two minor isomeric metabolites (Fig. 4) seem to contain two hydroxy groups, one situated on the phe-

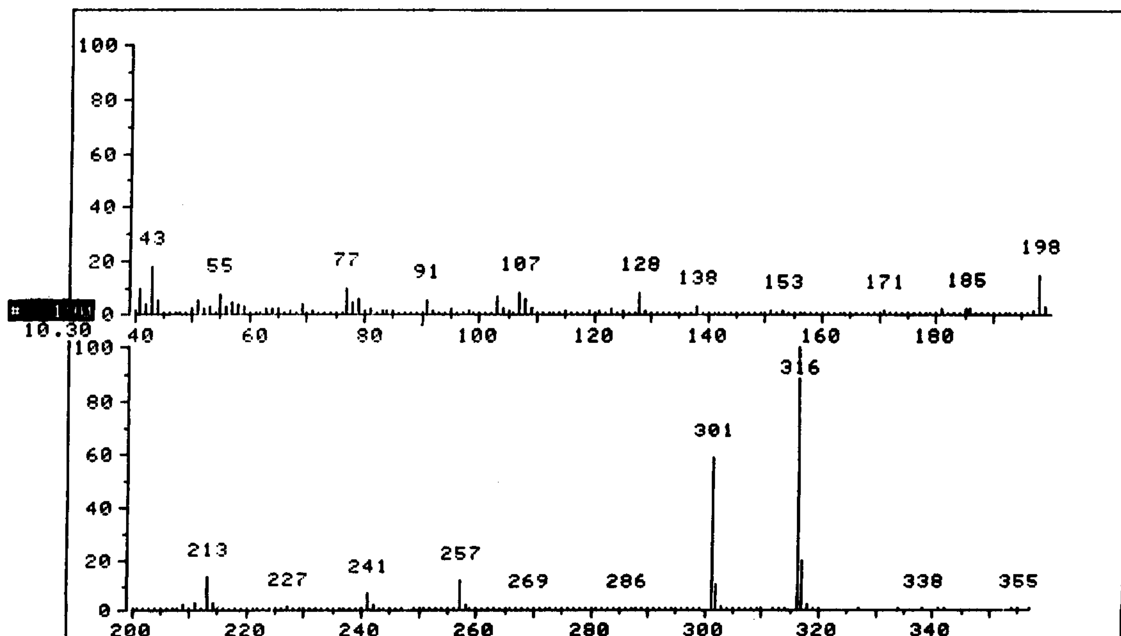

Fig. 4. Mass spectrum of a bishydroxylated metabolite in a methylated urine extract obtained 4 h after administration of fenoprofen.

noxyphenyl part of the molecule (198 u) and the other on the propionic moeity, as indicated by the molecular ion (316 u) and the fragment $M^+ - 15$ (301 u). Synthesis of hydroxyl substituted fenoprofen derivatives, however, is needed to fully characterise these metabolites.

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