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Short Communication

Identification of a tolfenamic acid metabolite in the horse by gas chromatography–mass spectrometry

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ABSTRACT

A tolfenamic acid metabolite, a hydroxylated product, has been identified in equine plasma and urine samples using gas chromatography–mass spectrometry in the electron-impact and chemical-ionization modes. The method also allows the qualitative monitoring of the elimination of the drug and its metabolites from plasma. The two compounds are detected up to 48 and 24 h, respectively, after a single oral administration of a 30 mg/kg dose. The simultaneous detection of the two products increases the reliability of anti-doping control analysis.

INTRODUCTION

Tolfenamic acid, N-(2-methyl-3-chlorophenyl)-anthranilic acid (Fig. 1), is a non-steroidal anti-inflammatory agent used for humans and recently introduced as a veterinary treatment. This drug is also used to dope horses because of its potent analgesic properties, which can mask lamenesses due to varied kinds of lesions. Its metabolism has been thoroughly studied in humans after oral administration [1–3], but has not been described in equine species. Because of this lack of information, it was decided to identify one phase I metabolite of tolfenamic acid. Indeed, the simultaneous detection of a drug and one of its metabolites is helpful in reporting a positive case in anti-doping control studies. It is also of great interest to define the period within which routine detection of the doping agent derivative in urine and plasma is possible. The second aim of this study was therefore to monitor the elimination of the metabolite from blood. Similar work has already been conducted in horse urine with flunixin, a non-steroidal anti-inflammatory drug structurally analogous to tolfenamic acid [4].

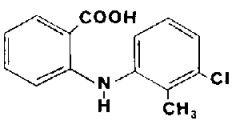

Fig. 1. Structure of tolfenamic acid.

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EXPERIMENTAL

Drug administration and sample collection

Four standard bred geldings aged between 9 and 16 years and weighing about 500 kg were used. After the oral administration of 30 mg/kg tolfenamic acid as a paste (Tolfedine, Vetoquinol, Lure, France), blood samples were collected by venous puncture at 0, 10, 30 min, 1, 1.5, 2, 2.5, 3, 4, 6, 10, 24 h and 2, 3, 4, 5, 6, 7 days. The samples were then centrifuged for 10 min at 900 *g* and the plasma was stored at −20°C until analysis. Urine samples were naturally collected by using an urinal at 0, 10 and 24 h after administration. Aliquots of 500 ml were taken for analysis after homogenization and were stored at −20°C.

Identification of the metabolite

Plasma sample preparation. A 2.5-ml plasma sample was adjusted to pH 5 with 1 *M* hydrochloric acid and extracted twice with 5 ml of diethyl ether. The organic layer, separated after centrifugation, was evaporated to dryness under a nitrogen stream at 60°C. The dry residue was then reconstituted with 100 μl of 25% trimethylanilinium hydroxide in methanol (Methelute Pierce Ref. 49301; Spiral, Dijon, France) and analysed by gas chromatography–mass spectrometry (GC–MS).

Urine sample preparation. A 2.5-ml urine sample was extracted and purified using a method described previously [4] and analysed by GC–MS.

Identification of the metabolite in plasma and urine. A 1-μl aliquot of each methylated sample was injected into the gas chromatograph of the GC–MS system (Hewlett-Packard 5970-5890). The chromatograph was equipped with a 25 m × 0.32 mm I.D. column (SE 30, Spiral). The operating temperatures were as follows: injector, 250°C; oven, from 75 to 215°C at 30°C/min, 215°C for 3 min, then from 215 to 280°C at 30°C/min, and finally 280°C for 4 min; interface, 250°C. The carrier gas was helium at a flow-rate of 20 cm/s. The mass spectra were obtained at 70 eV and scanned from 40 to 400 a.m.u. at a rate of 1.52 ms/a.m.u.

A 1-μl volume of each of the two methylated plasma extracts obtained from blood collected at 2 and 3 h were injected into the gas chromatograph of a GC–MS MS system (Finnigan TSQ 70). The column was a BP5 (S.G.E.) 25 m × 0.25 mm I.D. column and the GC parameters were identical to those used for GC–MS identification. The ion source conditions were: temperature 150°C and pressure 600 kPa (methane as reactant gas). The pressure in the collision cell Q2 was 0.2 kPa (argon) and the collision offset was fixed at −20 V. The TSQ 70 was set in the daughter ion mode, Q1 was tuned to *m/z* 306 and Q3 scanned from *m/z* 40 to 310 at a scan rate of 3.7 ms/a.m.u.

Detection times of drug and metabolite in plasma

Reconstructed ion chromatograms were plotted for the sum of the selected ions *m/z* 208 and 305, which represent the base peaks of tolfenamic acid and the metabolite mass spectra, respectively. Mass spectra of the drug and its metabolite were plotted for each time period.

RESULTS AND DISCUSSION

Identification of the metabolite in plasma and urine samples

The metabolite was first characterized in both urine and plasma with the normalized and background-substracted mass spectrum, obtained by electron-impact ionization, of its dimethylated derivative. This spectrum was compared with that of the monomethylated tolfenamic acid derivative (Fig. 2). The retention time of the dimethylated metabolite derivative was 8 min 45 s; that of the monomethylated tolfenamic acid derivative was 6 min 39 s.

Fig. 3 shows a fragmentation pathway of the tolfenamic acid metabolite, which is most probably a phenolic compound. The molecular ion seems to be ion *m/z* 305 (A). An interesting ion appears at *m/z* 273 (B). It could be formed by the loss of CH_3OH by the "*ortho* effect" involving ester and secondary amine functions. This effect has been already described with a hydroxylated flunixin metabolite [4]. Such a fragmentation suggests that the hydroxy function of the tolfenamic acid metabolite has been methylated in spite of the presence of the amino group. Ion *m/z* 273 can then lose CH_3 (*m/z* 258), CO [*m/z* 230 (C)] and

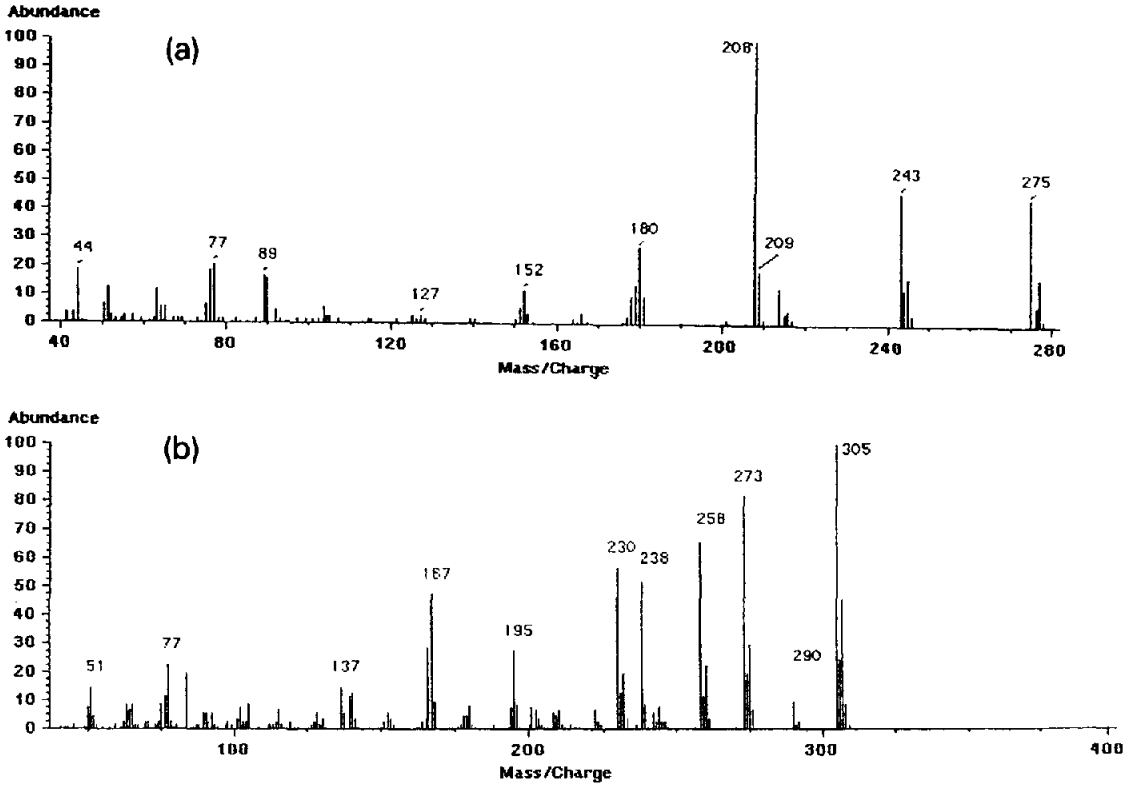

Fig. 2. Mass spectra of the dimethylated tolfenamic acid hydroxy metabolite derivative (b), compared with that of the monomethylated tolfenamic acid derivative (a).

Cl, resulting in *m/z* 195. Molecular ion demethylation can also occur, producing *m/z* 290. Ion *m/z* 273 can lose Cl, resulting in *m/z* 238. Finally, the fragment *m/z* 195 can also be formed from *m/z* 238 by the loss of $COCH_3$.

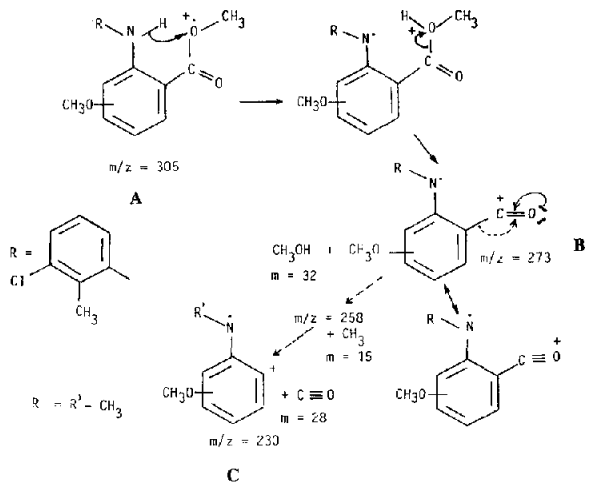

Fig. 3. Fragmentation pathway of the dimethylated metabolite derivative showing an *ortho* effect.

When the structure of a fragment ion includes a chloride atom, the parent peak, p, is followed by an isotopic peak, p + 2, whose abundance is around 33% of that of the parent peak. This phenomen can be observed with ions *m/z* 305, 273, 258 and 230. It seems that no intramolecular cyclization of the molecular ion occurs, with the loss of $COCH_3$, as is seen with the dimethylated flunixin hydroxy metabolite [4].

The mass spectrum obtained with the dimethylated metabolite derivative in the chemical-ionization mode (Fig. 4) provides evidence that the protonated molecular ion is the fragment *m/z* 306. Therefore, the previously supposed molecular ion is actually *m/z* 305. Ion *m/z* 334 is an adduct resulting from C_2H_4 binding on this fragment. Ions *m/z* 291 and 274 correspond, respectively, to ions *m/z* 290 and 273 formed by electron impact.

The spectrum obtained in chemical-ionization mode could be clarified using the MS–MS technique. The pseudo-molecular ion *m/z* 306 was se-

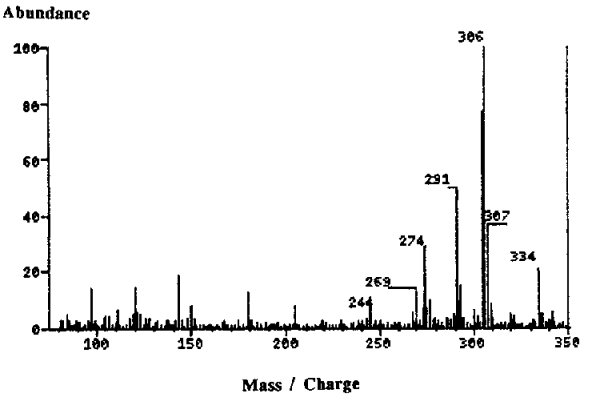

Fig. 4. Mass spectrum of the dimethylated tolfenamic acid hydroxy metabolite derivative, obtained by GC–MS with chemical ionization.

lected through Q1 and the daughter ions were recorded (Fig. 5). The labels of the ions can be seen in Fig. 3. The fragmentation pattern shows five major ions: m/z 306 is the unbroken pseudomolecular ion M+H corresponding to A; m/z 291 corresponds to the loss of one methyl group (M+H)−15; m/z 274 is the B+H ion; m/z 259 corresponds to the loss of CH_3 in the B+H ion; and m/z 231 is the C+H ion. This fragment should present a loss of a methyl group, but the intensity is too low to be recorded. This fragmentation pattern shows no discordance with the scheme proposed in Fig. 3.

Finally, the different mass spectra analysed show the presence of a phenolic-type function in the structure of the phase I metabolite found in both urine and plasma samples. However, they do not indicate which of the two aromatic rings of tolfenamic acid has been oxidized, and what

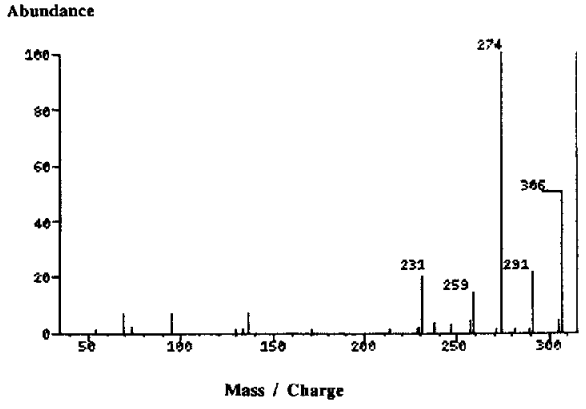

Fig. 5. Daughter ions of m/z 306, obtained by MS–MS.

position is occupied by the hydroxy group on the ring. These structural parameters should be determined in a further study using ^{1}H NMR spectroscopy. In the horse, as in humans [1,2], tolfenamic acid is partly converted into a hydroxylated metabolite.

The derivatization procedure with trimethylanilinium hydroxide allows a flash methylation into the injector of the chromatograph, but a polyfunctional molecule usually gives rise to several methylated products. In this experiment, the carboxylic, amine and phenolic functions of the metabolite were liable to react with the methylating agent. However, only one metabolite derivative was identified, in contrast to the results obtained with flunixin. Indeed, the main metabolite of flunixin was characterized by both its dimethylated and trimethylated derivatives [4]. Such a difference in reactivity may be explained by structural considerations, involving the replacing of the chlorine atom of the tolfenamic acid molecule by a trifluoromethyl group in flunixin. This hypothesis seems to be corroborated by the fact that no dimethylated derivative of tolfenamic acid could be identified during this experiment.

Detection times of the drug and metabolite in plasma samples

The elimination of tolfenamic acid and its metabolite from plasma was followed qualitatively with reconstructed ion chromatograms and mass spectra. A reconstructed ion chromatogram shows the two compounds in one horse 10 h after administration (Fig. 6). Tolfenamic acid can be detected up to 48 h and the metabolite up to 24 h after administration. After this time, the mass spectra contain only low-abundance ions. Therefore, the estimated detection times for the anti-inflammatory drug and its hydroxy metabolite in plasma, *i.e.* the period during which the molecules can be detected in this biological fluid using a definite analytical method [5], are two days and one day, respectively. Further experiments, using a quantitative method, should be carried out to define more accurately the detection times in urine and plasma. The simultaneous identification of tolfenamic acid and its hydroxy metabolite in biological samples is of real interest in anti-doping control.

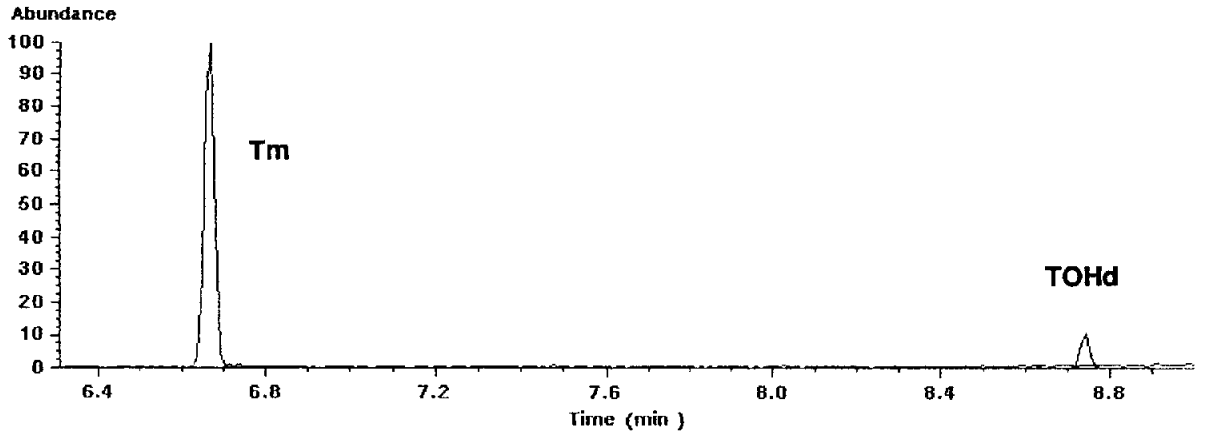

Fig. 6. Reconstructed ion chromatogram, plotted on the sum of the ions *m/z* 208 and 305, obtained with the 10-h plasma sample. The outline shows peaks of the monomethylated tolfenamic acid derivative (Tm) and the dimethylated metabolite derivative (TOHd). Retention times are in minutes.

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