Journal of Chromatography, 423 (1987) 123–130
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3886

IDENTIFICATION OF A FLUNIXIN METABOLITE IN THE HORSE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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(First received March 26th, 1987; revised manuscript received June 19th, 1987)

SUMMARY

The main metabolite of flunixin, a hydroxylated product, has been identified by gas chromatography–mass spectrometry and ^1H NMR spectroscopy in equine urine and plasma. The method also permits the qualitative monitoring of the urinary elimination of the drug and its metabolite. The two products are detected up to 175 and 54 h, respectively, after a single intravenous administration at the dose of 1 mg/kg. Simultaneous detection of the two compounds increases the reliability of anti-doping control analysis.

INTRODUCTION

Flunixin (Fig. 1) is a potent non-steroidal anti-inflammatory agent with analgesic activity [1, 2], widely used in equine medicine and for doping. Its pharmacokinetics have been thoroughly studied [2, 3] in this species, but its biotransformations have not been described, though conjugation reactions have been suggested [2]. Because of this lack of information, it was decided to identify one phase I metabolite of flunixin, as simultaneous detection of the drug and one of its metabolites can help to assure a positive result during antidoping control

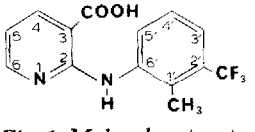

Fig. 1. Molecular structure of flunixin.

0378-4347/87/$03.50

procedures. It was necessary to define the period within which routine detection of the doping agent in urine would be possible, so the second aim of this study was to monitor the urinary excretion of flunixin and its phase I metabolite concurrently.

EXPERIMENTAL

Drug administration and sample collection

One standerbred gelding (500 kg) was used. After the intravenous administration of 1 mg/kg flunixin (Finadyne®, Rigaux Galena, Levallois-Perret, France), urine samples were collected naturally into a urinal at 0, 4, 7, 24, 30, 48, 54, 72, 127, 144, 151, 168, 175, 192, 199 and 216 h. Aliquots of 500 ml were taken for analysis after homogenization and stored at −20 °C.

A second horse, suspected to have been doped with flunixin, was also used. At the time of an antidoping control, 10 ml of blood were collected by venous punction and centrifuged, and 2.5 ml of plasma were taken off for analysis.

Identification of the metabolite

Urine sample preparation. A 25-ml volume of the 4-h urine sample was adjusted to pH 12 with 2.5 *M* sodium hydroxide, then centrifuged. The supernatant was adjusted to pH 1 with 6 *M* hydrochloric acid, then extracted twice with 50 ml of diethyl ether. The organic phase was added to 15 ml of aqueous saturated sodium bicarbonate solution, and shaken for 3 min. After dehydration with anhydrous sodium sulphate, the ether phase was filtered and evaporated to dryness under reduced pressure at room temperature. The residue was dissolved into 5 ml of diethyl ether, and then purified by thin-layer chromatography on silica gel preparative plates (60 F 254 S Merck, Darmstadt, F.R.G.), using chloroform–hexane–acetic acid (40:40:20) as mobile phase. The eluted products were visualized under UV at 254 nm. The two principal bands on the silica gel, corresponding to flunixin and its major metabolite, were separately scraped off and eluted with acetone. The solvent was then filtered and evaporated under reduced pressure at room temperature. The dry residue was extracted five times with 0.5 ml of acetone, and the extracts were recombined.

A 100-μl volume of each acetonic solution was evaporated to dryness under a nitrogen stream at 60 °C. Each of the two residues was then dissolved in 100 μl of Methelute Pierce® (25%; Ref. 49301, Spiral, Dijon, France), used as methylating agent to form the methyl derivatives for gas chromatographic–mass spectrometric (GC–MS) analysis. The remaining acetonic fraction obtained from the metabolite band was evaporated to dryness under a nitrogen stream at 60 °C; then the dry product was analysed.

Plasma sample preparation. A plasma sample (2.5 ml) was adjusted to pH 1 with 1 *M* hydrochloric acid, and extracted twice with 5 ml of diethyl ether. The ether phase, separated after centrifugation, was evaporated under a nitrogen stream at 60 °C. The dry residue was then reconstituted with 100 μl of Methelute Pierce (25%) and analysed by GC–MS.

Identification of flunixin in urine. A 1-μl volume of the methylated sample was injected into the gas chromatograph of the GC–MS system (Ribermag R-10-10). The chromatograph was equipped with a 25 m capillary column (SE 30, Spiral).

Operating temperatures were as follows: injector, 260°C; oven, from 165 to 210°C at 3°C/min; interface, 250°C; source, 120°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 50 to 450 a.m.u. at a rate of 0.5 a.m.u./ms. Flunixin in the extract was identified by comparison with the mass spectrum of an analytical standard.

Identification of the metabolite in urine. A 1-μl volume of the methylated sample was analysed by GC–MS under the same conditions. The dry product obtained by thin-layer purification was analysed by IR spectroscopy (sample compressed with KBr, Beckman Acculab 3 IR spectrophotometer) and by ^1H NMR spectroscopy (sample [$^2H_6$]acetone, Cameca 350-MHz spectrometer).

Flunixin spectra were plotted under the same conditions to allow comparison. A dry product aliquot was also characterized with the Folin-Ciocalteu reagent [4].

Identification of the metabolite in plasma. A 1-μl volume of the methylated sample was analysed by GC–MS under the same conditions.

Urinary elimination of flunixin and its metabolite

Sample preparation. A 2-ml urine sample was adjusted to pH 12 with 2.5 *M* sodium hydroxide, then centrifuged. The supernatant was separated, adjusted to pH 1 with 1 *M* hydrochloric acid, and extracted twice with 5 ml of diethyl ether. The ether layer was purified by shaking for 3 min with 1 ml of a saturated sodium bicarbonate solution. The organic phase was aspirated off and evaporated to dryness under a nitrogen stream at 60°C. The sample was reconstituted with 1 ml of methanol–diethyl ether (1:1). The solvent was then removed under a nitrogen stream at 60°C, and the dry residue was dissolved in 100 μl of Methelute Pierce (25%).

Analysis. A 1-μl volume of the methylated sample was analysed by GC–MS, under the same conditions as methylated urine extracts. Flunixin and its metabolite were identified by their mass spectra. Reconstructed ion chromatograms were plotted for the selected ions *m/z* 263, 295, 310, 323, 340 and 354, and for their sum. These ions correspond to fragments observed in the spectra of flunixin and its metabolite (Fig. 2).

RESULTS

Identification of the metabolite in urine

Mass spectrometry. The metabolite was first identified with the normalized and background-subtracted mass spectra of its dimethylated and trimethylated derivatives. These spectra were compared with that of the monomethylated flunixin derivative (Fig. 2).

The retention time of the dimethylated metabolite derivative is 11 min 6 s. Fig. 3 shows the various fragmentation pathways of this product. An interesting ion appears at *m/z* 293. It could be formed by the loss of CH_3OH by the "*ortho* effect" involving ester and secondary amine functions, after molecular ion demethylation (Fig. 4). This fragmentation would suggest that the hydroxy function of the

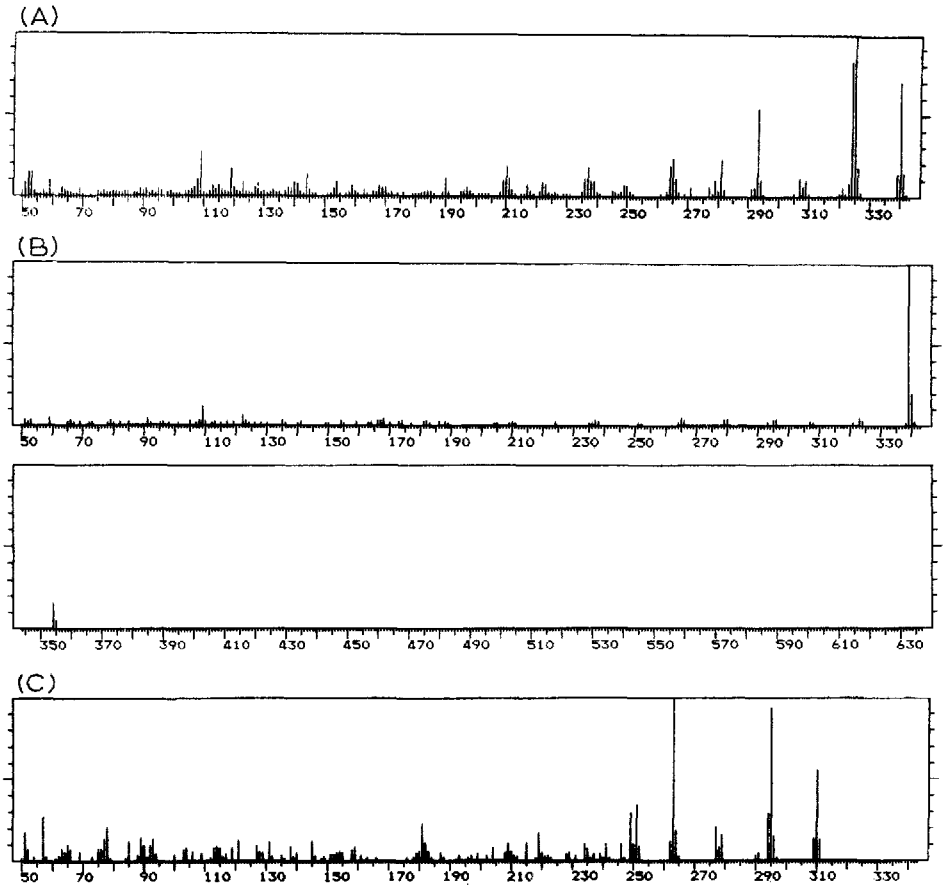

Fig. 2. Mass spectra of the dimethylated (A) and trimethylated (B) flunixin hydroxy metabolite derivatives, compared with that of the monomethylated flunixin derivative (C).

metabolite has been methylated in spite of the presence of the amino group. Ion *m/z* 293 can then lose CO, resulting in ion *m/z* 265 (Fig. 4).

Another ion at *m/z* 281 results from intramolecular cyclization of the molecular ion, with loss of $COOCH_3$. This fragmentation pathway, described previously for niflumic acid [5], seems to agree with the presence of ion *m/z* 251 in the mass spectrum of the monomethylated flunixin derivative (Fig. 3A).

The retention time of the trimethylated metabolite derivative is 7 min 55 s. The main fragments are shown in Fig. 3B.

Therefore, the mass spectra suggest the presence of a phenolic-type function in the structure of the metabolite, but they do not indicate whether the hydroxy group is in a pyridinic or a benzenic position. Indeed, unlike niflumic acid and its hydroxy metabolites [8], the loss of a substituted phenyl fragment by cleavage of the α position of the secondary amino group does not seem to occur.

^1H NMR spectroscopy. The aromatic proton chemical shifts of flunixin are given in Table I. The low chemical shift of the H 6 proton might be attributed to the diamagnetic anisotropy of nitrogen in the pyridine ring, in a favourable confor-

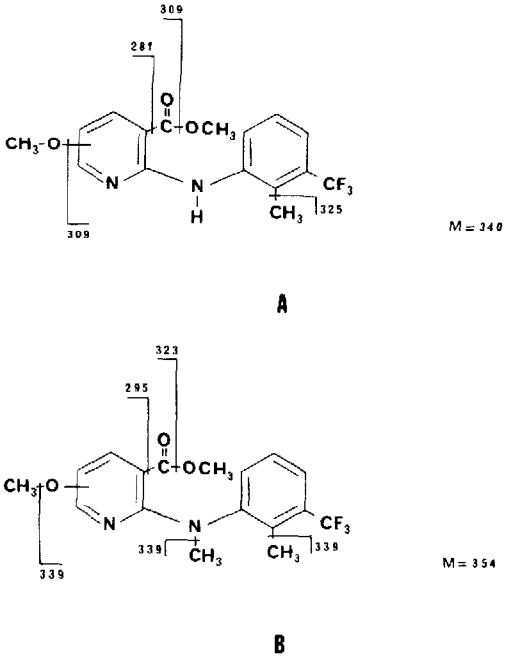

Fig. 3. Fragmentation of the dimethylated (A) and trimethylated (B) flunixin hydroxy metabolite derivatives.

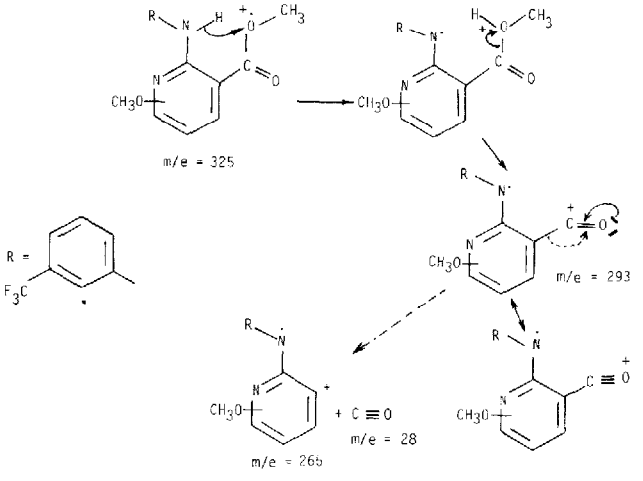

Fig. 4. Fragmentation pathway of the dimethylated metabolite derivative, showing an *ortho* effect.

mation according to radiocrystallography data of a structural analogue of flunixin, niflumic acid [6].

The metabolite was identified as 5-hydroxyflunixin by the different chemical shifts of the aromatic protons H-4 and H-6, which appear at δ 7.5 ppm. Taking the unsubstituted compound as reference, the theoretical values of these protons have been calculated by adding the difference in chemical shift induced by the hydroxy group at C-5 [7].

TABLE I

CHEMICAL SHIFTS IN THE ^1H NMR SPECTRUM OF FLUNIXIN IN DEUTERATED ACETONE (AROMATIC PROTONS ASSIGNMENTS)

Atom	δ (ppm)*
(C-4) H	8.30 (dd, 1 H, J (H-4-H-5) = 7.4 Hz)
(C-5) H	6.85 (dd, 1 H, J (H-5-H-6) = 4.9 Hz)
(C-6) H	8.37 (dd, 1 H, J (H-4-H-6) = 1.9 Hz)
(C-4′) H and (C-5′) H	7.35 (d , 2 H, J (H-4′-H-5′) = 4.3 Hz)
(C-6′) H	8.69 (t , 1 H)

*d = doublet; dd = doublet doublet; t = triplet.

IR spectroscopy. The main absorption bands present in the spectrum of flunixin (Table II) also appear in the spectrum of the hydroxy metabolite. Moreover, a strong band at ca. 3500 cm^{-1} is indicative of a hydroxy function.

Characterization by a chemical method. The phenolic nature of the metabolite was demonstrated by its positive reaction with the Folin-Ciocalteu reagent. This assay has been also used to characterize a phenolic metabolite of niflumic acid [8].

However, some signals in the NMR spectrum of the hydroxy metabolite show the presence of another product, which is now the subject of study.

Identification of the metabolite in plasma

The presence of the identified metabolite in plasma was demonstrated. During antidoping control, the same mass spectra were obtained as those plotted from urine extracts.

Urinary elimination of flunixin and its hydroxy metabolite

Elimination of the two products was followed qualitatively by GC–MS, with reconstructed ion chromatograms and mass spectra. The chromatograms plotted on the sum of the ions at 4, 54 and 175 h are shown in Fig. 5.

During the selected period, the intensities of the peaks corresponding to flu-

TABLE II

INFRARED SPECTRUM OF FLUNIXIN

Absorption band, ν (cm^{-1})	Assignment
3240	N-H
2500	C-H (aromatic)
2000-3100 (strong)	OH (COOH)
1670	C=O (COOH)
1440, 1500 and 1580	C=C (aromatic)
1230	C-O (COOH)
1300, 1160 and 1120	CF_3

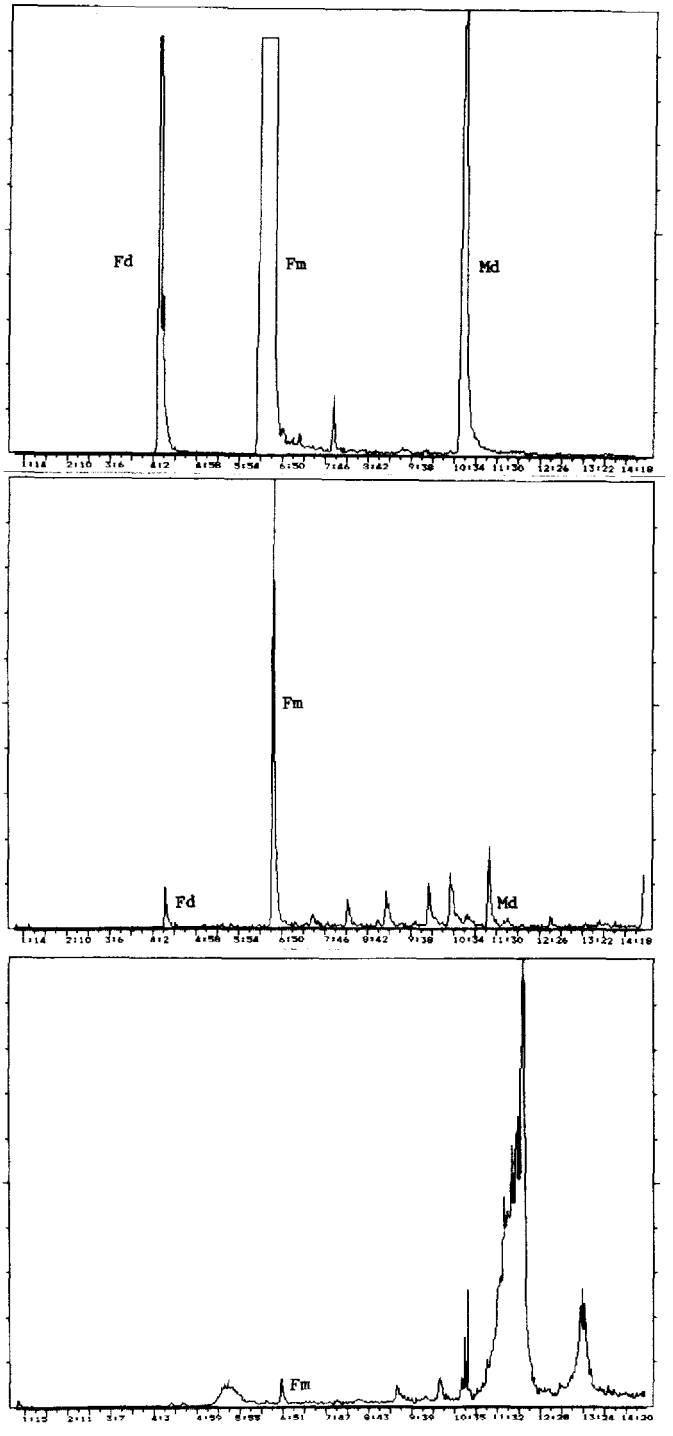

Fig. 5. Reconstructed ion chromatograms plotted at 4, 54 and 175 h on the sum of the ions, showing peaks of the monomethylated flunixin derivative (Fm), the dimethylated flunixin derivative (Fd) and the dimethylated metabolite derivative (Md). Retention times are given in minutes and seconds.

nixin and its metabolite decrease from 4 to 175 h. Flunixin can be detected at up to 175 h and the metabolite at up to 54 h. After these times, the mass spectra contain many interferences and low-abundance ions.

CONCLUSION

Oxidative flunixin metabolism in the horse seems very similar to that of niflumic acid in humans and dogs [8, 9]. In these species, niflumic acid is partly converted into hydroxylated metabolites at C-4′ or C-5. Therefore, we note some differences from the other structural analogue clonixin [10]. It does not seem that the flunixin methyl group is converted into a carboxylic and/or hydroxymethyl function. The GC–MS study has not indicated the existence of such metabolites.

These initial data showed that the maximum time available for detection of flunixin in urine by MS is 175 h. It is also apparent that in antidoping control, simultaneous detection of flunixin and its 5-hydroxy metabolite can give an approximate value of the administration time as less than 54 h before urine sampling.

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