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Identification of a flunixin metabolite in camel by gas chromatography–mass spectrometry

I.A. Wasfi*, A.A.A. Hadi, Nawal A. Alkatheeri, Ishar M. Barezaiq, M. ElGhazali, N.S. Boni, O. Zorob

Camelracing Forensic Laboratory, Forensic Science Laboratory, P.O. Box 253, Abu Dhabi, United Arab Emirates

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Abstract

A flunixin metabolite, a hydroxylated product, has been identified in camel urine and plasma samples using gas chromatography–mass spectrometry (GC–MS) and GC–MS–MS in the electron impact and chemical ionization modes. Its major fragmentation pattern has been verified by GC–MS–MS in daughter ion and parent ion scan modes. The method could detect flunixin and its metabolite in camel urine after a single intravenous dose of 2.2 mg of flunixin/kg body weight for 96 and 48 h, respectively, which increases the reliability of antidoping control analysis.

Keywords: Flunixin

1. Introduction

Flunixin is a potent non-steroidal anti-inflammatory drug (NSAID) which acts by inhibiting the synthesis of prostanoids and their unique effect is mediated through prostaglandins, thromboxanes or leukotrienes [1]. Flunixin has been used extensively to treat a number of conditions in veterinary medicine [2–5]. Its pharmacokinetics has been studied in horses, cattle, dogs, cats and camels [6–11]. Identification of a phase 1 flunixin metabolite has been reported in the horse [12] and the dog [13] but not in camel.

The objective of the current study was to identify a phase 1 flunixin metabolite in the camel, verify its major fragmentation pattern and to establish the detection time for flunixin and its metabolite in urine following intravenous (i.v.) administration. The latter piece of information would be helpful for camel trainers and veterinarians as this would enable them when to discontinue flunixin use before racing in order to avoid penalties imposed by the camelracing commissioner if a camel tests positive for a foreign substance.

2. Experimental

2.1. Drug administration and sample collection

Four clinically healthy male camels, 8–10 years old and ranging in body weight from 400–550 kg were used. They were kept in open pens. None had received any drug for at least eight weeks prior to the

*Corresponding author.

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study. Good quality hay and Lucerne were fed once daily and water was provided ad libitum. Flunixin meglumine (Finadyne, 50 mg/ml, Schering-Plough, Sante Animal, France) was administered as a bolus i.v. dose of 2.2 mg/kg of body weight. Blood samples (10 ml) were collected from the opposite jugular vein at hourly intervals for 60 h. The blood samples were allowed to clot, serum was separated by centrifugation (2000 *g* for 10 min) and was stored at −20°C pending analysis within 10 days. Voided urine samples were collected in fractions for 12 h as reported previously [14] and then daily at 8 a.m. for 12 days and immediately stored at −20°C pending analysis within 10 days.

2.2. Identification of flunixin metabolites by GC–MS and by GC–MS–MS

2.2.1. Urine sample preparation

The procedure used by Jaussand et al. [12] was modified as follows: a 10-ml urine sample, 7 h after flunixin administration, was adjusted to pH 12.0 with NaOH (2.5 *M*) and was left for 15 min. The sample was centrifuged for 10 min at 2000 *g* and the supernatant was separated and adjusted to pH 1.0 with 6.0 *M* HCl and was extracted twice with 10-ml aliquots of diethyl ether. The ether aliquots were combined and were added to 10 ml aqueous saturated sodium bicarbonate solution and was shaken for 3 min. The ether phase was then separated, dehydrated with anhydrous sodium sulfate and was evaporated to dryness at 45°C under a stream of nitrogen. The residue was dissolved in 1 ml diethyl ether and was then purified by use of thin-layer chromatography on silica gel plates (60 F 254, E. Merck, Darmstadt, Germany) using a mobile phase of chloroform–hexane–acetic acid (40:40:20). The eluted products were visualized under UV light at 254 nm. Two major bands were observed, one corresponding to flunixin (R_F=0.45) and the other

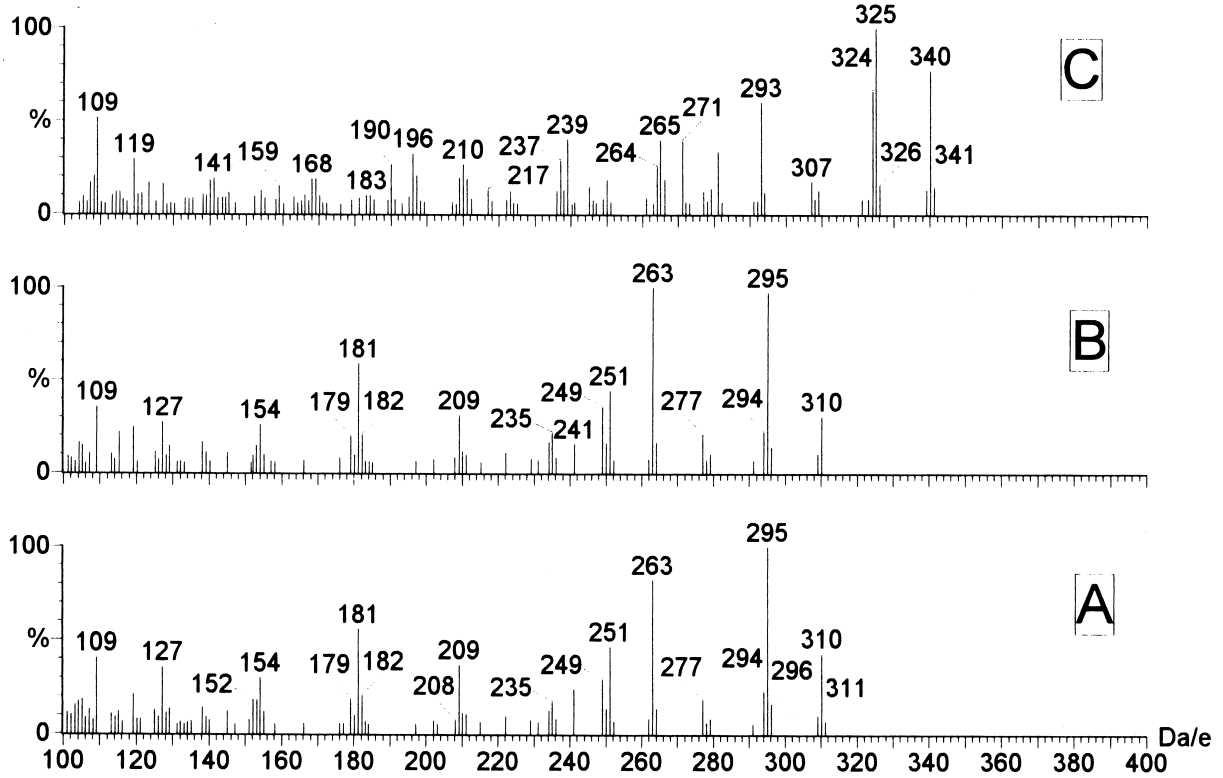

Fig. 1. Positive EI mass spectrum of methylated flunixin isolated from a camel urine 7 h after i.v. administration of flunixin at a dose of 2.2 mg/kg/body weight (A), flunixin standard (B), hydroxy flunixin metabolite (dimethylated, C). The latter was isolated from i.v. treated camel with flunixin (2.2 mg/kg body weight). The drug and metabolite were first purified using thin-layer chromatography.

with a lower R_F value (0.30) as major flunixin metabolites. These bands were separately scraped off and were eluted twice with 5 ml acetone. The solvent was then filtered and evaporated at 45°C under a gently stream of nitrogen. The residues were then methylated by dissolving in 100 μl of methelute (Pierce, Rockford, IL, USA) which is used as an on column methylating agent to form methyl derivatives for GC–MS and GC–MS–MS analysis.

The system used was a tandem mass spectrometer (Quattro, Fisons, Manchester, UK) interfaced to a gas chromatograph (GC 8060 MS, Milan, Italy). Injections were made in the splitless mode onto a 30 m×0.25 mm I.D. HP-5MS (Hwelett-Packard, Palo Alto, CA, USA). The initial column temperature was 75°C and was programmed at 25°C/min to 290°C and the final temperature was maintained for 5 min. Injection and interface temperature were 250°C and 280°C, respectively. Helium was used as a carrier gas at a flow-rate of 20 cm/s. In the electron impact (EI) mode, spectra were obtained at 70 eV and scanned from 100 to 400 a.m.u. at a scan rate of 1.88 a.m.u./ms. The source temperature was 180°C. In chemical ionization (CI) positive mode, spectra were obtained at 30 eV and methane was used as a reagent gas at a source pressure of $1.0 \cdot 110^{-4}$ Bar. The source temperature was 150°C. When data were obtained in EI positive MS–MS modes (neutral loss, daughter and parent scans) argon was used as a collision gas at analyser pressure of $1.1 \cdot 10^{-5}$ mBar. A collision energy of 15 eV was used.

2.2.2. Serum sample preparation

A serum sample (5 ml) was extracted and derivatized as reported elsewhere [12] and was analyzed as described in Section 2.2.1.

2.2.3. Determination of detection time

One of the objectives of this study was to determine the withdrawal time for flunixin in racing camels. This was done by collecting urine samples from treated camels daily at 8 a.m. for 12 days. These urine samples were then subjected to our routine procedure for screening acidic and neutral drugs in post race urine samples by extractive methylation [16] and were analyzed by GC–MS as

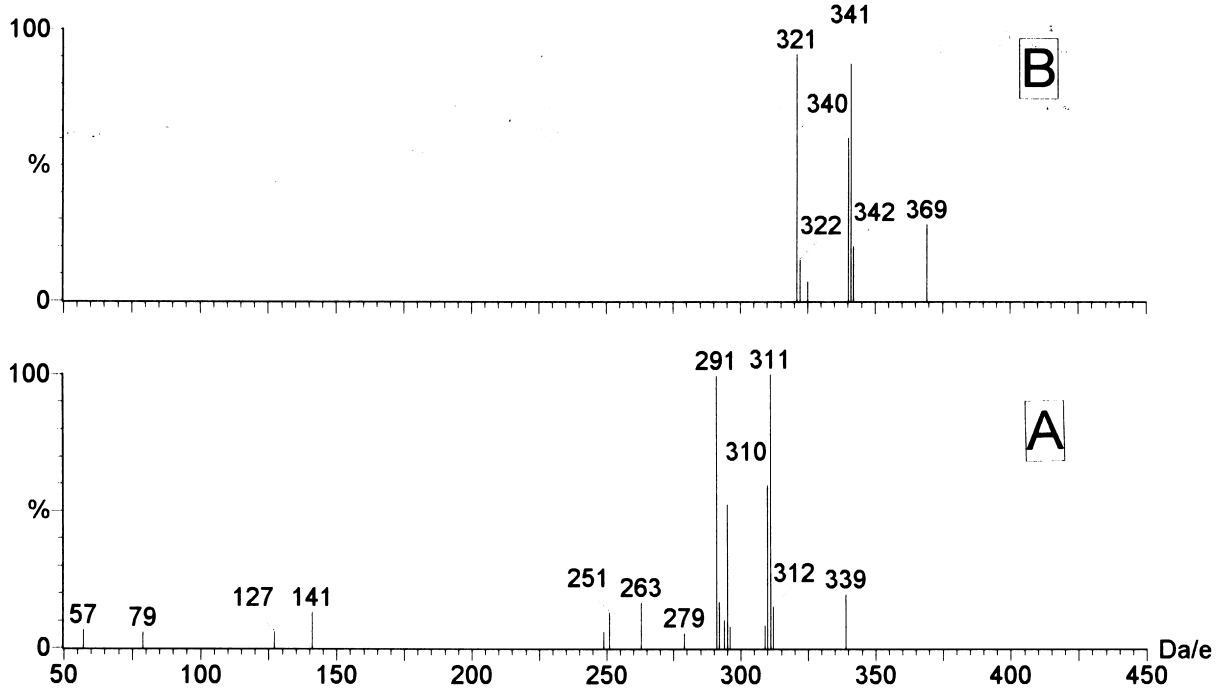

Fig. 2. Positive CI mass spectrum of methylated flunixin (A), and hydroxy flunixin (B) isolated from camel urine after treatment with an i.v. dose of flunixin (2.2 mg/kg body weight). The drug and metabolite were first purified using thin-layer chromatography.

follows: The initial column temperature, 70°C, was maintained for 1 min. The oven was programmed at 15°C/min to 290°C which was maintained for 5 min. Data was acquired in selective ion mode (EI), monitoring three to five ions for each compound in selected time windows according to their retention times. The ions monitored for flunixin were *m/z* 310, 295 and 263 and for flunixin metabolite, were *m/z* 340, 325 and 293.

3. Results and discussion

The spectrum (Fig. 1A) of the eluted band with the higher R_F value compared well with the spectrum of methylated flunixin standard (Fig. 1B) with a retention time of 9.07 min which is further confirmed with a positive CI scan where there is an intense *m/z* 311 [M+1] ion (Fig. 2A). The major EI fragmentation pathway involved demethylation of the methylated molecular ion (*m/z* 310) resulting in ion *m/z* 295 and then a loss of ion 32 a.m.u. to form ion *m/z* 263 with subsequent loss of 28 a.m.u. giving ion *m/z* 235. A neutral loss scan of *m/z* 32 was then performed on the eluted band with the lower R_F value (metabolite). One major peak at retention time 10.43 min was observed. The EI spectra of this peak showed the ions *m/z* 340, 325 and 293 which is suggestive of hydroxylated flunixin (Fig. 1C). In CI scan mode (Fig. 2B) the pseudomolecular ion at *m/z* 341 is due to [M+1] while the ion at *m/z* 369 is due to [M+H+C_2H_4] adduct. The MS–MS scan modes

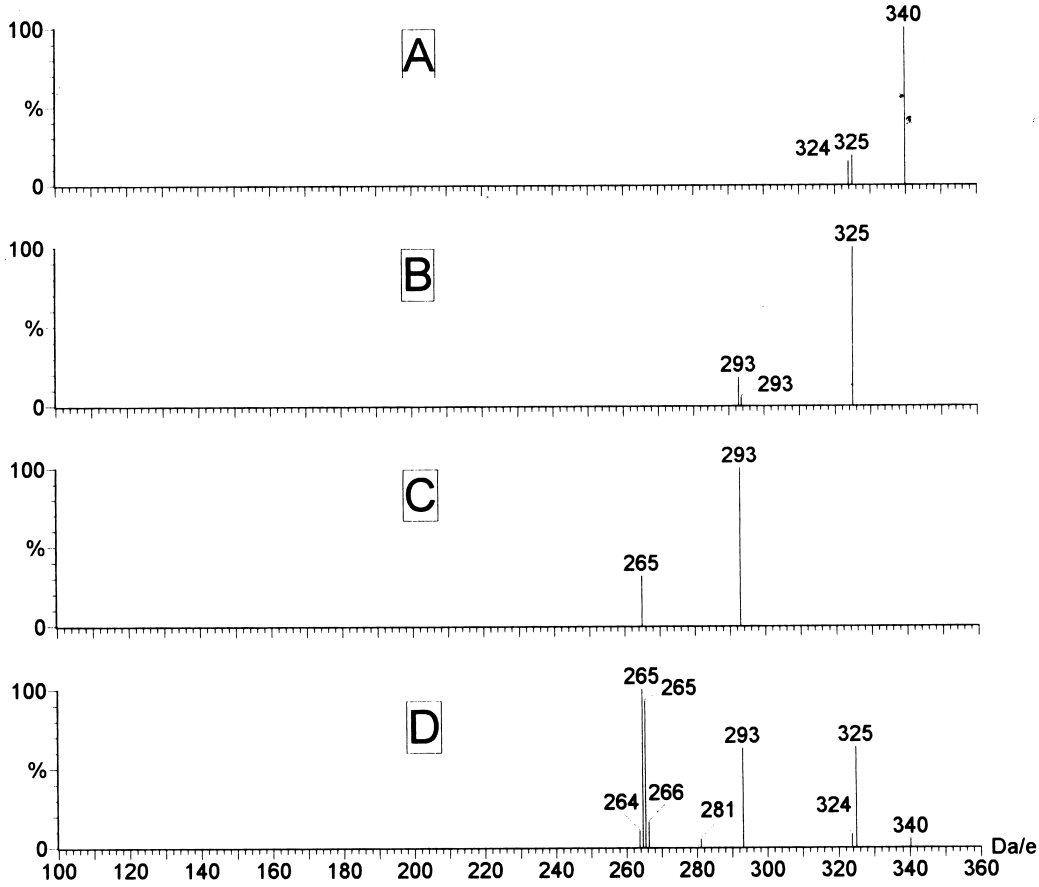

Fig. 3. Positive EI daughter scan of the molecular ion of methylated hydroxy flunixin metabolite (*m/z* 340) showing loss of CH_3 group to *m/z* 325 (A), daughter scan of *m/z* 325 showing loss of CH_3OH to *m/z* ion 293 (B), daughter scan of *m/z* 293 showing loss of CO to ion *m/z* 265 (C) and a parent scan of *m/z* 265 confirming the fragmentation pathway pattern (D).

(neutral loss, parent and daughter) have clearly demonstrated that the major fragmentation pattern of flunixin metabolites are similar to that of flunixin viz.; demethylation of the molecular ion, neutral loss of 32 a.m.u. followed by a neutral loss of 28 a.m.u. As an example a daughter ion scan of the molecular ion of the dimethylated metabolite (*m*/*z* 340) resulted in a daughter of *m*/*z* 325 (Fig. 3A). Then a daughter ion scan of ion *m*/*z* 325 gave ion *m*/*z* 293 (Fig. 3B) and a daughter ion scan of ion *m*/*z* 293 gave ion *m*/*z* 265 (Fig. 3C). Finally a parent scan of ion *m*/*z* 265 confirmed the sequential major fragmentation pathway of the metabolite (Fig. 3D). The suggested major EI fragmentation pattern of the dimethylated metabolite is shown in Fig. 4.

Using our routine method for screening post race urine samples, we were able to detect flunixin and its major metabolite (hydroxy flunixin) for 96 and 48 h, respectively (Fig. 5).

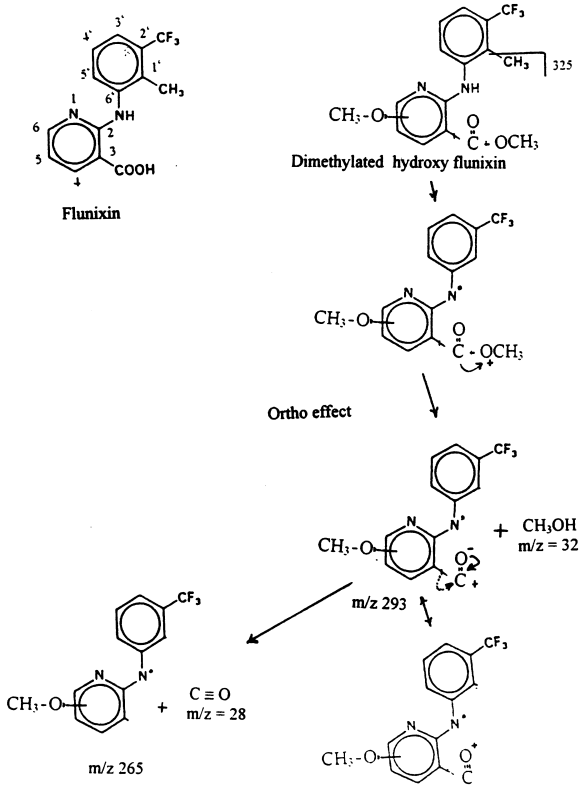

Fig. 4. Flunixin and methylated dihydroxy flunixin showing *ortho* effect and major EI fragmentation pattern of the latter.

The utility of MS–MS for rapid identification of drug metabolites proved to be quick and efficient. This is based on the fact that most drug metabolites retain much of the original drug molecular structure or some substructures of the drug [17]. This was clearly demonstrated in this study in which the fragmentation pattern of pure flunixin standard was used to identify flunixin metabolite in camels. By performing a series of daughter and parent ion scans, the major fragmentation pattern was identified and found to be similar to that of flunixin, namely, demethylation, followed by loss of 32 a.m.u. (CH_3OH), most probably due to the ''*ortho* effect'' involving ester and secondary amine functions, and then loss of CO (28 a.m.u.). The exact location of the hydroxyl group whether on the pyridine or benzene ring cannot be ascertained from this study. This, however could be determined by use of ^1H NMR spectroscopy. Thus flunixin metabolism in camels was found to be similar to that reported in horses [12]. It appears that camels eliminate flunixin by two pathways. The first one being direct conjugation of flunixin with glucuoronic acid, possibly a C-1 β-configuration on C-1 glucuronic acid [15]. The second pathway of elimination is by oxidizing flunixin with subsequent elimination. The first pathway seems to be the major one because the relative concentration of free flunixin was greater than that of flunixin metabolite in hydrolysed urine sample. In addition we were able to detect flunixin and flunixin metabolite in urine for 96 and 48 h, respectively after i.v. administration of flunixin. Clearly the simultaneous detection of flunixin and its metabolite increases the reliability of anti-doping control analysis.

Because the camelracing commissioner in UAE has adopted a zero medication regulation, veterinarians and camel trainers would be advised to discontinue flunixin administration for a minimum period of five days before racing.

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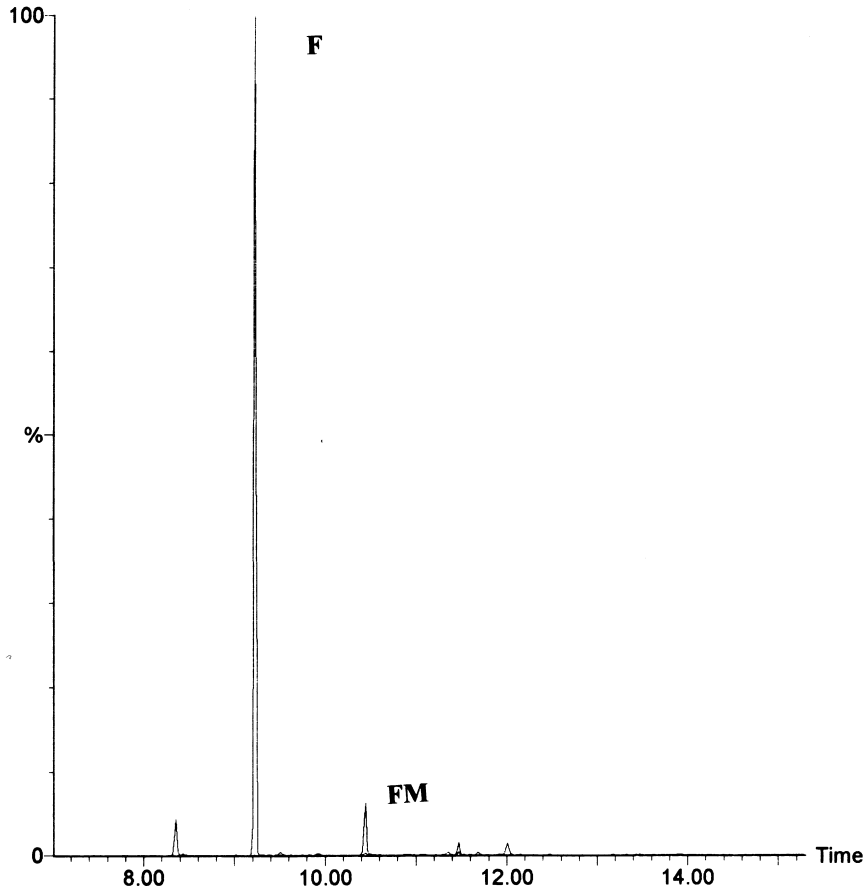

Fig. 5. Reconstructed ion chromatogram (SIM mode) of urine 48 h after flunixin administration. Urine was extracted by use of our routine screening procedure for acidic and neutral drugs. F, flunixin; FM, flunixin metabolite.

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