ELSEVIER

Journal of Chromatography B, 732 (1999) 299–306

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Identification and confirmation of 3-hydroxy metabolite of ketoprofen in camels by gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy

Nawal A. Alkatheeri[a], I.A. Wasfi[a,*], P. Kvanagh[b], M. Lambert[b]

[a]*Camelracing Forensic Laboratory, Forensic Science Laboratory, P.O. Box 253, Abu Dhabi, United Arab Emirates*
[b]*Department of Pharmacology and Therapeutics, Trinity College, Dublin 2, Ireland*

Received 23 November 1998; received in revised form 25 May 1999; accepted 22 June 1999

Abstract

The metabolites of ketoprofen were investigated in five camels following intravenous administration of a dose of 2.0 mg/kg body weight. Two metabolites were identified. The first one was purified with thin-layer chromatography. It was identified by gas chromatography–mass spectrometry (GC–MS) in comparison with authenticated reference standard and was found to be hydroxyketoprofen due to reduction of the ketone group of ketoprofen. The second metabolite was purified by high-performance liquid chromatography. It was identified with GC–MS and nuclear magnetic resonance spectroscopy as 3-hydroxybenzolketoprofen resulting from oxidation of the aromatic ring. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Ketoprofen; 3-Hydroxyketoprofen; 3-Hydroxybenzolketoprofen

1. Introduction

Ketoprofen, 2-(3-benzolphenyl)propanoic acid, is a non-steroidal anti-inflammatory drug (NSAID) used in human and veterinary medicine [1,2]. As with other NSAIDs, the primary mechanism of action is believed to be via inhibition of the cyclo-oxygenase [3] and the lipoxygenase pathways [4]. Its pharmacokinetics has been studied in horses, cattle, donkeys, camels, rats and humans [5–11]. We have found that ketoprofen in camels is eliminated in the free and conjugated form, possibly with glucuronide, and as a hydroxy metabolite due to reduction of the ketone group [12]. These elimination pathways, however, account for only a small portion of the administered dose which suggest the existence of other metabolic pathway(s). In laboratory animals and man, hydroxylation of the aromatic ring of ketoprofen to 3- and 4-hydroxybenzolketoprofen has been reported [13].

The purpose of the present study was to investigate if hydroxylation of the aromatic ring occurs in camels. Clearly, in antidoping control, simultaneous detection of a metabolite(s) with the parent compound increases the reliability of antidoping control analysis.

*Corresponding author. Fax: +971-463-470.
E-mail address: iawasfi@emirates.net.ae (I.A. Wasfi)

0378-4347/99/$ – see front matter © 1999 Published by Elsevier Science B.V. All rights reserved.
PII: S0378-4347(99)00297-2

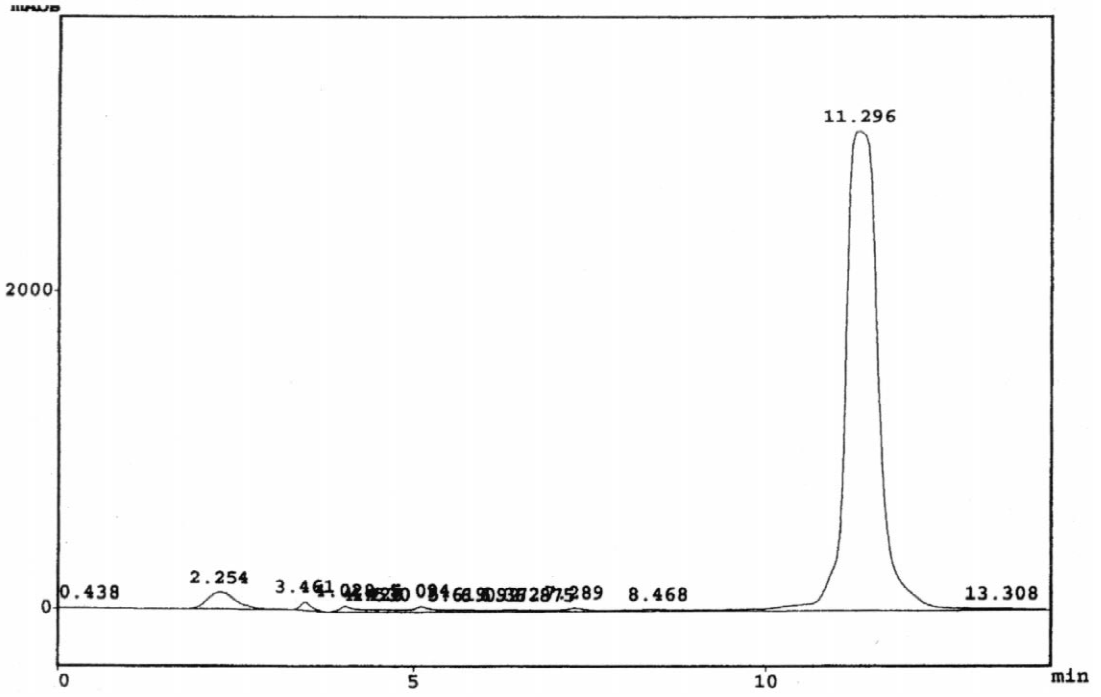

Fig. 1. HPLC chromatogram of the 3- or 4-hydroxybenzolketoprofen peak isolated from extracted camel urine after i.v. administration of ketoprofen dose of 3 mg/kg body weight.

2. Experimental

2.1. Drug administration and sample collection

Five male camels, 8–10 years old and weighing 300 to 450 kg were used. They were kept in open pens. Good quality hay and Lucerne were fed once daily and water was provided ad libitum.

Ketoprofen (ketoprofen, 100 mg/ml, Nature Vet, Richmond, Australia) was administered as a bolus intravenous (i.v.) dose of 2.0 mg/kg of body weight, urine samples were collected in fractions for 24 h as reported previously [14] and immediately stored at −20°C. The samples were freeze–dried within seven days.

2.2. Purification and identification of hydroxyketoprofen

This was done as reported earlier [15,16]. Briefly, a 10.0-ml urine sample, 6 h after ketoprofen 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 3.0 with 2.0 *M* HCl and was extracted twice with 10.0-ml aliquots of diethyl ether. The ether aliquots were combined and were added to 10.0 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 (60:40:15), the eluted products were visualized under UV light at 254 nm.

2.3. Purification and identification of 3-hydroxybenzolketoprofen by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS)

A 24 g amount of the freeze–dried urine sample was dissolved in 200 ml distilled water. The pH was

adjusted to 5.0 with acetic acid and 20 000 units of β-glucuronidase (Sigma, St. Louis, MO, USA) were added and the whole was incubated for 3 h at 50°C. The pH was then adjusted to 3.0 with 2.0 *M* HCl and was extracted three times with 200 ml dichloromethane. The organic layers were combined after centrifugation for 10 min at 2000 *g* and the solvent was concentrated in vacuum at 40°C using a rotary evaporator. The obtained residue was used for HPLC separation.

2.4. HPLC separation of the ring hydroxylated metabolite

The HPLC system consisted of a Shimadzu pump (LC-10A) equipped with a UV–Vis detector (SPD-10A). Sample residues (25 mg) were dissolved in 100 μl of the mobile phase (0.2% acetic acid–acetonitrile, 65:35) and were analyzed on a JVA analytical ODS C_{18} column (25 cm×4.6 mm I.D.). The flow-rate was 0.7 ml/min and the detector was monitoring at 255 nm. Metabolites were isolated by collecting eluents as they eluted off the column, then evaporated under a stream of nitrogen at 40°C (Fig. 1).

Tentative identification of the metabolite was made by GC–MS analysis following on-column methylation with methelute (0.2 *M* trimethylanilinium hydroxide in methanol; Pierce, Rockford, IL, USA). Several injections of the sample extract were made on the HPLC system to obtain sufficient amount of the metabolite for mass spectral identification. The fractions were then combined and were evaporated under a stream of nitrogen at 40°C. The residue was then dissolving in hexadeuterioacetone for nuclear magnetic resonance (NMR) spectral analysis.

2.5. GC–MS analysis

A small amount of the metabolite was dissolved in 100 μl methanol in addition to 20 μl methelute and 2 μl was analyzed on the GC–MS system. The system used was a Hewlett-Packard 5973 mass-selective detector interfaced to a HP (6890) gas chromatograph with HP6890 autoinjector and sample tray. Injection was made in the splitless mode onto a 30 m×0.25 mm I.D. DP-5MS column.

The initial column temperature was 90°C and was programmed at 15°C/min to 280°C, where the temperature was held for 6 min then was programmed to 300°C at 10°C min and the final temperature was maintained for 5 min. Helium was used as a carrier gas at a flow-rate of 1 ml/min. In the electron impact (EI) scan mode, spectra were scanned from 50 to 500 u.

2.6. NMR spectroscopy

NMR spectroscopy [^{1}H and total correlation spectroscopy (TOCSY)] was performed on a Bruker Avance DPX400 spectrometer (400 MHz) using hexadeuterioacetone as solvent. All chemical shifts are reported in ppm using tetramethylsilane as reference.

3. Results and discussion

3.1. Mass spectrometry

All mass spectra were background subtracted before evaluation.

Two metabolites of ketoprofen were isolated from camel urine. The first one, which confirms our previous findings [12], was a hydroxy metabolite resulted from reduction of the ketone group of ketoprofen. It gave identical chromatographic and spectral characteristics to authenticated reference standard (Fig. 2). Because of the derivatization nature of methelute, both the carboxylic and hydroxyl group are liable to methylation to give mono- and dimethylated hydroxyketoprofen with respective molecular ions of *m/z* 270 and *m/z* 284. Their retention times were 11.9 and 11.3 min, respectively. Hydroxyketoprofen has been reported in horses [6,17]. The reduction of ketones to alcohols is a common metabolic pathway for several compounds which has been reported in several species [18,19].

The second metabolite of ketoprofen appears to be due to oxidation of the aromatic ring to 3- or 4-hydroxybenzolketoprofen. Again a mono- and a dimethylated derivative would be expected with molecular ions of *m/z* 284 and *m/z* 298, respectively (Fig. 3). Their retention times were 14.0 and 13.3 min, respectively. Comparing the fragment ions *m/z* 107 and 135 *m/z* of the dimethylated metabolite (Fig. 3) with fragment ions *m/z* 77 and *m/z* 105 of

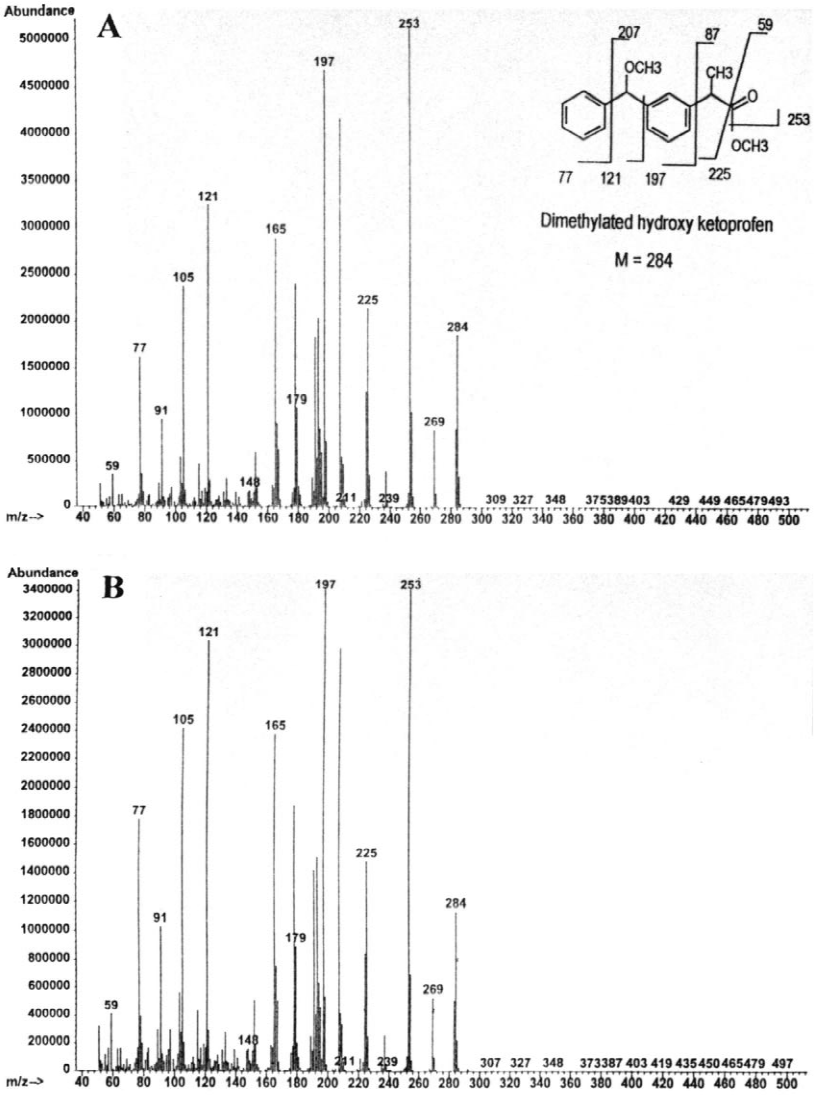

Fig. 2. Positive EI mass spectrum of dimethylated hydroxyketoprofen where (A) dimethylated hydroxyketoprofen standard and (B) dimethylated hydroxyketoprofen isolated from camel urine 6 h after i.v. administration of ketoprofen dose of 3 mg/kg body weight.

methylketoprofen (Fig. 4) suggest the oxidation of the aromatic ring of ketoprofen.

3.2. ^{1}H-NMR spectroscopy

The EI mass spectrum of the hydroxy metabolite, derivatized with methelute, clearly shows a molecular ion of m/z 298 (dimethylated) with fragment ions m/z 135 and 191 indicating that the hydroxy group is on ring B (Fig. 3B).

The position of the hydroxy group was elucidated using ^{1}H-NMR spectroscopy which showed (i) the presence of eight aromatic hydrogens in the molecule, consistent with mono-hydroxylation, and (ii) that the hydroxyl group was on the 3 position.

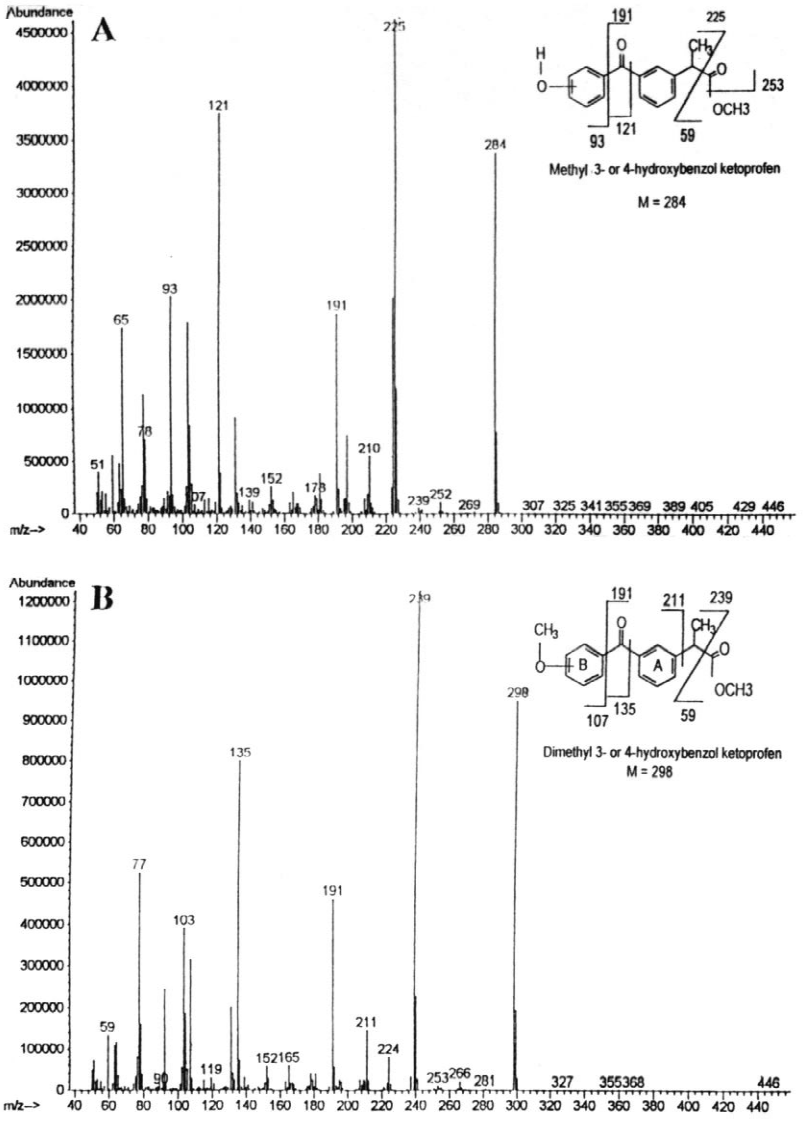

Fig. 3. Positive EI mass spectrum of methylated 3- or 4-hydroxybenzolketoprofen isolated from camel urine 6 h after i.v. administration of ketoprofen at dose of 3 mg/kg body weight, where (A) monomethylated and (B) dimethylated 3- or 4-hydroxybenzolketoprofen.

The $CH(CH_3)$ moiety was also observed to be intact. Structural assignments for the aromatic hydrogens were made using two-dimensional $^1H-^1H$ TOCSY. The triplet at 7.80 ppm ($J=2$ Hz, di-*meta* coupling) was assigned to H-2′. Hydrogens H-4′ and 6′ (both *ortho*/di-*meta*) are co-incidental in the spectrum and occur as a ''distorted triplet'' at 7.67 ppm. The di-*ortho* coupling for H-5 is evident in the triplet ($J=7$ Hz) at 7.53 ppm. All the assignments are shown in Table 1 and Fig. 5.

3.3. Overall ketoprofen metabolism in the camel

Results obtained from this study and from our previous work [12] show that the camel eliminates ketoprofen in urine in several ways (Fig. 6); in free

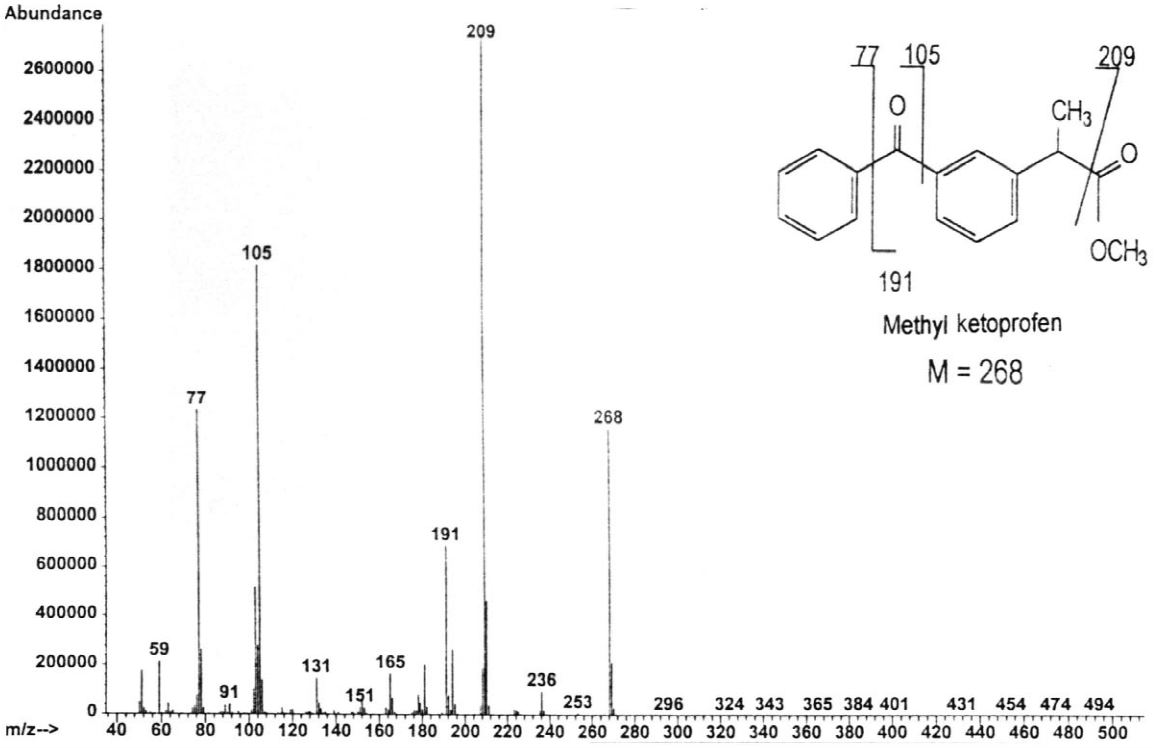

Fig. 4. Positive EI mass spectrum of methylated ketoprofen standard.

form and as a conjugate (glucuronide and/or sulfate). In addition to this, the camel metabolizes ketoprofen in two ways viz.; by reduction of the ketone group to form hydroxyketoprofen and by oxidation of the aromatic ring to form 3-hydroxybenzolketoprofen. Both of the latter metabolites, however, were present in low concentrations. These routes of elimination in urine, however, do not seem to account for the administered dose of ketoprofen which suggest the existence of other route(s) of

Table 1
^{1}H-NMR chemical shift of 3-hydroxybenzolketoprofen

Assignment	No. of hydrogens	Chemical shift (ppm)	Multiplicity	Coupling constants (Hz)
H-2′	1	7.80	Triplet	2
H-4′, H-6′	2	7.66	‘‘Distorted triplet’’	–
H-5′	1	7.53	Triplet	7
H-5	1	7.38	Triplet	7
H-2	1	7.28	Triplet	2
H-4 or H-6	1	7.24	Double triplet	7, 2
H-4 or H6	1	7.13	Double double doublet	7, 2, 7, 2
$\underline{CH}(CH_3)$	1	3.90	Quartet	9
CH_3	3	1.50	Doublet	9

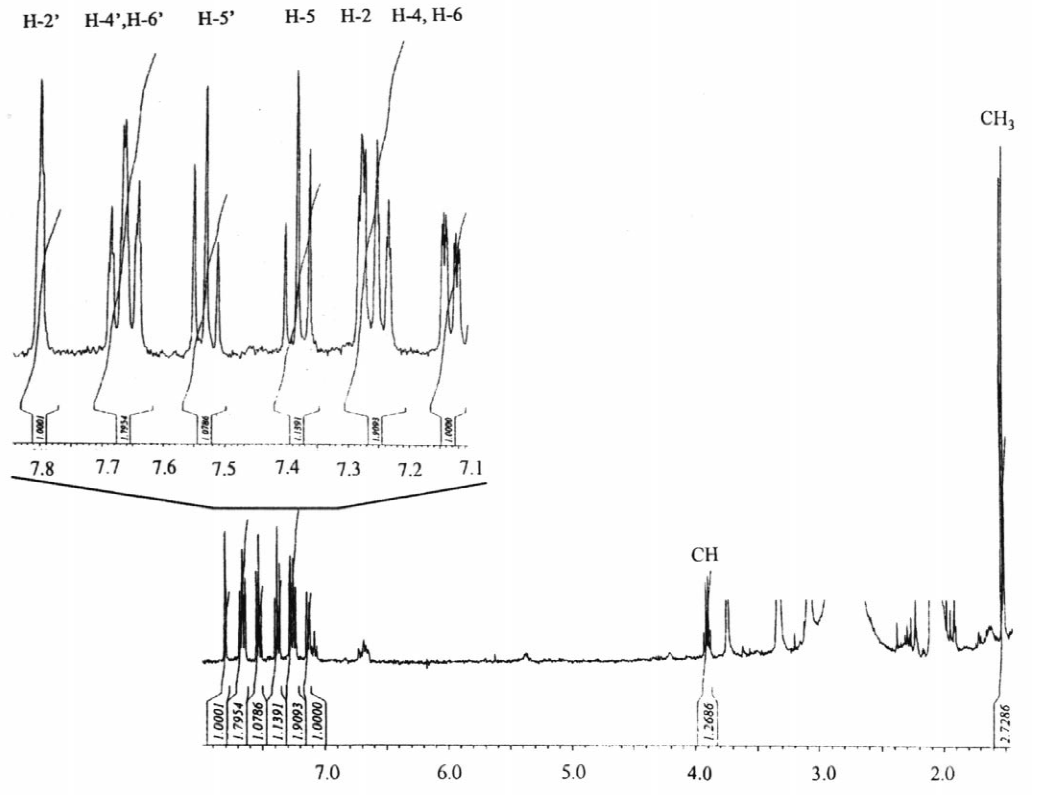

Fig. 5. ^{1}H-NMR of the 3-hydroxybenzolketoprofen.

elimination. Biliary excretion might be expected as it has been reported to be the major route of elimination of caprofen, a member of the 2-arylpropionic family, in dogs, rats, horses and humans [20,21]. In the latter species glucuronidation with subsequent biliary excretion was enantioselective for *S*-carprofen.

Simultaneous detection of a doping agent together with its metabolite(s) undoubtedly increases the reliability of antidoping control analysis. We have previously reported that we could detect ketoprofen and hydroxyketoprofen in camels for one and three days, respectively [12]. Although 3-hydroxybenzolketoprofen is a minor metabolite in camels, still it could be detected for one day after administration of ketoprofen which will add more reliability to positive cases. The detection of presumably polar metabolites in the camel is of particular importance. Because of the unique renal physiology and water conservation mechanism, these compounds appear to be eliminated in a slow manner. We have previously reported that we could detect the hydroxy metabolites of flunixin and tolfenamic acid for a period longer than the parent compounds themselves [16,22].

Acknowledgements

This study was supported by Lt. Col. Ibrahim Al-Howsani Director of Forensic Science Laboratory. The authors thanks Dr. B Agha and S. Mousa for technical assistance. Thanks are also due to Dr. Ismail Yousif at the tradition Reviving Society for housing and care of the camel and Mohammed Osman, Food and Environment Control Centre, Abu Dhabi for freeze–drying the samples.

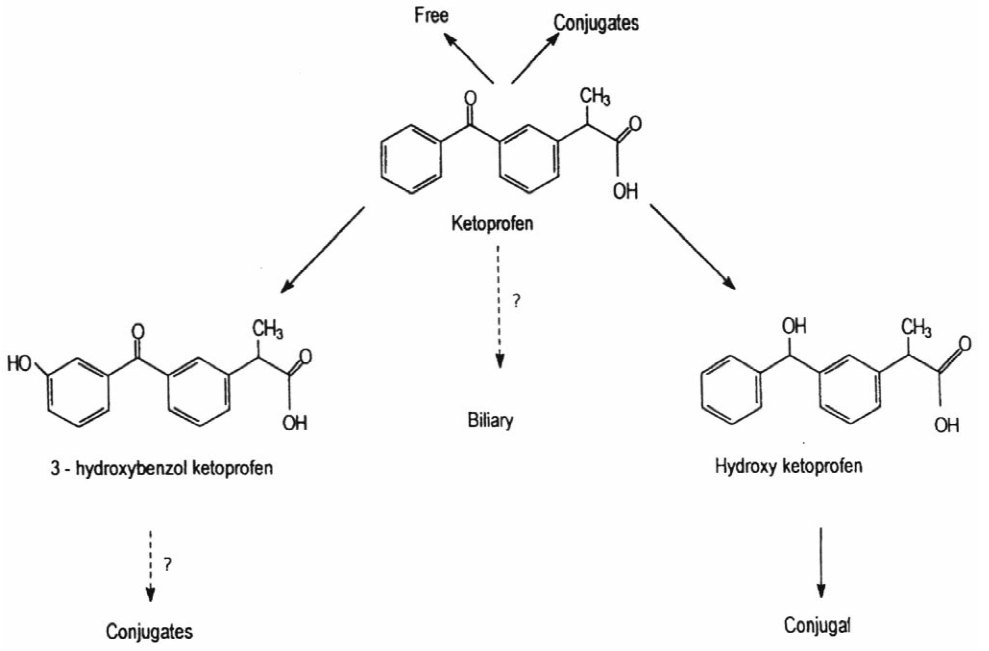

Fig. 6. Ketoprofen elimination pathways in camels after i.v. administration.

References

[1] M.G. Rurarius, P. Suominen, G.A. Baer, O. Romppanen, R. Tuimala, Br. J. Anaesth. 70 (1993) 293.
[2] J.G. Owens, S.G. Kamerling, S.R. Stanton, M. Keowen, Equine Vet. J. 27 (1995) 296.
[3] J.R. Vane, Nature (New Biol.) 231 (1971) 232.
[4] J.L. Walker, in: B. Samuelsson, P.W. Ramwell, R. Paoletti (Eds.), Advances in Prostaglandin and Thromboxane Research, Vol. 6, Raven Press, New York, pp. 115–119.
[5] R. Sams, D.F. Gerken, S.M. Ashraft, J. Vet. Pharmacol. Ther. 18 (1995) 108.
[6] M. Kurosawa, S. Yamashita, T. Tsuji, I. Ohtake, in: Proceedings of the 10th International Conference of Racing Analysts and Veterinarians, Stockholm, 1994, p. 17.
[7] F.J. DeGraves, M.G. Riddell, J. Schumacher, Am. J. Vet. Res. 57 (1996) 1031.
[8] M. Oukessou, F. Bouljihad, G.F. Van, M. Alvinerie, J. Vet. Med. 43 (1996) 423.
[9] M. Oukessou, R. Zin-Filali, G.F. Van, M. Alvinerie, Indian Vet. J. 72 (1995) 233.
[10] J.H. Satterwhite, F.D. Boudinot, Biopharm. Drug Dispos. 13 (1992) 197.
[11] P. Netter, B. Bannwarth, F. Lapicque, J.M. Harrewyn, A. Frydman, J.N. Tomisier, A. Gaucher, R.J. Royer, Clin. Pharmacol. 42 (1987) 555.
[12] N.A. Alkatheeri, I.A. Wasfi, M. Lambert, J. Vet. Pharm. Ther., in press.
[13] P. Populaire, B. Terlain, S. Pascal, B. Decouvelaere, A. Renard, J.P. Thomas, Ann. Pharm. Francaises 31 (1973) 735.
[14] I.A. Wasfi, A.A.A. Hadi, O. Zorob, M.O. Alghazali, N.S. Boni, Am. J. Vet. Res. 58 (1997) 636.
[15] P. Jaussaud, D. Courtot, L. Guyot, J. Chromatogr. 423 (1987) 123.
[16] I.A. Wasfi, A.A.A. Hadi, N.A. Alkatheeri, I.M. Barezaiq, M. Elghazali, N.S. Boni, O. Zorob, J. Chromatogr. B 709 (1998) 209.
[17] E. Benoit, P. Jaussaud, S. Besse, B. Videmann, D. Courtot, P. Delatour, J. Chromatogr. 583 (1992) 167.
[18] C. Behm, R. Cornish, C. Bryant, Res. Vet. Sci. 34 (1983) 37.
[19] C. Braithwaite, M. Roberts, R. Allan, T. Watson, Eur. J. Clin. Pharmacol. 22 (1982) 161.
[20] A. Soraci, E. Benoit, P. Jaussaud, P. Less, P. Delatour, Am. J. Vet. Res. 56 (1995) 358.
[21] F. Rubio, S. Seawall, R. Pocelinko, B. DeBarbieri, W. Benz, L. Berger, L. Morgan, J. Pao, T.H. Williams, B. Koechlin, J. Pharm. Sci. 69 (1980) 1245.
[22] I.A. Wasfi, M.A. Osman, A.A.A. Hadi, O. Zorob, N.S. Boni, N.A. Alkatheeri, I.M. Barezaiq, Am. J. Vet. Res. 59 (1998) 1451.