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Identification of a benzhydrolic metabolite of ketoprofen in horses by gas chromatography–mass spectrometry and high-performance liquid chromatography

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ABSTRACT

A benzhydrolic metabolite of ketoprofen, formed by reduction of the keto group of the drug, has been identified by gas chromatography–mass spectrometry in equine plasma and urine. After partial synthesis, its structure has been confirmed by UV, IR and ^{1}H NMR spectroscopy. The kinetics of ketoprofen and this metabolite have been monitored in plasma by high-performance liquid chromatography. The two products were quantified in plasma up to 4 and 3 h, respectively, and were detected in urine up to 72 and 24 h, respectively, after a single intravenous administration to horses at the dose of 2.2 mg/kg. Simultaneous detection of both compounds increases the reliability of antidoping control analysis.

INTRODUCTION

Ketoprofen [2-(3-benzoylphenyl)propionic acid] (Fig. 1) is a non-steroidal anti-inflammatory drug widely used in humans. It has been recently introduced in equine medicine, in the form of a solution injected intravenously. This drug is also used to dope horses because of its potent analgesic properties, which can mask lameness due to varied kinds of lesions. Its metabolism has been studied in humans, rats, dogs and rabbits after oral administration [1,2], but has not been described in equine species. Because of this lack of information, it was decided to explore the metabolism of ketoprofen in this animal species. Indeed, the simultaneous detection of the unchanged drug and metabolites can help to support a positive result during antidoping control procedures. The second aim of this study was to monitor the elimination of the metabolite from blood and urine. This procedure can define the period within which routine detection of the dop-

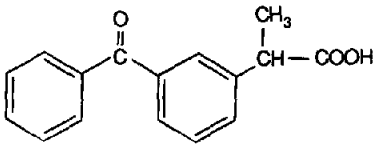

Fig. 1. Molecular structure of ketoprofen.

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ing agent and its metabolite in plasma and urine is possible.

As shown in a comprehensive review [3], ketoprofen is currently determined in biological extracts by GC, GC–MS or HPLC. If the drug is directly analysed by GC, partial decomposition results. This can be overcome by working with the methyl ester [4] or the trimethylsilyl ester [5], prepared quantitatively from ketoprofen. An OV-17 phase is often used [4–7]. GC–MS is also of great interest for dosing the compound in biological extracts [8–10]. HPLC analysis of ketoprofen is usually performed on a reversed phase [3,11]. Recently, this last method has been largely applied to separate ketoprofen enantiomers in plasma or urine extracts, using a chiral [12] or non-chiral [13] stationary phase. The choice of a non-chiral phase involves derivatization of the extracts with chiral reagents, before HPLC analysis.

EXPERIMENTAL

Drug administration and sample collection

Four healthy geldings aged between 15 and 16 years, and weighing 450, 480, 530 and 550 kg, were selected for the study. They were dosed intravenously with a single dose of 2.2 mg ketoprofen/kg body weight (Ketofen; Rhône Mérieux, Lyon, France). Blood samples were collected by jugular venipuncture 0, 2, 4, 8, 15, 20, 30, 40, 50, 60, 70, 80 and 90 min after drug administration. The samples were immediately centrifuged for 10 min at 900 *g*, and the plasma was stored at −20°C until analysis. Urine samples were naturally collected by using a urinal at 0, 3, 6, 12, 24, 48, 72 and 96 h. Aliquots of 500 ml were taken for analysis after homogenization and were stored at −20°C.

Plasma and urine sample preparation

Plasma and urine samples of 2.5 ml were extracted, purified and methylated using previously described methods [14,15].

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 5890–5970). The chromatograph was equipped with a 25 m × 0.32 mm I.D. column, in which the film thickness of the stationary phase was 0.2 μm (SE 30, Spiral). The operating temperatures were as follows: injector, 250°C; oven, from 75°C to 160°C at 30°C/min, then from 160°C to 250°C at 4°C/min, then from 250°C to 280°C at 30°C/min, and finally 280°C for 3 min; interface, 250°C; source, 280°C. The carrier gas was helium at a flow-rate of 1.8 ml/min. 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. The limit of detection was 0.4 μg/ml.

Partial synthesis of the metabolite

Ketoprofen (Sigma K 1751, Saint-Quentin-Fallavier, France), 1.97 mmol, was placed in suspension in 25 ml of water, and 27 mmol of $NaBH_4$ were slowly added. The mixture was shaken for 5 min at room temperature. The resulting solution was progressively acidified with 12 *M* HCl, and twice extracted with 40 ml of diethyl oxide. The organic phase was finally evaporated under a nitrogen stream at 60°C, and the dry product was collected. The yield of the reaction was 98%. The analytical purity of the compound was evaluated by HPLC, using the method described below. The product was characterized by its melting point, its UV spectrum (Shimadzu MPS 2000), by TLC, IR spectroscopy (sample compressed with KBr, Perkin-Elmer 1600 IR spectrophotometer), ^1H NMR spectroscopy [sample in dimethyl sulphoxide (DMSO) and ^2H_2O + DMSO, AM 300-MHz Bruker spectrometer], and by GC–MS (under the previously described conditions). The spectra of ketoprofen were obtained under the same conditions to allow comparison.

Kinetics and determination of ketoprofen and its metabolite in plasma

Sample preparation. A 200-μl plasma sample was added to 200 μl of 100 m*M* H_3PO_4 in water. The mixture was then adsorbed onto Vac Elut system (Varian) C_8 cassettes, previously activat-

ed with 750 μl of methanol, then with 750 μl of 100 m*M* H_3PO_4 in water. The cassettes were successively washed with 750 μl of 100 m*M* H_3PO_4 in water, 750 μl of methanol–100 m*M* H_3PO_4 in water (20:80), and 750 μl methanol–water (20:80). They were then introduced into an advanced automated sample processor (AASP, Varian).

HPLC analysis. The analytes were eluted and injected into an HPLC system (Beckman 110 b pumps coupled to a Varian 2050 UV detector and a 3393 A Hewlett-Packard integrator). The chromatograph was equipped with a 25 cm × 4 mm I.D. column (LiChrospher RP 18, 5 μm particle diameter, Merck). The mobile phase was acetonitrile–100 m*M* H_3PO_4 in water, with a flow-rate of 2 ml/min. A gradient was established, according to the following sequence for H_3PO_4: from 70 to 50% in 10 min, then from 50 to 40% in 2 min, then 40% for 2 min, then from 40 to 75% in 1 min, and finally 70% for 5 min. The UV detection wavelength was 220 nm. Ketoprofen and its metabolite were quantified in the samples by comparison with a spiked plasma. The analytical standards used for this purpose were ketoprofen (Sigma K 1751, Saint-Quentin-Fallavier, France) and the synthetized metabolite. For the two compounds, the extraction rate was 95% and the method was linear between 0.5 μg/ml and 10 μg/ml. The limit of quantification for ketoprofen and its metabolite was 0.5 μg/ml, and the limit of detection 0.1 μg/ml. The inter-assay and intra-assay precisions, at the concentration of 2 μg/ml, were 6.5% and 2.2%, respectively. The accuracy of the method was 5%.

Determination of ketoprofen and its metabolite in urine

Reconstructed ion chromatograms were plotted for the sum of the selected ions *m/z* 209 and 197, which represent the base peaks of ketoprofen and the metabolite mass spectra, respectively. The mass spectra of the drug and its metabolite were plotted for each time period.

RESULTS

Identification of the metabolite

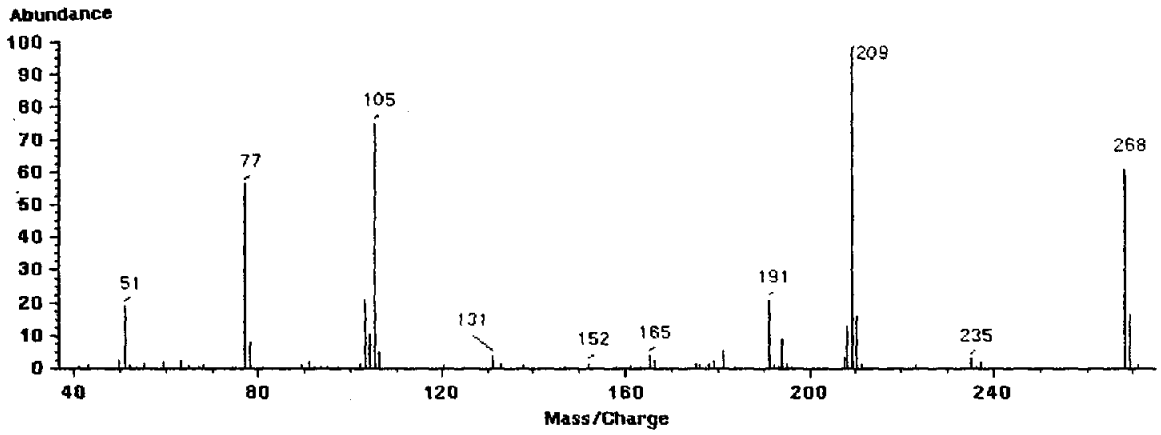
Mass spectrometry. The metabolite was first characterized in both urine and plasma with the normalized and background-subtracted mass spectrum, obtained by electron-impact ionization, of its dimethylated derivative. This spectrum was compared with that of the monomethylated ketoprofen derivative (Fig. 2), whose fragmentation pathway has been described [3]. The retention time of the dimethylated metabolite derivative was 8 min 45 s, and that of the monomethylated ketoprofen derivative was 10 min 30 s.

Fig. 3 shows the various fragmentation pathways of the ketoprofen metabolite, which is most probably a benzhydrolic compound. The molecular ion seems to be *m/z* 284, which can lose CH_3, resulting in *m/z* 269. Two interesting ions appear at *m/z* 121 and 77. The comparison of these ions with the corresponding ions at *m/z* 105 and 77 of the ketoprofen spectrum seems to indicate a metabolic reduction of the keto function of the drug to an alcoholic group. The ion at *m/z* 197 can lose CH_3OH, resulting in *m/z* 165. The fragmentation pathway proposed for the metabolite suggests that the secondary alcohol function has been methylated as well as the carboxylic function.

The mass and UV spectra, as well as the HPLC retention time of the synthesized product, in which structure the keto group of ketoprofen has been reduced by $NaBH_4$, were found to be identical with those obtained with the extracted compound. Therefore, the two compounds certainly have the same structure. The metabolite was also characterized by studying the following physical properties of the synthesized product.

Melting point and analytical purity. The melting point of the metabolite is 106–107°C. Comparison with the lower values, 91–96°C, obtained for ketoprofen [3,16] accords with a benzhydrolic structure for the metabolite. The analytical purity of the product, evaluated by HPLC, was 99.8%.

UV spectroscopy. In methanol, λ_{min} and λ_{max} of the alcohol metabolite were found to be 245 and



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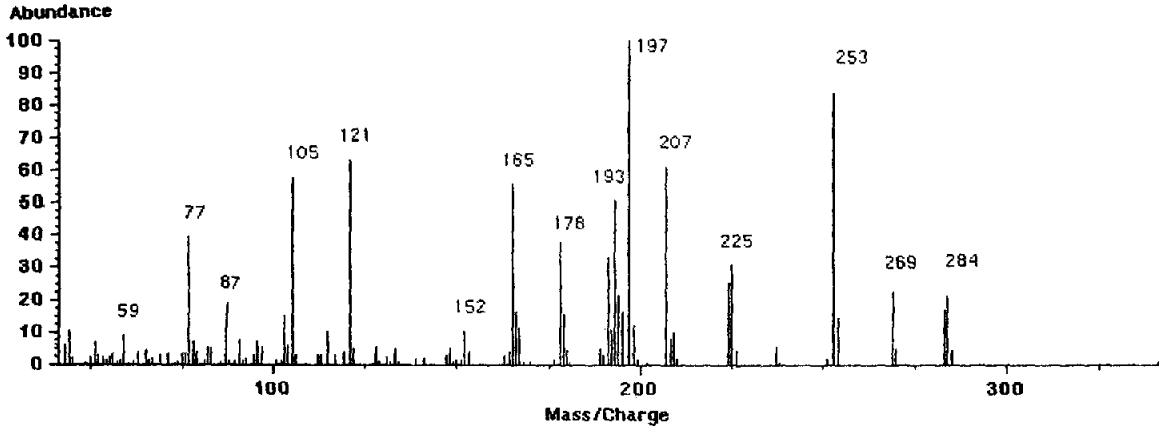

B

Fig. 2. Mass spectra of (A) the monomethylated ketoprofen derivative, and (B) the dimethylated ketoprofen benzhydrolic metabolite derivative.

260 nm, respectively. The corresponding values for ketoprofen were 235 and 252 nm.

IR spectroscopy. The main absorption bands present in the spectrum of the metabolite are at 3415, 1708, 1600, 1489 and 1453 cm^{-1}. A strong band at 3415 cm^{-1} is indicative of an hydroxy function. The band at 1655 cm^{-1} present in the spectrum of ketoprofen, which is indicative of a keto function, is absent from the spectrum of the metabolite.

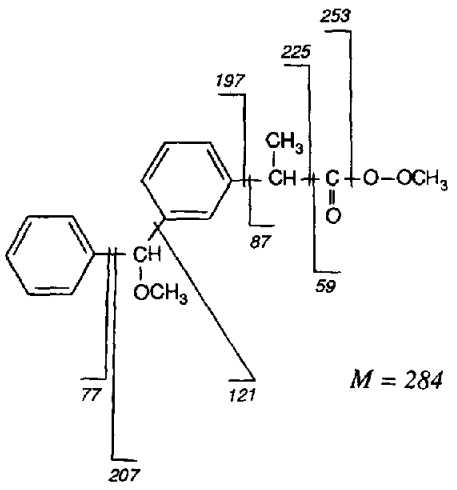

Fig. 3. Fragmentation of the dimethylated ketoprofen benzhydrolic metabolite derivative.

^1H *NMR spectroscopy.* The metabolite spectrum shows the shift of an OH proton, which appears at 5.7 ppm and is absent from the spectrum of ketoprofen. This spectrum is in accord with the literature data [5,17]. For both compounds, the shift of the carboxylic OH proton appears at 12.5 ppm. Both these signals disappear when ^2H_2O is added to the metabolite sample.

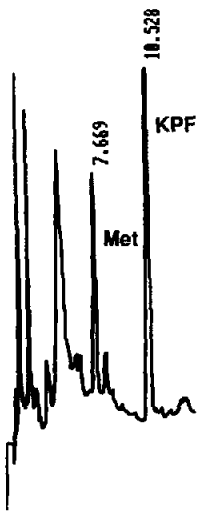

Fig. 4. HPLC of a plasma extract sample, 50 min after an intravenous dosage of 2.2 mg ketoprofen/kg body weight. The figure shows the peaks corresponding to ketoprofen (KPF) and its benzhydrolic metabolite (Met).

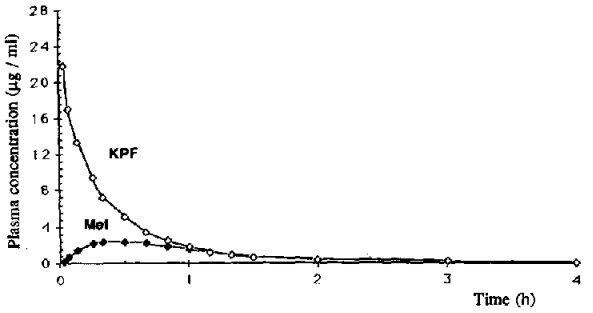

Fig. 5. Plasma profiles of ketoprofen (KPF) and its benzhydrolic metabolite (Met), obtained by HPLC analysis, after an intravenous dosage of 2.2 mg ketoprofen/kg body weight.

Therefore, the studied metabolite can be identified as 2-(3-(α-hydroxybenzyl))phenylpropionic acid.

Determination of ketoprofen and metabolite in plasma and urine

Kinetics and determination of ketoprofen and its metabolite in plasma. Under the HPLC conditions, the retention times of ketoprofen and its metabolite were 10.5 and 7.7 min, respectively, as indicated in Fig. 4, showing a chromatogram of a plasma sample extract. Fig. 5 shows the arithmetic plots of ketoprofen and its metabolite plasma concentrations *vs.* time. The metabolite levels increase up to 30 min, reaching 2.3 µg/ml, then decrease. The ketoprofen concentration profile shows a logarithmic decrease *vs.* time. Ketoprofen is quantified by HPLC up to 4 h, and its metabolite up to 3 h, with a limit of quantification of 0.1 µg/ml.

Determination of ketoprofen and its metabolite in urine. The elimination of ketoprofen and its metabolite from urine was followed qualitatively with reconstructed ion chromatograms and mass spectra. The reconstructed ion chromatogram in Fig. 6 shows the two compounds in a sample taken from a horse 6 h after administration. Ketoprofen can be detected up to 72 h and the metabolite up to 24 h after administration. After these times, the mass spectra contain only low-abundance ions. Therefore, the estimated detection times for the anti-inflammatory drug and its metabolite in urine, *i.e.* the period during which the

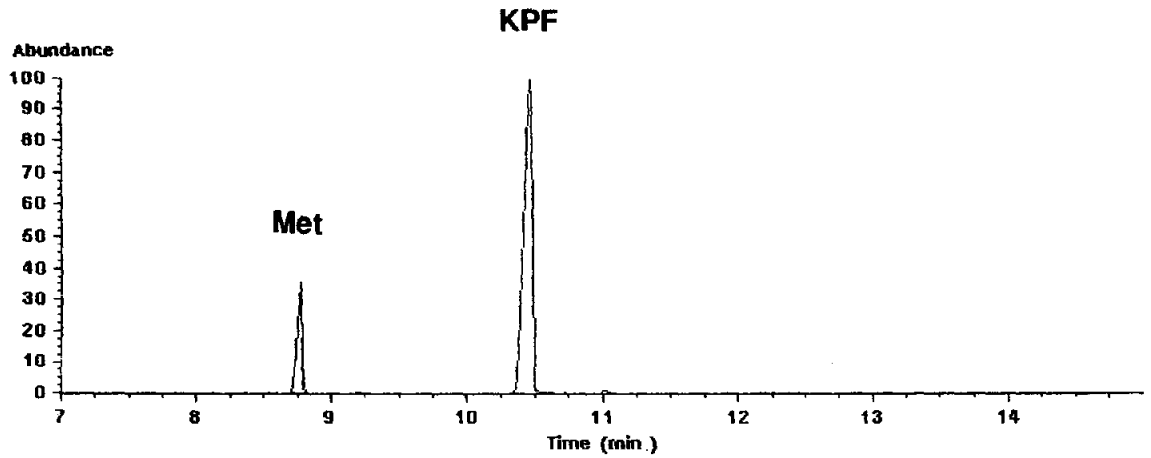

Fig. 6. Reconstructed ion chromatogram, plotted on the sum of the ions *m/z* 197 and 209, obtained with the 6-h urine sample. Peaks: KPF = monomethylated ketoprofen derivative (9 μg/ml); Met = dimethylated benzhydrolic metabolite derivative (3.4 μg/ml). Retention times are given in minutes.

molecules can be positively detected in this biological fluid using a definite analytical method [18], are 3 days and 1 day, respectively.

DISCUSSION

The benzhydrolic metabolite of ketoprofen has not been described in previous publications concerning other species: only phenolic and hydroxylated products of the propionic group have been described [1,2]. Nevertheless, the GC–MS study has indicated the existence of such metabolites in horse urine, but these compounds seemed to be present at lower concentrations than the benzhydrolic metabolite.

The derivatization procedure used [14] allows a flash methylation into the injector of the gas chromatograph, but a polyfunctional molecule usually gives rise to several methylated products. In this experiment, the carboxylic and alcohol functions of the metabolite were liable to react with the methylating agent. However, only one metabolite derivative was identified, according to the results obtained with tolfenamic acid, and in contrast with those obtained with flunixin [14,15]. Such variations may be explained by differences in the reactivity of the different functions. Indeed, the alcohol group is more easily methylated that the amino group present in flunixin and tolfenamic acid, which reacts erratically.

The metabolic reduction of ketones to alcohols is a common pathway that has been described for numerous substances, such as 5-keto-substituted benzimidazoles [19–22], fenofibric acid [23] and oxisuran [24]. This process occurs in laboratory animals as well as in humans [22] and sheep [21]. In the specific case of ketoprofen, this reduction is of special interest because, as with fenofibric acid [25], two different alcohol stereomers (product stereospecificity) exist, possibly dependent on the parent *R*(−) or *S*(+) ketoprofen enantiomer (substrate stereospecificity). This leads theoretically to four diastereomeric alcohol metabolites. We already know that, after administration of the racemate, the dispositions of the individual *R*(−) and *S*(+) enantiomers of ketoprofen are obviously different in horses [26].

Regardless of the chiral aspect of the metabolism of ketoprofen, the present data showed that the maximum time available for detection of this drug in urine by GC–MS is 72 h. It is also apparent that, in antidoping control, simultaneous detection of ketoprofen and its reduced metabolite can provide an approximate value of the administration time, at least within 24 h before urine sampling.

REFERENCES

1 L. Julou, J.-C. Guyonnet, R. Ducrot, J. Fournel and J. Pasquet, *Scand. J. Rheumatol.*, 5 (Suppl. 14) (1976) 33.
2 P. Populaire, B. Terlain, S. Pascal, B. Decouvelaere, A. Renard and J.-P. Thomas, *Ann. Pharm. Fr.*, 31 (1973) 735.
3 G. Liversidge, *Anal. Profiles Drug Subst.*, 10 (1981) 443.
4 P. Populaire, B. Terlain, S. Pascal, B. Decouvelaere, A. Renard and J.-P. Thomas, *Ann. Pharm. Fr.*, 31 (1973) 679.
5 N. Blazevic, M. Zinic, T. Kovac, V. Sunjic and F. Kajfez, *Acta Pharm. Jugosl.*, 25 (1975) 155.
6 A. Bannier, J.-L. Brazier and B. Ribon, *J. Chromatogr.*, 155 (1978) 371.
7 A. Bannier, J.-L. Brazier and C. Quincy, *Feuil. Biol.*, 20 (1979) 91.
8 J. De Graeve, C. Frankinet and J. E. Gielen, *Biomed. Mass Spectrom.*, 6 (1979) 249.
9 D. Heusse and L. Raynaud, *Ann. Pharm. Fr.*, 36 (1978) 631.
10 P. Stenberg, T. E. Joensson, B. Nilsson and F. Wollheim, *J. Chromatogr.*, 177 (1979) 145.
11 A. Bannier, J.-L. Brazier, B. Ribon and C. Quincy, *J. Pharm. Sci.*, 69 (1980) 763.
12 T. A. Noctor, G. Felix and I. W. Wainer, *Chromatographia*, 31 (1991) 55.
13 R. T. Foster and F. Jamali, *J. Chromatogr.*, 416 (1987) 388.
14 P. Jaussaud, D. Courtot, J.-L. Guyot and J. Paris, *J. Chromatogr.*, 423 (1987) 123.
15 P. Jaussaud, D. Guieu, D. Courtot, B. Barbier and Y. Bonnaire, *J. Chromatogr.*, 573 (1992) 136.
16 A. Allais, G. Rousseau, J. Meier, R. Deraedt, J. Benzoni and L. Chifflot, *Eur. J. Med. Chem.*, 9 (1974) 381.
17 B. Unterhalt, *Pharm. Ztg.*, 123 (1978) 1801.
18 T. Tobin, J. Crombie and T. Nugent, *J. Vet. Pharmacol. Ther.*, 5 (1982) 195.
19 H. Van den Bossche, F. Rochette and C. Horig, *Adv. Pharmacol. Chemother.*, 19 (1982) 67.
20 B. Mayo, R. Brodie, L. Chasseaud and D. Hawkins, *Drug Metab. Dispos.*, 6 (1978) 518.
21 C. Behm, R. Cornish and C. Bryant, *Res. Vet. Sci.*, 34 (1983) 37.
22 P. Braithwaite, M. Roberts, R. Allan and T. Watson, *Eur. J. Clin. Pharmacol.*, 22 (1982) 161.
23 J. Caldwell, M. Strolin Benedetti and A. Weil, *Br. J. Clin. Pharmacol.*, 22 (1986) 219.
24 F. Di Carlo, *Drug Metab. Rev.*, 10 (1979) 225.
25 A. Weil, J. Caldwell, J.-P. Guichard and G. Picot, *Chirality*, 1 (1989) 197.
26 P. Jaussaud, C. Bellon, S. Besse, D. Courtot and P. Delatour, *J. Vet. Pharmacol. Ther.*, 15 (1993) in press.