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HPLC on Chiralcel OJ-R for Enantiomer Separation and Analysis of Ketoprofen, from Horse Plasma, as the 9-Aminophenanthrene Derivative*

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Abstract

Racemic ketoprofen is a non-steroidal anti-inflammatory drug used to treat musculoskeletal and colic conditions in horses. The enantioselective chiral inversion of ketoprofen administered to horses has been studied by use of cellulose tris(4-methylbenzoate), also known as Chiralcel OJ-R, as chiral stationary phase; acetonitrile – 0·02 M perchlorate buffer (pH 2·0) – methanol, 60 : 15 : 25 (v/v/v) was used as mobile phase. Before chromatography, to effect adequate chiral interaction with the chiral stationary phase ketoprofen was derivatized with 9-aminophenanthrene, under acid conditions, after solid-phase (C_{18}) extraction and then liquid–liquid extraction, to ensure effective removal of endogenous plasma materials. The 9-aminophenanthrene derivative of *S*-ibuprofen was used as internal standard. The enantiomers of ketoprofen were separated to baseline (Rs = 6·44, $\alpha = 1{\cdot}76$) within a short analysis time.

The results indicate that the bio-inversion of *R*-ketoprofen to the *S* isomer is significant in equine species. However, considerable differences in pharmacokinetic parameters were observed, indicating large inter-animal variation.

Ketoprofen is a widely used non-steroidal anti-inflammatory drug; in horses its analgesic properties are used for the treatment of colic conditions and its anti-inflammatory properties are used for the treatment of musculoskeletal conditions such as arthritis and tendinitis. Anti-inflammatory drugs of the profen type are of particular interest because of their specific pharmacokinetic behaviour. Depending on the species and the drug studied, characteristic bio-inversion occurs whereby the *R* enantiomer (distomer) is inverted to its *S* antipode (eutomer). Ketoprofen is administered as a racemate and numerous studies have dealt with the enantioselective pharmacokinetics of ketoprofen in various animal species, including man (Abas & Meffin 1987; Foster et al 1988a, b; Sallustio et al 1988; Iwakawa et al 1991; Delatour et al 1993; Mauleon et al 1994; Aberg et al 1995; Landoni et al 1995a; Boisvert et al 1997; Jamali et al 1997). The horse is an attractive species for investigation of this drug because, in contrast with man, significant stereo-inversion occurs. The enantioselective pharmacokinetics of ketoprofen in equine species were first reported by Jaussaud et al (1993) and since ketoprofen was approved by the US FDA for use in horses, closer attention has been paid to its stereoselective pharmacokinetic and dynamic processes (Landoni & Lees 1995b, c).

A newly developed cellulose-based chiral stationary phase, Chiralcel OJ-R, has been studied to determine its enantioselective properties towards non-steroidal anti-inflammatory profen drugs. Although the chemical structures of Chiralcel OJ and Chiralcel OJ-R, tris(4-methylbenzoate) cellulose, are the same, the chiral discriminative features of the phases are different. Both the polymer and

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the silica support of the Chiralcel OJ-R phase are optimized to accommodate reversed-phase application without swelling of the cellulose derivative (Daicel 1995). Racemic ketoprofen has been resolved on Chiralcel OJ but effective chiral interaction was not possible on Chiralcel OJ-R (Daicel 1994; Van Overbeke et al 1995; Tang 1996; Van Overbeke et al 1996).

Derivatization of the carboxylic acid group of the drug provides the molecule with additional interaction sites and improved detectability. This paper describes the enantioselective separation of ketoprofen enantiomers on Chiralcel OJ-R after derivatization with an achiral reagent, 9-aminophenanthrene. Furthermore, the application of the method to the analysis of ketoprofen enantiomers in horse plasma has also been demonstrated.

Materials and Methods

Chemicals

Racemic ketoprofen, 2-(3-benzoylphenyl)propionic acid, *S*-ibuprofen, 2-(4-isobutylphenyl)propionic acid and 9-aminophenanthrene were purchased from Sigma-Aldrich (Bornem, Belgium). Sodium perchlorate, perchloric acid 70% aqueous solution, methanol, acetonitrile, *n*-hexane, and ethyl acetate were of analytical or HPLC grade. Deionized water was used throughout.

Instrumentation

HPLC was performed with a Varian 9010 SDS pump (Varian Associates, Walnut Creek, CA) equipped with a Rheodyne injector with a 20-μL loop. Detection was achieved at 254 nm with a Hewlett-Packard (Waldbronn, Germany) 1050 diode-array detector equipped with an 8-μL flow-cell and 8-μm slits. Integration of the chromatographic signal was performed with Hewlett-Packard software.

The elution time of methanol was used to determine t_0 (dead time) for the Chiralcel OJ-R column (15 cm × 4·6 mm i.d., 5 μm particle size), a kind gift from Dr Oda of Daicel Chemical Industries (Tokyo, Japan). The analytical column was protected with a LiChrocart 4–4 guard column packed with 5-μm LiChrospher 100 RP 18 (Merck), and by a 0·62 × 0·25 μm PEEK frit (Upchurch Scientific); the column was maintained at 35°C in a water bath. The mobile phase was acetonitrile – 0·02 M perchlorate buffer, pH 2·0 – methanol, 60:25:15 (v/v/v); it was pumped at a flow rate of 0·8 mL min^{-1} for 18 min and and then at 1·2 mL min^{-1} up to a total analysis time of 30 min. The buffer solution was prepared by acidification of 0·02 M sodium perchlorate solution to pH 2·0 with 0·02 M perchloric acid. The buffer was degassed by ultrasonication and filtered under vacuum through a 0·45-μm membrane filter. Although, for safety reasons, phosphate systems tend to be preferred to perchlorate buffers (Daicel 1995), the use of phosphate systems is limited by the poor solubility of phosphate salt solutions in combination with high concentrations of acetonitrile.

Under these conditions, the 9-aminophenanthrene derivatives of *R*- and *S*-ketoprofen and *S*-ibuprofen eluted after 6·5, 10·1 and 12·7 min, respectively. The resolution Rs between the enantiomers of ketoprofen was 6·44; the capacity factor α was 1·76. Peak identity of the two enantiomers was confirmed by on-line UV-scanning (absorption maxima at 254, 206 and 294·5 nm) and LC-MS.

Sample preparation

The study was performed on two mares, 5 and 15 years, 675 and 517 kg respectively. They were given standard food concentrate and had free access to hay, straw and water. A parenteral formulation of 10% (w/v) ketoprofen in 10·0 mL aqueous solution (ketofen 10%; Rhone Mérieux, Lyon, France) was used for intravenous administration. The solution was injected into the jugular vein opposite to that from which the samples were collected. Blood samples (5 mL) were taken shortly before drug administration and then at 2, 5, 10, 15, 20, 30, 45 min and at hourly intervals until 12 h after administration. Blood samples were collected in tubes containing heparin as anti-coagulant. Plasma was separated at 2000 *g* for 10 min and stored at −18°C until analysis.

Standard stock solutions in methanol of ketoprofen (0·40 mg mL^{-1}) and the internal standard (IS) *S*-ibuprofen (0·32 mg mL^{-1}) were prepared and serially diluted with methanol to furnish standards of concentration 64 μg mL^{-1} and 10·24 μg mL^{-1} for ketoprofen and 51·2 μg mL^{-1} and 8·19 μg mL^{-1} for *S*-ibuprofen.

Plasma calibration standards were prepared by adding hydrochloric acid (0·18 M; 0·85 mL) and then ketoprofen solution and the appropriate *S*-ibuprofen standard solution to drug-free horse plasma (1·0 mL). Three sets of calibration standards were prepared by adding 10, 30, 50, 70, or 100 μL ketoprofen solution and 50 μL *S*-ibuprofen solution. Each sample was diluted to 150 μL with methanol.

Internal standard solution (50 μL) and methanol (100 μL) were added to the study samples.

The main clean-up stage involved solid-phase extraction. The samples were extracted on disposable C_{18ec} BondElut cartridges (Varian), pre-

viously conditioned with methanol (2 × 1 mL), water (1 mL) and hydrochloric acid (0·1 M; 1 mL). The plasma sample was applied to the cartridge which was then washed with water (2 × 1 mL) and, to promote drying of the cartridges under vacuum, methanol–water, 70:30 (v/v) (0·1 mL). Elution was performed with methanol (4 × 0·25 mL). The extract was dried under nitrogen at 25°C and the residues were dissolved in pH 10 sodium carbonate buffer (1·0 mL) and the solution was washed with *n*-hexane (3 × 2 mL). The aqueous layer was acidified with hydrochloric acid (1·0 M; 0·2 mL) and extracted with ethyl acetate (3 × 2 mL). Each time the *n*-hexane and ethyl acetate layers were removed after centrifugation at 2000 rev min^{-1} for 3 min. The combined ethyl acetate layers were evaporated under nitrogen at 30°C and the residue was derivatized.

Plasma calibration standards were prepared and analysed with each set of experimental plasma samples. The analysis of each series was performed twice on separate days and calculated concentrations in each experimental plasma sample were averaged. Some experimental plasma samples were checked in relation to different calibration sets because of the mutual diverging concentrations of the ketoprofen enantiomers.

Derivatization procedure

The 9-aminophenanthrene derivative of ketoprofen was prepared by use of 1-ethyl-3-dimethylaminopropylcarbodiimide as coupling agent in combination with 1-hydroxybenzotriazole. The reagent solutions were prepared daily and shielded from daylight.

1-Hydroxybenzotriazole solution (0·127 mg mL^{-1} in chloroform containing 1% (v/v) pyridine; 0·5 mL), 1-ethyl-3-dimethylaminopropylcarbodiimide (0·181 mg mL^{-1} in chloroform; 0·5 mL), and 9-aminophenanthrene (0·270 mg mL^{-1} in chloroform; 0·5 mL) were added successively to the residue from extraction of the plasma samples. The mixture was vortex mixed, left for approximately 1 h, evaporated to dryness under a stream of nitrogen and, before injection, dissolved in methanol.

Identification of 9-aminophenanthrene derivatives

The 9-aminophenanthrene derivatives of ketoprofen and *S*-ibuprofen were synthesized on a larger scale and purified by recrystallization from methanol. The identities of the reaction products were confirmed by NMR and MS analysis. ^{1}H NMR spectra were obtained with a Bruker WH 360 spectrometer, mass spectra with a Hewlett–Packard 5988A spectrometer in electron impact (EI) mode with an electron energy of 70 eV.

Results and Discussion

Extraction yield

Studies of the recovery of ketoprofen and of *S*-ibuprofen were conducted at two different concentrations (n = 4). Hydrochloric acid (0·18 M; 0·85 mL), ketoprofen solution (100 μL containing either 40 μg or 6·4 μg) and *S*-ibuprofen (50 μL containing either 6·4 μg or 2·56 μg) were added to drug-free horse plasma (1·0 mL). Areas under the plasma concentration–time curves (AUC) were compared with those of solutions obtained by drying volumes equal to the amount added to the plasma and re-dissolving them in 100 μL methanol.

The extraction yield was tested on the Chiralcel OJ-R column, using 88:12 (v/v) methanol – 0·1 M perchlorate buffer, pH 2·0, as mobile phase at a flow rate of 0·5 mL min^{-1} at 30°C. Detection was performed at 230 nm (retention times: ketoprofen 6·85 min, ibuprofen 8·15 min). For samples to which the larger amounts had been added the recoveries were 100·3 ± 0·9% for ketoprofen and 91·7 ± 1·4% for *S*-ibuprofen; for samples to which the smaller amounts had been added the respective values were 102·0% ± 1·1% and 93·8% ± 2·3%.

Derivatization yield

Derivatization yield was calculated by comparing the AUC value obtained from a methanolic solution of purified derivative (considered 100% pure) with that obtained after derivatization of 40 μg ketoprofen or 32 μg *S*-ibuprofen by the method described above (n = 10). For *S*-ketoprofen the yield was 87 ± 2·8% (calculated for the second eluting enantiomer), for *S*-ibuprofen it was 85 ± 2·2%.

The additional extraction procedure after solid-phase extraction was necessary to eliminate substances that seemed to interfere with the subsequent derivatization procedure, reducing the yield by approximately 40%. It improved the total yield for derivatization of the plasma samples to approximately 77%.

Determination of order of elution of the enantiomers

To obtain a solution with enhanced enantiomeric excess, a solution (50 mg mL^{-1}) of racemic ketoprofen in isopropanol was analysed by HPLC on a Chiralcel OJ column (25 cm × 4·6 mm i.d.) using 95:5:0·5 (v/v/v) *n*-hexane-isopropanol-trifluoroacetic acid as mobile phase at a flow rate of 1·0 mL min^{-1}. Fractions corresponding to a single

enantiomer were collected at the outlet of the detector, set at 254 nm. Fractions from successive injections were combined and re-injected repeatedly until no detectable signal of the chiral antipode was observed. Both fractions were evaporated under a stream of nitrogen and derivatized with 9-aminophenanthrene as described above. The derivatives were analysed by HPLC on the Chiralcel OJ-R phase. The *R* enantiomer eluted before the *S*.

Limit of quantification

The limit of quantification of the second eluting peak was determined to be approximately 13·2 ng of the 9-aminophenanthrene derivative of the ketoprofen enantiomer, on column; this corresponded to approximately 8 ng *S*-ketoprofen. However, because of incomplete derivatization and interferences in the plasma, the quantification limit in plasma was approximately 20 times higher.

Validation assay

Intra-day and inter-day variation are summarized in Table 1. Best-fit calibration plots of peak-area ratios against concentration were determined by least-square regression analysis. Adequate linearity ($n = 5$) was obtained for ketoprofen added to plasma samples ($r > 0{\cdot}999$ for the highest and the middle calibration ranges and $r > 0{\cdot}995$ for the lowest concentration range).

Pharmacokinetic results

The *S*:*R* concentration ratio increased progressively with time, but the rate of increase was less pronounced as the time interval after administration was prolonged. Clear predominance of *S*-ketoprofen over the *R* enantiomer was apparent in the second experimental plasma sample (5 min after dosage) and lasted. Whereas the enantiomer ratio 2 min after drug administration was still approximately 50 : 50, after 5 min the *S* : *R* enantiomer ratio was 52 : 48 and after 20 min it was 66 : 34. As the major part of the racemic dose administered was recovered as the *S* enantiomer, considerable bioinversion of *R*-ketoprofen to the *S* isomer was confirmed for equine species (Figure 1). Unfortunately, the extent of inversion could not be adequately estimated as only racemic ketoprofen was administered and not the pure *R* enantiomer, which was not available to us.

Figure 2 shows chromatograms obtained from a blank plasma sample and from a drug-free plasma sample to which 20 μg racemic ketoprofen and 16 μg *S*-ibuprofen had been added.

Calculation of pharmacokinetic parameters

R- and *S*-ketoprofen concentration–time profiles were analysed by use of the Kinfit program in the MW/PHARM 3.15 software package (Mediware, Utrecht, the Netherlands). By applying a relative error independent of the concentration method, the data were fitted to a biexponential function $C = Ae^{-\alpha t} + Be^{-\beta t}$, where C is the plasma concentration at time t, A and B are ordinate intercepts and α and β are slope values. The apparent elimination half-life ($t\frac{1}{2}_\beta$) was calculated from $\ln 2/\beta$. The $AUC_{0\rightarrow t}$ was determined by the linear trapezoidal rule and total clearance (CL) was calculated from the dose ($A/\alpha + B/\beta$). The apparent volume of distribution (Vd_β) was calculated from CL/β ($= dose/AUC_\beta$). The mean residence time (MRT) is an estimate of the mean transit time of one intact molecule through the body and is calculated from the equation:

$$MRT = \left({}_0\sum^{\infty} tC_p dt\right)/\left({}_0\sum^{\infty} C_p dt\right) \quad (1)$$

Table 1. Intra-day and inter-day precision and accuracy for analysis of *R*-ketoprofen and *S*-ketoprofen added to horse plasma.

Concentration added ($\mu g\,mL^{-1}$)	*R*-Ketoprofen		*S*-Ketoprofen	
	Mean (%; n = 4)	Coefficient of variation (%)	Mean (%; n = 4)	Coefficient of variation (%)
Intra-day				
20	99·8	2·01	98·7	1·66
3·2	97·8	4·81	98·5	1·77
0·512	102·1	6·81	99·4	9·7
Inter-day				
20	99·6	2·13	98·3	2·18
3·2	96·9	6·79	97·2	8·60
0·512	104·2	7·34	106·1	10·4

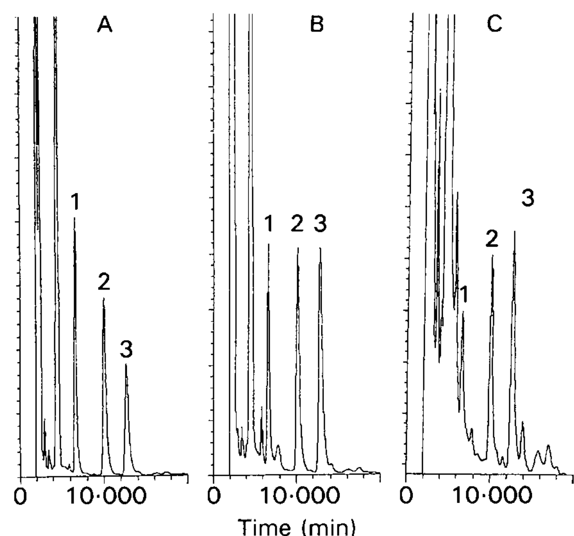

Figure 1. Chromatographic analyses of samples taken 2 min (A), 15 min (B) and 75 min (C) after intravenous administration of racemic ketoprofen: 1, *R*-ketoprofen; 2, *S*-ketoprofen; 3, *S*-ibuprofen.

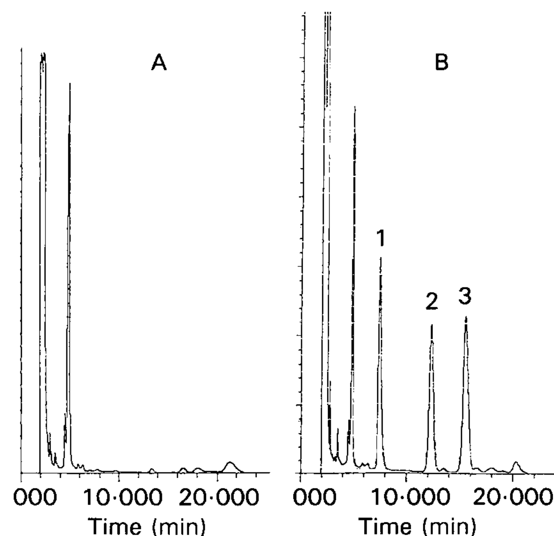

Figure 2. Chromatographic analysis of a drug-free plasma (A) and of a plasma sample to which has been added 20 μg racemic ketoprofen and 16 μg *S*-ibuprofen (B): 1, *R*-ketoprofen; 2, *S*-ketoprofen; 3, *S*-ibuprofen.

where ${}_0\sum^{\infty} tC_p dt$ is the area under the plasma concentration–time curve AUC and ${}_0\sum^{\infty} C_p dt$ is the first moment of the plasma concentration–time curve, AUMC (Table 2).

The data obtained revealed substantial differences between the parameters for the two horses, indicating that there might be large inter-animal differences between drug pharmacokinetics. In both animals used for this study *S*-ketoprofen had lower clearance CL and a higher volume of distribution Vd_β than *R*-ketoprofen. In contrast, Jaussaud et al (1993), reported a mean Vd_β that was higher for the *R* enantiomer (88·6 L → 143·4 L) compared to its antipode (59·7 L → 96·1 L). The different volumes of distribution of the enantiomers are probably a consequence of different plasma-protein binding. However, possible stereoselective binding to plasma proteins (especially albumin) has not been investigated and no specific data for horses are available in the literature. Hence, the apparent elimination half-life $t^{1/2}_\beta$ is higher for the *S* enantiomer than for the *R*. The mean $t^{1/2}_\beta$ found (0·56 h and 1·28 h for *R*- and *S*-ketoprofen, respectively) were between those reported in the literature. They were shorter than those measured by Landoni & Lees (1995a) (0·70 ± 0·13 h and 1·09 h for *R*-ketoprofen, 0·99 ± 0·14 h and 1·51 h for *S*-ketoprofen), but longer than those given by Jaussaud et al (1993), who obtained similar $t^{1/2}_\beta$ values for both enantiomers.

Conclusions

The bioinversion of *R*-ketoprofen to the *S* isomer in horses is an example of significant metabolic activation of this anti-inflammatory drug, and its susceptibility to inter-animal variation.

The method described illustrates the advantages of derivatization with an achiral reagent to broaden the applicability of the chiral stationary phase and effect enantioselective separation of the enantiomers of ketoprofen. The combined use of methanol and acetonitrile in the mobile phase enabled complete baseline resolution of the enantiomers; they

Table 2. The main pharmacokinetic parameters for both horses.

Parameter (unit)	*R*-Ketoprofen		*S*-ketoprofen		Ratio *R* : *S*	
	Horse 1	Horse 2	Horse 1	Horse 2	Horse 1	Horse 2
Area under plasma concentration-time curve (h mg L^{-1})	6·36	10·4	11·0	17·8	0·58	0·59
Clearance (L h^{-1})	72·8	47·7	42·5	28·9	1·71	1·65
Clearance (L h^{-1} kg^{-1})	0·14	0·07	0·08	0·04		
Volume of distribution (L)	69·8	31·4	82·3	47·4	0·85	0·66
Volume of distribution (L kg^{-1})	0·14	0·05	0·16	0·07		
Apparent elimination half-life (h)	0·66	0·46	1·34	1·14	0·49	0·40
Mean residence time (h)	0·46	0·41	0·77	0·59	0·60	0·70

were well separated from *S*-ibuprofen and there was no interference from the fast-eluting derivatization reagents. Because λ_{max} is 254 nm for both 9-aminophenanthrene and ketoprofen, the detectability of the derivative is improved; this is of benefit in clinical applications, for example the analysis of ketoprofen enantiomers in horse plasma. The pharmacokinetic results indicate that the bio-inversion of *R*- to *S*-ketoprofen in horses is significant.

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