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Stereospecific high-performance liquid chromatographic assay of pirprofen enantiomers in rat plasma and urine

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ABSTRACT

A stereospecific high-performance liquid chromatographic method was developed for the assay of pirprofen enantiomers in rat plasma and urine. Following addition of internal standard (ketoprofen) and acidifier (L-ascorbic acid) to biological fluids, pirprofen was extracted into an isopropanol–isooctane (5:95) mixture. Diastereomers of pirprofen enantiomers, which were formed using L-leucinamide, were separated on a reversed-phase column with ultraviolet detection at 275 nm using 0.06 *M* KH_2PO_4–acetonitrile–triethylamine (64:36:0.1) as mobile phase. The limit of quantitation was 0.1 μg/ml for each enantiomer, based on 100 μl of rat plasma. No spontaneous oxidation of pirprofen to its pyrrole metabolite occurred during sample preparation and analysis. In three female rats which were dosed with 10 mg/kg racemic pirprofen orally, plasma concentrations of the enantiomers could be followed for 24 h. Pirprofen enantiomers in plasma were virtually unconjugated, and negligible concentrations of pyrrole metabolites were observed. Less than 10% of the total dose was recovered in urine as intact drug and its glucuroconjugates. The assay was found suitable for the study of the pharmacokinetics of pirprofen enantiomers in the rat.

INTRODUCTION

Pirprofen (PR), (±)-2-[3-chloro-4-(3-pyrrolinyl)phenyl]propionic acid, is a member of the 2-arylpropionic acid (2-APA) class of non-steroidal anti-inflammatory drugs (NSAIDs) and contains a chiral center (Fig. 1). PR, which was recently withdrawn from the pharmaceutical market, was previously available as the racemate for the treatment of pain and inflammation associated with rheumatoid arthritis [1–3]. Similar to other chiral 2-APAs [4,5], the therapeutic benefits of PR can be attributed almost exclusively to the *S*-enantiomer [6]; the respective *R*-enantiomer has little if any analgesic or anti-inflammatory activity.

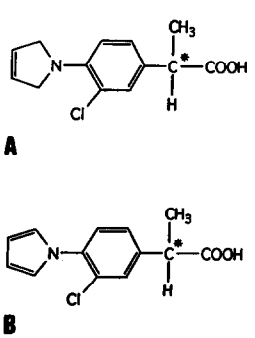

Fig. 1. Structures of pirprofen (A) and its pyrrole metabolite/by-product (B). The asymmetric carbon is denoted by an asterisk.

A stereospecific high-performance liquid chromatographic (HPLC) assay for the determination of PR enantiomers has been reported which required a somewhat lengthy sample preparation time [7]. Because PR can spontaneously oxidize to its pyrrole (PYR) analogue (Fig. 1) during sample manipulation [8], a short sample preparation time is most desirable.

In this report we describe a rapid and convenient stereospecific HPLC method for the assay of PR enantiomers in rat plasma and urine, and

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its application in a preliminary study of the pharmacokinetics of PR enantiomers in the rat.

EXPERIMENTAL

Chemicals

Racemic PR was kindly provided by Ciba-Geigy (Mississauga, Canada) and the internal standard (I.S.), racemic ketoprofen, was purchased from Sigma (St. Louis, MO, USA). L-Ascorbic acid and ethyl chloroformate were supplied by Fisher Scientific (Edmonton, Canada) and BDH (Edmonton, Canada), respectively. L-Leucinamide and 2,3-dicyano-5,6-dichloro-1,4-benzoquinone (DDQ) were obtained from Sigma. Water was of HPLC grade while acetonitrile, toluene, acetic acid, isopropanol, isooctane and methanol were of analytical grade (BDH, Toronto, Canada). Laboratory-grade H_2SO_4, KH_2PO_4, and NaOH were purchased from BDH. Analytical-grade toluene and triethylamine (TEA) were purchased from Mallinckrodt (Paris, KY, USA).

Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Scientific (Mississauga, Canada) Model M-45 pump, Waters Model 712 WISP automatic sample processor, Waters Model 481 UV spectrophotometer with the analytical wavelength set at 275 nm, and a 100 mm × 4.6 mm I.D. reversed-phase column (Partisil ODS-3, 5 μm particle size; Phenomenex, Torrance, CA, USA) which was attached to a 50 mm × 4.6 mm I.D. guard column packed with 37–53 μm C_{18} material (Whatman, Rose Scientific, Edmonton, Canada). A Model 3390A Hewlett Packard integrator (Avondale PA, USA) was used to determine peak areas; the peak-area ratios of PR enantiomer/I.S. enantiomer were used to quantify PR enantiomers.

The mobile phase, 0.06 *M* KH_2PO_4–acetonitrile–TEA (64:36:0.1; pH 6.8) was degassed by filtering through a 0.45-μm membrane filter (Scientific Products & Equipment, Rexdale, Canada). It was pumped at a flow-rate of 1.0 ml/min.

Standard solutions

Ethyl chloroformate (60 m*M*) was prepared in acetonitrile, and L-leucinamide (1 *M*) was dissolved in 1 *M* TEA in methanol. Aqueous solutions of L-ascorbic acid (50 mg/ml) were freshly prepared before use in amber glass bottles. A stock solution of 100 μg/ml PR as the racemate was prepared in 10 ml of methanol and sufficient 0.01 *M* NaOH to make 100 ml, while the I.S. was dissolved in 0.01 *M* NaOH. All solutions were stored at 5°C except PR, which was frozen at −20°C. Aqueous working solutions were prepared using HPLC water.

Sample preparation

To 100 μl of rat plasma, 50 μl of I.S. (10 μg/ml) and 0.5 ml of L-ascorbic acid (50 mg/ml) were added. To 0.5 ml of urine, 50 μl of I.S. (50 μg/ml) and 1 ml of 50 mg/ml L-ascorbic acid were added. The constituents were then extracted using 3 ml of isooctane–isopropanol (95:5). Following vortex-mixing for 20 s and centrifugation (Adams Dynac centrifuge, Clay-Adams, Parsipanny, NJ, USA) at 1800 *g* for 3 min, the organic layer was transferred to clean tubes and evaporated to dryness (Savant Speed Vac concentrator–evaporator, Emerston Instruments, Scarborough, Canada).

The derivatization procedure was similar to that used in a previously developed assay for ketoprofen enantiomers [9] in which three solutions were added in the following sequence. The residue was first reconstituted in 100 μl of 50 m*M* TEA in acetonitrile, followed by the addition of 50 μl of ethyl chloroformate solution. After 30 s, 50 μl of L-leucinamide solution were added, and the derivatization was allowed 2 min to proceed at room temperature. Water (50 μl) was then added. Aliquots of 2–45 μl were injected into the HPLC system.

To measure glucuronide conjugates in plasma and urine, 125 μl of 1 *M* NaOH were added to hydrolyse the conjugates. After vortex-mixing for 5 s, 100 μl of 0.6 *M* H_2SO_4 and 1 ml of 50 mg/ml L-ascorbic acid were added to adjust the pH to between 3 and 4. The samples were then treated as described for non-hydrolysed samples. The

difference in PR concentrations between alkali-treated and non-alkali-treated samples was considered as representing glucuronide conjugates of PR.

Standard calibration curves

The enantiomers were quantified by spiking aliquots of 0.1 ml blank plasma and 0.5 ml blank urine with racemic PR to yield enantiomeric concentrations of 0.1, 0.5, 1.0, 5.0, 10, 20 and 40 μg/ml. The solutions were analyzed according to the procedure described above.

Extraction and extraction efficiency

To determine the optimal conditions for extraction of PR, blank plasma was spiked with racemic PR (20 μg/ml) and 50 μl of I.S. (10 μg/ml). This mixture was acidified with varying amounts of H_2SO_4 (0.0006, 0.006, 0.06 and 0.6 *M*) and/or L-ascorbic (5, 50 and 100 mg/ml). The pH of the mixtures were measured with a pH meter (Model 632 Brinkmann, Metrohm Herisau, Switzerland), and the constituents were extracted into 3 ml of isooctane–isopropanol (95:5). The peak area of PR enantiomer was used to determine the best extraction conditions.

To assess the efficiency of extraction, 0.1 ml of blank plasma ($n=3$) and 0.5 ml of blank urine ($n=3$) were spiked with both 1 and 10 μg/ml racemic PR, in the absence of I.S. Following extraction from plasma, 2 ml of the organic layer were evaporated, and the residue was reconstituted with methanol (200 μl) and 10 μg/ml I.S. (50 μl). The residue from the extracted urine was reconstituted with methanol (500 μl) and 50 μg/ml I.S. (50 μl). The analysis of underivatized PR was carried out using a mobile phase consisting of 0.06 *M* KH_2PO_4–acetonitrile–TEA (75:25:0.1). The peak-area ratios PR/I.S. were compared with those of solutions directly injected into the HPLC column.

Derivatization yield

Plasma samples ($n=10$) containing 20 μg/ml of each PR enantiomer were subjected to extraction as described under *Sample preparation*. In five samples, PR was derivatized to the amide derivatives, while the remaining samples were dissolved in 200 μl of 50 m*M* TEA in acetonitrile. Derivatization yield was calculated by comparing the total peak areas of the *S*- and *R*-PR in the derivatized and underivatized samples. The analysis was accomplished using the method described for extraction efficiency.

Accuracy and precision

Various concentrations of PR (Table I) were added to plasma, and the enantiomers were quantified individually using calibration curves. Accuracy was calculated based on the mean percentage error [(mean measured concentration − expected concentration)/expected concentration × 100%], while precision was evaluated by calculating intra- and inter-day coefficients of variation (C.V.).

Pyrrole analogue synthesis

Because pure PYR was not available, DDQ was used to oxidize PR to PYR. A 0.1% solution of DDQ in toluene was prepared [8]. A volume of 0.2 ml DDQ solution was added to a minute quantity of racemic PR powder; this was allowed to react at room temperature for 24 h to allow for complete oxidation. The PYR analogue was derivatized and analyzed with a mobile phase of 0.06 *M* KH_2PO_4–acetonitrile–TEA (64:36:0.1).

Pharmacokinetic studies

Three female Sprague–Dawley rats were studied (weight, 262 ± 5.8 g). Surgery was performed as previously described [10]. Racemic PR was dispersed in 2% methylcellulose and given as 10 mg/kg oral doses to the rats by gastric intubation. Blood (0.1–0.2 ml) was collected from the jugular vein cannula at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after drug administration. After each blood sample collection, the catheter was filled with 100 U/ml heparin. Cumulative 24 h urine was collected. Each blood sample was immediately centrifuged for 3 min to permit separation of plasma. All plasma and urine samples were stored at −20°C until analyzed.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY FOR *R*- AND *S*-PIRPROFEN ENANTIOMERS

Expected concentration (mg/l)	*n*	Mean measured concentration (mg/l)		Mean absolute error (%)		Coefficient of variation (%)	
		R	*S*	*R*	*S*	*R*	*S*
Intra-day							
0.1	5	0.11 ± 0.023	0.12 ± 0.011	13.9	19.5	20.0	10.4
0.5	5	0.52 ± 0.041	0.56 ± 0.031	4.1	11.2	7.9	6.2
1.0	11	1.1 ± 0.10	1.1 ± 0.09	4.8	6.4	9.7	8.3
5.0	5	5.3 ± 0.16	5.3 ± 0.15	5.6	5.4	3.0	2.9
20.0	11	19.4 ± 0.89	19.4 ± 0.86	3.0	3.1	4.6	4.5
Inter-day							
0.1	3	0.12 ± 0.007	0.12 ± 0.005	15.3	23.7	5.9	4.1
0.5	3	0.45 ± 0.075	0.51 ± 0.013	9.3	0.93	16.5	2.5
1.0	7	1.1 ± 0.11	1.1 ± 0.11	7.2	14.3	10.2	10.0
5.0	7	5.1 ± 0.44	5.1 ± 0.43	1.3	2.5	8.6	8.4
20.0	7	20.0 ± 0.97	20.1 ± 1.0	0.050	0.51	4.8	4.8

Data analysis

Pharmacokinetic data for the area under the plasma–concentration *versus* time curve from 0 h to infinity (AUC), oral clearance (Cl/F) and volume of distribution (V_d/F) were calculated as previously described [11], assuming no chiral inversion. Statistical significance was evaluated using Student's paired t-test at $\alpha = 0.05$. Values are expressed as mean ± S.D.

RESULTS

The chromatograms of blank samples, drug-spiked standards and *in vivo* samples are depicted in Fig. 2. Because optically pure PR enantiomers were unavailable to us, the order of elution of the PR enantiomers was presumed to be the same as that of other 2-APA analogues, derivatized and chromatographed using similar techniques [9,12–16]. Therefore, the peaks representing I.S. diastereomers eluted at 8.8 and 10.1 min, whereas those of the *R*- and *S*-PR diastereomers eluted at 12.4 and 14.2 min, respectively. Each of the I.S. and PR peaks were free of interference from plasma and urine constituents.

The PYR product generated using DDQ was

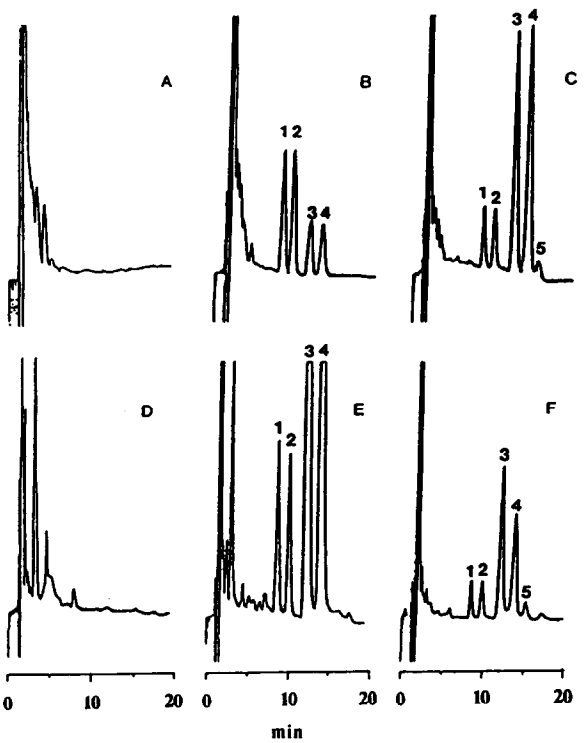

Fig. 2. HPLC profiles of (A) blank plasma, (B) plasma spiked with 1 mg/ml racemic PR, (C) an 8-h rat plasma sample, (D) blank urine, (E) urine spiked with 5 mg/l racemic PR and (F) a 24-h cumulative rat urine sample. Peaks: 1 and 2 = I.S.; 3 = *R*-PR; 4 = *S*-PR; 5 = *S*-PYR.

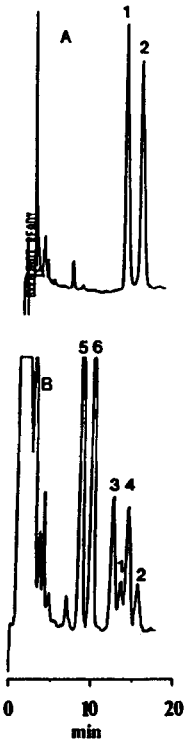

Fig. 3. Chromatograms of (A) pyrrole metabolite and (B) a small amount of pyrrole derivative added to plasma spiked with 1 mg/l PR. Peaks: 1 = *R*-PYR; 2 = *S*-PYR; 3 = *R*-PR; 4 = *S*-PR; 5 and 6 = I.S.

chromatographed after undergoing the same sample preparation mentioned above. The diastereomeric derivatives of *R*- and *S*-PYR eluted at 13.6 and 15.8 min, respectively. As seen in Fig. 3, *R*-PYR eluted between the peaks of the PR enantiomers.

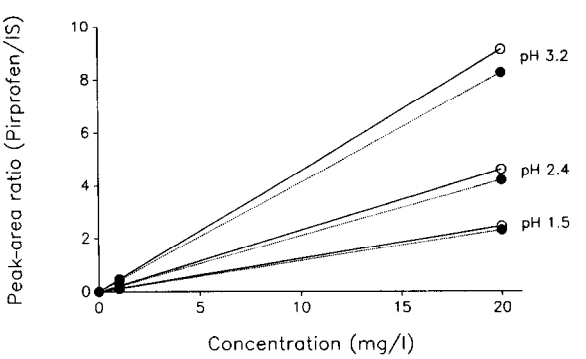

Fig. 4. Effects of the addition of ascorbic acid (pH 3.2), sulfuric acid (pH 2.4), and both acids (pH 1.5) on the extraction of PR enantiomers. Extraction was studied at 1 and 20 mg/l of each PR enantiomer, and 5 mg/l I.S. Key: ● = *S*-PR; ○ = *R*-PR.

The influence of extraction with L-ascorbic acid, and the sample pH, is depicted in Fig. 4. The most favourable extraction conditions were seen when ascorbic acid was added to plasma alone. When ascorbic acid was added to the samples in combination with H_2SO_4, the extraction efficiency of the PR enantiomers was reduced. The peak areas of the I.S. peaks were not affected by the conditions depicted in Fig. 4. The optimal pH for extraction occurred at 4.1; at pH values outside the range 3.1–4.1, the peak areas of PR enantiomers decreased markedly.

Under the conditions stated above for determination of the extraction and derivatization yield, the underivatized I.S. and PR peaks eluted at 4.9 and 6.3 min, respectively. The yield of (*S* + *R*)-PR from plasma samples after extraction was 81 ± 4.8 and 85 ± 3.5% at 1.0 and 10 μg/ml racemate, respectively. The derivatization reaction took place at ambient temperature with 97.02 ± 2.7% overall derivatization yield within 3 min.

Excellent linear correlations ($r^2 > 0.999$) were present between the peak-area ratios (*R*-PR/I.S. and *S*-PR/I.S.) and the corresponding plasma and urine concentrations over the range of concentrations examined (Table I). A typical plasma standard curve could be described by $y = 0.616x - 0.024$ and $y = 0.574x - 0.021$ for the *R*- and *S*-PR, respectively, where y represents peak-area ratio (PR enantiomer/I.S.) and x represents PR concentration. In urine, the standard curves were best described by $y = 0.526x - 0.129$ and $y = 0.489x - 0.112$ for *R*- and *S*-PR, respectively.

The method was accurate and precise. At different concentrations, the associated error ranged from 0.05 to 23.7% and the C.V. was between 2.0 and 17.9% (Table I). Based on 100 μl of rat plasma, the limit of quantitation was 0.1 μg/ml for each PR enantiomer.

No derivatized PYR enantiomers were detected in any of the PR-spiked plasma or urine samples (Fig. 2). However, a small peak was seen near the position where the *S*-PYR usually elutes in the biological samples (Fig. 2). This peak first appeared in the 3–4 h plasma samples, and increased in size over time.

TABLE II

PHARMACOKINETIC INDICES OF PR ENANTIOMERS AFTER ORAL ADMINISTRATION OF 10 mg/kg RACEMATE TO FEMALE RATS

No.	$t_{1/2}$ (h)		AUC (mg h l^{-1})		S/R AUC	Cl/F (ml h kg^{-1})		V_d/F (ml kg^{-1})		ΣXu[a] (% of dose)	
	R	*S*	*R*	*S*		*R*	*S*	*R*	*S*	*R*	*S*
1	6.2	7.0	198	240	1.21	25.3	20.8	226	210	5.0	3.8
2	4.0	4.7	149	197	1.32	33.4	25.3	193	172	1.4	1.4
3	4.6	4.8	136	153	1.13	37.6	33.4	250	231	3.9	2.7
Mean	4.9	5.5	161	197[b]	1.22	32.1	26.5[b]	223	204	3.4	2.6
S.D.	1.1	1.3	32.7	43.5	0.095	6.25	6.39	28.6	29.9	1.9	1.3

[a] Urinary recovery of conjugated and intact drug.
[b] Significantly different from the *R* enantiomer.

The pharmacokinetic indices of PR in the rats are depicted in Table II and Fig. 5. The AUC was significantly greater for *S*-PR as compared with *R*-PR (mean $S/R = 1.19$). After alkali hydrolysis the plasma concentration *versus* time profiles were almost identical to the unhydrolysed samples, indicating no accumulation of acylglucuroconjugates of PR. The mean total recoveries in urine of intact and glucuroconjugated parent drug were 3.4 ± 1.9% (range 1.4–5.0%) and 2.6 ± 1.2% (range 1.4–3.8%) for *R*-PR and *S*-PR, respectively. A negligible amount of PYR was recovered in urine.

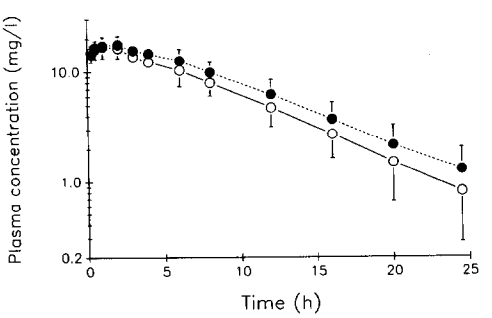

Fig. 5. Mean plasma concentration *versus* time profiles for *R*-PR (○) and *S*-PR (●) in three female rats dosed with 10 mg/kg racemate orally.

DISCUSSION

The assay described here was sensitive, specific and suitable for the study of PR enantiomers in rat plasma. Derivatization of the drug with L-leucinamide resulted in well resolved peaks of the PR diastereomers, the resolution factor being 1.6. Furthermore, the assay represented an improvement over the previously published assay by Sioufi *et al.* [7], as the time required for derivatization was only 2.5 min, rather than 35 min.

In general the *R* enantiomers of 2-APA NSAIDs seem to elute before the corresponding *S* enantiomers when L-leucinamide is used to derivatize the drug, and reversed-phase HPLC separation is employed [9,12–16]. Unfortunately, optically pure PR enantiomers were not available to us. However, Sioufi *et al.* [7] had reported that the mean post-distributive plasma concentrations of *S*-PR were greater than those of *R*-PR in eleven human volunteers given 200 mg of racemic PR. In our hands, 7 h after administration of 200 mg of racemic PR to a normal, healthy, consenting male volunteer (age, 23 years), the second eluted PR peak was 22% greater than the first peak. This confirms that the *R*-PR derivative elutes before the *S*-PR derivative.

Due to the possibility of spontaneous oxidation to the PYR by-product [8], it is important to

prepare PR samples in the shortest possible time. Under the conditions described in this paper, it took less than 40 min to fully prepare a set of twelve samples.

To minimize decomposition of PR during extraction [17], L-ascorbic acid was used to acidify the samples. L-Ascorbic acid yielded a higher extraction efficiency than either 0.06 or 0.6 *M* H_2SO_4. To assess whether this was due to an antioxidant effect, or to pH, L-ascorbic acid was added to biological samples which were already acidified with H_2SO_4 (Fig. 4). A decrease occurred in the PR/I.S. ratio, indicating that the greater extraction of PR afforded by L-ascorbic acid was not due to its antioxidant properties, but rather to a favourable extraction pH of 3.2. The maximal range of pH for extraction (3.1–4.1) encompassed the isoelectric point of PR [1], which is 3.8.

The stability of PR during the assay procedure was confirmed as no PYR was detected in the standard plasma and urine samples (Fig. 2). More importantly, the absence of PYR in the 15-min rat samples supported that PR is stable during sample collection, storage and analysis. Hence the small *S*-PYR peak, beginning to appear in the 3–4 h plasma samples, can be attributed to metabolic oxidation of PR in the rat. The amount of *S*-PYR formed, however, was relatively low in comparison with the concentrations of *S*-PR seen in the rat.

Although we could not rule out the possibility of racemization during derivatization, its occurrence would not seem likely because racemization of other NSAIDs in assays using a similar derivatization reaction has not been observed [9,12–16].

The assay was suitable for use in a pharmacokinetic study of PR in the rat, because, while the minimum quantifiable concentration was 0.1 mg/l (Table I), the 24-h concentrations of PR enantiomers in the rat ranged from 0.42 to 1.4 mg/l for *R*-PR and 0.59 to 2.1 mg/l for *S*-PR (Fig. 5). Similar to humans, in female rats the concentrations of *S*-PR exceeded those of its antipode (Table II; Fig. 5).

Our data do not provide information as to the possibility of chiral inversion of the inactive *R* enantiomer to active *S*-PR. In the rat this novel metabolic pathway is shared by other 2-APA NSAIDs, such as fenoprofen [18] and ketoprofen [19]. The difference between the enantiomers of PR may also be due to other factors, such as stereoselectivity in other routes of metabolism or protein binding, which is the case for the enantiomers of flurbiprofen [11] and etodolac [10]. The excretion of unchanged and conjugated PR was limited in urine. This is in line with the work of Egger *et al.* [20], who found that the urinary excretion of PR and PYR in the rat accounted for about 5% of the radioactively labelled dose of PR. Pirprofen has been reported to undergo extensive metabolism in the rat, mainly to the diol and amino metabolites [20].

It should be noted that the enantiomers of PR could also be derivatized and separated using a method previously applied to stereospecific analysis of etodolac enantiomers [21]. That procedure involves formation of PR diastereomers by derivatization with ethyl chloroformate and *R*-(+)-α-phenylethylamine, and separation using normal-phase chromatography. Although the resolution afforded by that method was greater than that reported here (resolution factor > 2), the use of L-leucinamide appeared to provide a greater sensitivity, based on a more stable baseline in the region where the PR diastereomers eluted, for PR enantiomers in the small volumes of plasma obtained from the rat.

In conclusion, the assay was sensitive, rapid, reproducible and suitable for the study of the pharmacokinetics of PR enantiomers in the rat. Although PR was recently removed from the pharmaceutical market owing to several known cases of hepatotoxicity [22], its study may nevertheless contribute to our knowledge of the mechanisms of enantioselective pharmacokinetics.

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