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Direct high-performance liquid chromatographic resolution of the enantiomers of tiaprofenic acid using immobilized human serum albumin

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ABSTRACT

Resolution of racemic tiaprofenic acid (TA) has been performed using immobilized human serum albumin as the stationary phase. The eluent was phosphate buffer–acetonitrile–*n*-octanoic acid (90:10:0.015, v/v). Detection was achieved at 305 nm. The pharmacokinetics of the enantiomers were studied following oral administration into humans and after subcutaneous injection in rats. Plasma concentrations of (+)-TA were much greater than those of (−)-TA. For the rat, the pharmacokinetic parameters between (−)-TA and (+)-TA were all statistically different ($p < 0.005$).

INTRODUCTION

The non-steroidal anti-inflammatory drugs (NSAIDs) encompass a variety of structural classes, but despite this structural diversity, chirality is a feature that distinguishes certain NSAIDs from all others [1]. Some NSAIDs contain a chiral atom and therefore exist as two enantiomers. Most of these chiral agents belong to the class of the arylpropionic acids. It was reported that the anti-inflammatory activity of chiral NSAIDs, through the inhibition of prostaglandin synthesis, is exerted *in vitro* by the (*S*)-enantiomer only [2], whereas analgesic effects may be related to both enantiomers [3]. Moreover, enantioselective interactions with chiral biological receptors may lead to pharmacodynamic differences between enantiomers [4,5] and may also be involved in stereoselective processes such as distribution, metabolism and excretion. Several chiral NSAIDs undergo unusual metabolic events for which substrate stereochemistry plays a crucial role: inversion of configuration [6–9] and incorporation into triacylglycerols [10,11]. For these NSAIDs, the (*R*)-enantiomer, inactive

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in vitro, is transformed into the corresponding (*S*)-enantiomer, active *in vitro*, in humans and different animal species [12–14].

The need to distinguish between enantiomers and determine them accurately in biological media is essential [15–17]. Resolution methods for enantiomers, achieved principally by chromatographic techniques, can be classified as direct or indirect. Indirect methods are based on the reaction of a racemic mixture with a chiral reagent to form a pair of diastereoisomers [18]. These diastereoisomers have different physicochemical properties, unlike enantiomers, and may be separated. Direct resolution of enantiomers may be performed either with a chemically bonded chiral stationary phase or with a conventional stationary phase eluted by a chiral component [19,20].

Tiaprofenic acid (TA) is an effective anti-inflammatory drug and an analgesic agent in the treatment of osteoarthritis, rheumatoid arthritis and acute pain states resulting from trauma or minor surgery [21]. Owing to the presence of a chiral carbon atom on the propionic acid side-chain, TA exists as a mixture of two enantiomers. A survey of relevant literature revealed indirect stereoselective methods, relying on gas chromatography [22] or high-performance liquid chromatography (HPLC) [8].

Resolution of TA enantiomers was first performed using L-leucinamide to form diastereoisomeric amide derivatives of TA, which were separated by reversed-phase HPLC [8]. Unfortunately, using each enantiomer separately, the two peaks of the racemate were also present, indicating complete racemization during the derivatization, because the ratio of the diastereoisomers obtained was 0.97 ± 0.02. Several coupling agents, including N,N′-dicyclohexylcarbodiimide, 1,1′-carbonyldiimidazole, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine and 1-hydroxybenzotriazole, were tested [23]. The racemization was reduced but not suppressed.

Since racemization was always observed during the derivatization, a direct resolution method was performed, using a chiral stationary phase (CSP). Stereoselective HPLC analysis often involves the use of proteins immobilized on a solid support as the chiral selector [9]. Several direct resolutions of underivatized 2-arylpropionic acid NSAIDs using this class of CSP, based on either bovine serum albumin [24], ovomucoid [25] or second-generation silica-bound α_1-acid glycoprotein (AGP) [26–30], are described in the literature.

In this study, we developed a technique using human serum albumin (HSA) as the chiral macromolecule of the stationary phase to separate the enantiomers of TA, according to the well known stereoselective protein binding of NSAIDs to this protein [4]. The immobilization of the protein to the silica surface does not significantly affect the drug binding properties [31] or the conformational mobility of the protein native form [32]. This CSP has already been employed for the stereochemical resolution of several chiral 2-arylpropionic acid NSAIDs [33]. In an analogous manner, we applied this technique to the separation of the optical isomers of TA.

Dansylsarcosine (DS) was chosen as the internal standard (I.S.) because it is bound to the same primary site as TA [23] and was thus expected to be eluted under the same conditions.

EXPERIMENTAL

Chemicals and reagents

TA (racemate and enantiomers) was a gift from Roussel (Paris, France). DS and *n*-octanoic acid (99 + %) were purchased from Sigma (L'Isle d'Abeau, France). Acetonitrile of chromatographic purity was obtained from SDS (Villeurbanne, France). All the products used were of analytical grade, and water was bidistilled (Merck, Nogent sur Marne, France).

DS and TA solutions were prepared in methanol in 1 mg/ml and stored at 4°C. Working solutions were obtained daily after dilution in water.

Biological samples

Human blood samples were collected after a single ingestion of 200 mg of racemic TA by two drug-free volunteers at 1, 3.50 and 5.50 h. Plasma was immediately harvested from the blood by centrifugation and kept at −80°C until analysis.

Four male Wistar rats (300–320 g) were purchased from Iffa Credo (L'Arbresle, France). The animals were allowed free access to food and water throughout the study. Racemic TA was prepared in mannitol (10%, 25 mg/ml) before injection. This suspension was administered subcutaneously to rats at a dose of 5 mg per rat. After slight anaesthesia with diethyl ether, blood samples (0.3 ml per puncture) were collected by cardiac puncture at 0.50, 1, 1.50, 3, 4 and 6 h. The samples were centrifuged immediately and plasma was stored at −80°C.

Apparatus

HPLC was performed using a Waters (Saint-Quentin en Yvelines, France) apparatus consisting of a Model 510 pump, a Model 490E variable-wavelength UV spectrophotometer with a four-channel detection device. Acquisitions and calculations were achieved with Maxima software (Waters) installed on an IBM-AT microcomputer. The separation was performed on a HSA-CSP column (SFCC-Shandon, Eragny, France) after injection with a Rheodyne Model 7125 injector equipped with a 50-μl loop.

Chromatographic conditions

The mobile phase was sodium dihydrogenphosphate–disodium hydrogenphosphate (0.05 *M*, pH 7)–acetonitrile–*n*-octanoic acid (90:10:0.015, v/v). A flow-rate of 1 ml/min was applied, and the UV detection wavelength was 305 nm, at room temperature.

Extraction

An acidic liquid–liquid extraction of the samples with 3 ml of diethyl ether was achieved after addition of 1 *M* HCl as described previously [34]. The dry residue was dissolved in the chromatographic eluent (200 μl), and 20 μl were injected at the top of the column.

Pharmacokinetic analysis

The peak concentration (C_{max}) and the corresponding time (t_{max}) were obtained from examination of the individual data.

Pharmacokinetic parameters were established, using a personal software installed on a IBM-AT after interpolation by use of third-degree Lagrange polynomials for each time interval, and terminal monoexponential extrapolation using at least three final points. The area under the plasma concentration–time curve (AUC), the mean residence time (MRT) and its variation (VRT) were calculated from 0 to 6 h. Elimination half-life ($t_{1/2}$) and elimination rate constant (k_e) were estimated from the terminal extrapolation.

Significance of the observed differences between enantiomers was carried out using Student's paired *t*-test ($\alpha = 0.05$). Statistical analysis of optical stability with time in various conditions of temperature and pH was performed using ANOVA for multiple comparisons with a single control group (initial conditions) (Statview SE plus graphics, Abacus concepts, Berkeley, CA, USA, 1988, running on a Macintosh).

RESULTS AND DISCUSSION

Validation of the technique

Retention time. Under the stated conditions, DS was eluted with a retention time of 4.2 min. The elution order of the TA isomers was determined with enantiomers assayed individually: the levorotatory form is eluted prior (8.3 min) to the dextrorotatory isomer (10.3 min) (Fig. 1).

Optical purity. When (−)-TA was directly injected it was found to contain a maximum of 6% of the (+)-enantiomer. Similarly, (+)-TA contained 6% of (−)-TA. This proportion was due to an optical impurity and not to racemization occurring in the eluent: it was verified that this proportion did not vary with the time of presence in this eluent over a period of 2 h, as would be the case if inversion had occurred.

Extraction efficiencies. The extraction efficiency of TA enantiomers and of DS was measured by comparison of their peak areas after injection of extracted samples into the HPLC system with those obtained after direct injection. The extraction recoveries obtained for both (−)-TA and (+)-TA (10 μg/ml) were 94.4%, in water and plasma, *i.e.* the same as previously obtained [34]. The recovery of DS was 54%. Each enantiomer

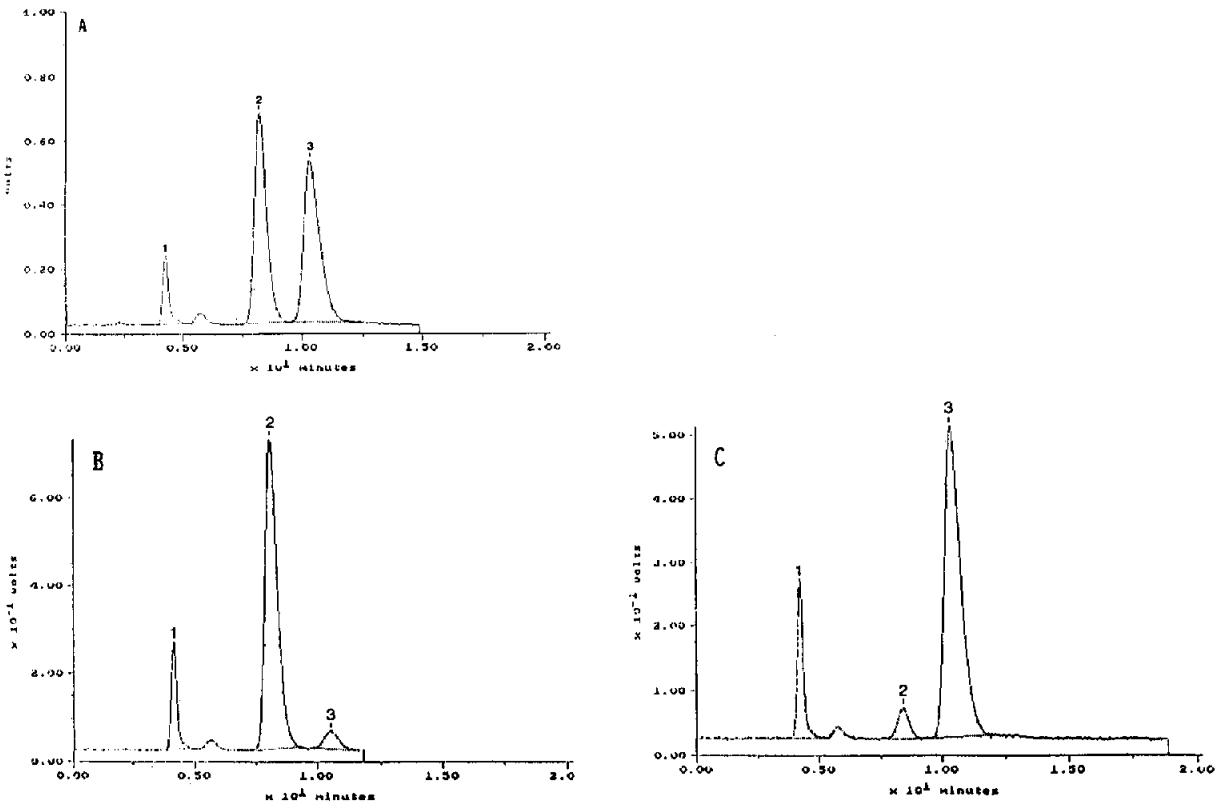

Fig. 1. Resolution of TA: (A) racemic TA (10 μg injected); (B) levorotatory TA (5 μg injected); (C) dextrorotatory TA (5 μg injected). Chromatographic conditions: detection wavelength, 305 nm; flow-rate, 1 ml/min; room temperature; column, HSA-CSP. Peaks: 1 = DS; 2 = (−)-TA; 3 = (+)-TA.

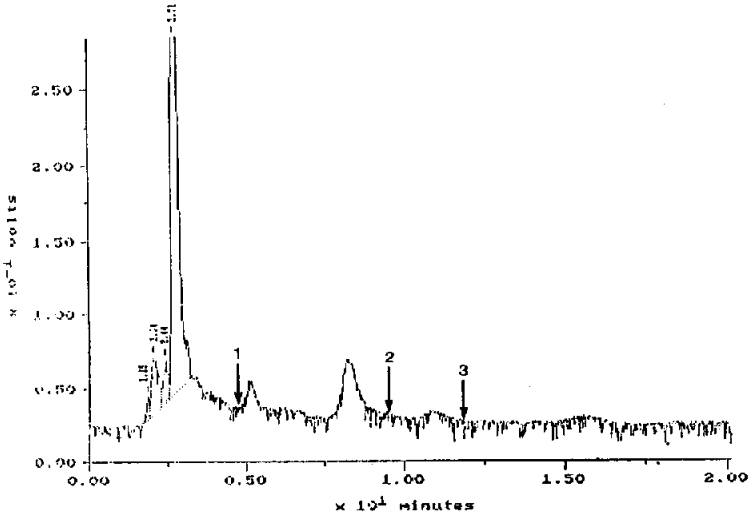

Fig. 2. Interference of a blank plasma sample. Chromatographic conditions as in Fig. 1.

TABLE I

STANDARD CURVE IN PLASMA

Concentrations in plasma were calculated with a calibration curve established in water. $y = ax + b$, where y = total amount of TA [(−) and (+)]; x = peak-area ratio between TA [(−) or (+)] and DS; a and b = regression parameters; r = correlation coefficient (n = 8).

Racemic TA added (μg)	(−)-TA (μg)	(+)-TA (μg)
5	2.49	2.49
2	0.996	0.996
1	0.497	0.497
0.5	0.249	0.249
0.25	0.125	0.125
0.1	0.0492	0.0495
0.05	0.0251	0.0252
0.025	0.0125	0.0127
a	0.996	0.996
b	−0.0001	−0.000002
r	1	1

was extracted alone: the extraction coefficient was the same and the optical purity was preserved.

Interferences. The extraction of a blank plasma sample did not produce any interfering peaks (Fig. 2). All the NSAIDs marketed were examined under the same conditions. None gave a chromatographic peak close to those of the TA enantiomers.

Linearity. The assay linearity for each enantiomer was determined by performing linear regression analysis on the plot of the peak-area ratio of (−)-TA or (+)-TA over DS *versus* amounts of racemic TA in the range 0.025–5 μg. Correlation coefficients obtained were typically 0.99995. A standard curve in water could be described by $y = 0.428x + 0.00457$ and $y = 0.428x + 0.00454$, for the (−) and (+) enantiomers, respectively, where y is the peak-area ratio [(−)- or (+)-TA/DS] and x is the TA racemic amount. The same curve over a range varying from 0.025 to 5 μg was also obtained in plasma; a perfect correlation was observed with the calibration curve established in water, allowing all subsequent determinations to be made in water (Table I).

Assay precision. Control standards were prepared by adding known amounts of racemic TA to water. Precision and intra-series reproducibility over at least six assays were determined for 0.025, 0.1, 1 and 10 μg of racemic TA. The coefficients of variation (C.V.) ranged from 1.63% at the highest level to 5% at the lowest concentration (Table II). The reproducibility of the determination in the plasma of a treated patient was also evaluated over eight assays (Table II) and the C.V. was of the order of 0.3% (0.31% for (+)-TA and 0.40% for (−)-TA). The inter-day variability was established in water by adding 10 μg of racemic TA (eight assays; C.V. *ca.* 3.8%) and in a patient's plasma assayed regularly over six months: these results show the stability of the sample during the storage period at −80°C (Table III).

Limit of detection. Using the criterion of mini-

TABLE II

INTRA-SERIES REPRODUCIBILITY OF KNOWN AMOUNTS OF RACEMIC TA ADDED TO WATER AND OF THE PLASMA OF ONE TREATED PATIENT

Amount of racemic TA added (μg)	n	Amount found (mean ± S.D.) (μg)		C.V. (%)		Precision (%)	
		(−)-TA	(+)-TA	(−)-TA	(+)-TA	(−)-TA	(+)-TA
10	9	5.11 ± 0.084	5.11 ± 0.084	1.63	1.64	2.28	2.24
1	9	0.494 ± 0.026	0.494 ± 0.027	5.35	5.42	1.27	1.22
0.1	9	0.0483 ± 0.001	0.0484 ± 0.001	2.17	2.01	1.22	3.11
0.025	6	0.0122 ± 0.0005	0.0120 ± 0.0007	4.43	5.50	2.40	2.40
Patient plasma	8	0.829 ± 0.003	0.958 ± 0.003	0.40	0.31	–	–

TABLE III

INTER-SERIES REPRODUCIBILITY OF A KNOWN AMOUNT OF RACEMIC TA ADDED TO WATER AND OF THE PLASMA OF ONE TREATED PATIENT

The amount of racemic TA added was 10 μg.

	In added racemic TA		In patient plasma	
	(−)-TA	(+)-TA	(−)-TA	(+)-TA
	4.43	4.43	6.12	7.21
	4.74	4.74	6.17	7.22
	4.51	4.51	6.16	7.22
	4.90	4.90	6.52	7.64
	4.40	4.40		
	4.75	4.75		
	4.61	4.60		
	4.72	4.72		
Mean (μg)	4.63	4.63	6.24	7.32
S.E. (μg)	0.062	0.062	0.093	0.106
C.V. (%)	3.78	3.79	2.98	2.89

mum detectability as three times the system noise, the detection limit for each TA enantiomer was 10 ng in water (Fig. 3). This value is also the lower limit of the assay, because for the addition of 25 ng of racemic TA to water, the C.V. of six determinations was near 5% (Table II). The difference in the retention times of the enantiomers between Figs. 1 and 4 can be attributed to the evolution of the protein column; however, the eluted peaks were validated with samples containing greater concentrations of racemate.

Optical stability. In order to verify that no spontaneous racemization, as observed during derivatization with L-leucinamide, occurred when the enantiomers of TA were exposed at several conditions, the stability of (−)- and (+)-TA (2 μg/ml) was evaluated at different temperatures and three pH values (6, 7.4, 8.5). The data are expressed as the percentage of optical impurity (Tables IV and V). The initial conditions correspond to the determination of the optical impurity immediately after addition of each enantiomer. For each isomer, the results obtained at various temperature and pH conditions were not statistically different from this initial state [for (−)-TA, $p > 0.80$; for (+)-TA, $p > 0.30$], suggesting that the enantiomers are very stable.

Application: stereoselective pharmacokinetics of TA

The curves of mean TA enantiomer plasma concentration *versus* time following the subcutaneous administration of the racemic drug to four rats are shown in Fig. 4. The pharmacokinetic parameters calculated from the individual data are compiled in Table VI. Fig. 4 indicates a

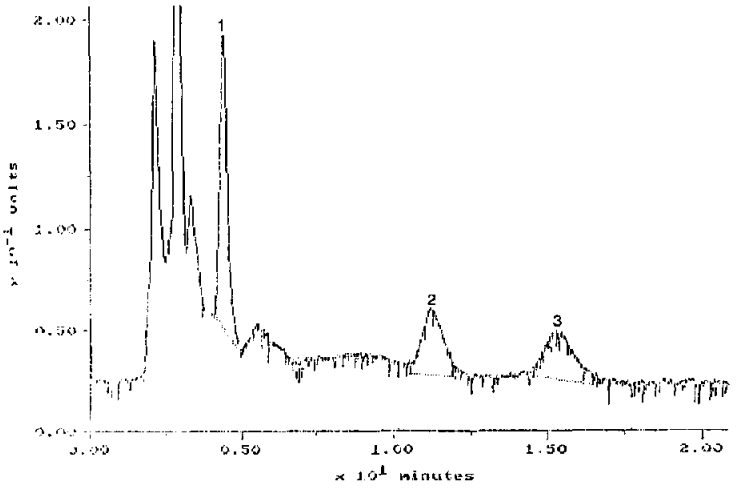

Fig. 3. Limit of detection of each enantiomer in water (10 ng of each TA enantiomer). Chromatographic conditions as in Fig. 1.

TABLE IV

STABILITY ASSAY OF (−)-TA AT VARIOUS TEMPERATURES AND pH VALUES

Initial conditions (% of optical impurity): 7.281, 6.000, 5.497 and 4.949 (mean = 5.932; S.E. = 0.499).

pH	Assayed optical impurity (%)			
	37°C, 3 h	20°C, 3 h	4°C, 24 h	−20°C, 72 h
6	5.643	6.072	5.767	6.020
7.4	5.402	5.945	5.869	5.858
8.5	5.627	5.634	5.784	6.301

TABLE V

STABILITY ASSAY OF (+)-TA AT VARIOUS TEMPERATURES AND pH VALUES

Initial conditions (% of optical impurity): 6.371, 5.045 and 5.157 (mean = 5.524; S.E. = 0.421).

pH	Assayed optical impurity (%)			
	37°C, 3 h	20°C, 3 h	4°C, 24 h	−20°C, 72 h
6	6.227	6.109	5.848	5.956
7.4	5.640	5.921	5.426	5.804
8.5	5.549	5.956	5.483	5.764

monoexponential decline, corresponding to a one-compartment model, for each individual enantiomer kinetics.

The plasma concentrations of the (+)-enantiomer were larger than those of (−)-enantiomer; this difference was statistically significant from the first hour after drug administration. The mean value of the peak plasma concentrations (C_{max}) (33.1 μg/ml) for the (+)-enantiomer was significantly greater than that of the (−)-enantiomer (27.29 μg/ml; $p < 0.0015$). For all the pharmacokinetic parameters, the value for (+)-TA was significantly greater than for its optical antipode ($p < 0.0002$, $p < 0.0001$, $p < 0.0027$ and $p < 0.0024$, for AUC, MRT, VRT and $t_{1/2}$, respectively).

In humans, the same difference was observed between enantiomers (Fig. 5): plasma concentra-

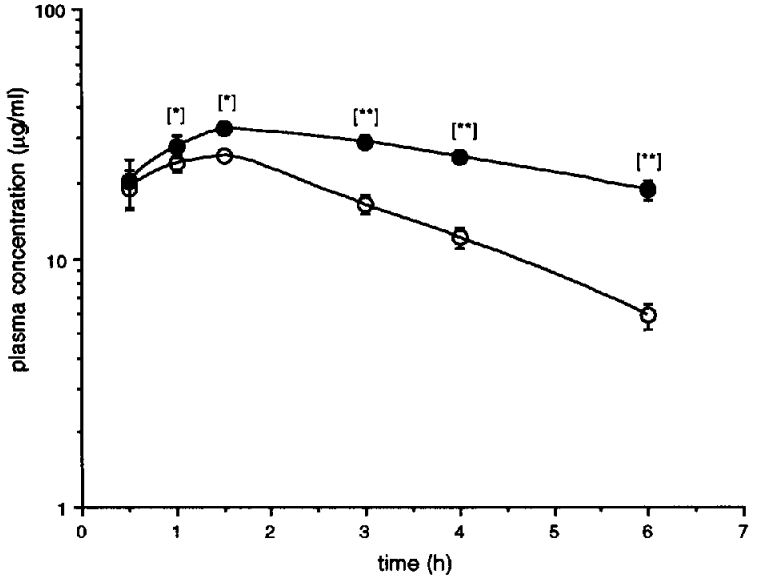

Fig. 4. Mean TA enantiomer log plasma concentration–time curves following s.c. administration of the racemic drug (5 mg) to four rats: (○) (−)-TA; (●) (+)-TA; (*) $p < 0.05$; (**) $p < 0.005$. Values are mean ± standard error.

TABLE VI

PHARMACOKINETIC PARAMETERS IN RATS OF EACH ENANTIOMER FOLLOWING SUBCUTANEOUS ADMINISTRATION OF RACEMIC TA

Mean and S.E. are expressed in the units corresponding to each parameter. Dose of racemic TA, 5 mg.

Rat No.	C_{max} (μg/ml)			t_{max} (h)		AUC_{6h} (μg/ml)			MRT (h)			VRT (h)			$t_{1/2}$ (h)		
	(−)-TA	(+)-TA	(+)/(−)	(−)-TA	(+)-TA	(−)-TA	(+)-TA	% (+)	(−)-TA	(+)-TA	(+)/(−)	(−)-TA	(+)-TA	(+)/(−)	(−)-TA	(+)-TA	(+)/(−)
1	26.06	33.25	1.276	1.5	1.5	96.354	158.38	62.17	2.648	3.066	1.158	2.165	2.370	1.095	2.444	5.440	2.226
2	29.62	35.34	1.193	1.5	1.5	105.79	165.21	60.96	2.497	2.973	1.191	2.166	2.458	1.135	2.121	5.620	2.650
3	25	29.69	1.188	1.5	1.5	86.83	138.07	61.39	2.515	3.007	1.196	2.066	2.308	1.117	1.960	5.219	2.663
4	28.48	34.12	1.198	0.5	1	84.87	147.11	63.42	2.221	2.691	1.212	2.211	2.549	1.153	1.930	4.024	2.085
Mean	27.29	33.1	1.214	1.25	1.375	93.461	152.193	61.985	2.470	2.934	1.189	2.152	2.421	1.125	2.114	5.076	2.406
S.E.	1.065	1.215	0.021	0.25	0.125	4.814	6.007	0.54	0.09	0.083	0.011	0.031	0.053	0.012	0.118	0.360	0.147

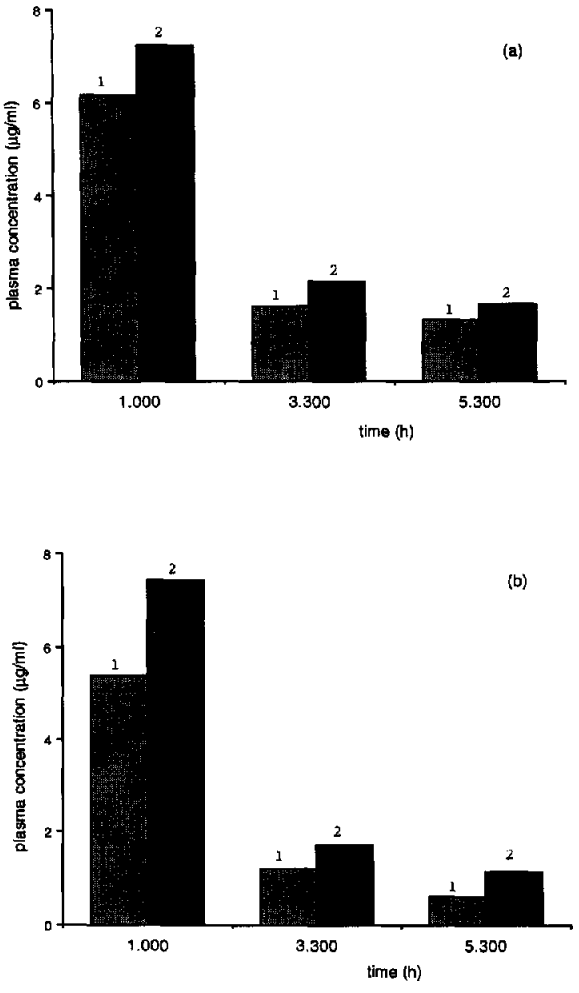

Fig. 5. TA enantiomer plasma concentrations following oral administration of the racemic drug (200 mg) to two healthy volunteers (a and b): (1) (−)-TA; (2) (+)-TA.

tions of (+)-TA were much greater than those of (−)-TA. The ratio (+)/(−) varied from 1.2 to 1.9.

Our results indicate that, like most 2-arylpropionic acid anti-inflammatory drugs, TA enantiomers have different disposition kinetics [35,36]. These data are not consistent with those described by Mehvar *et al.* [8], who observed no stereoselective pharmacokinetics owing to the unsuitability of their technique. The higher concentration of the dextrorotatory form could be due to chiral inversion or to stereoselectivity in other metabolic routes or to protein binding, which is the case for the optical isomers of flurbiprofen [35] or etodolac [37].

CONCLUSION

This assay is sensitive, reproducible and convenient for the study of the pharmacokinetics of TA enantiomers. Moreover, it highlights the need to use optically pure isomers, when possible, to verify the absence of racemization during derivatization, because it could result in erroneous conclusions concerning the stereoselective disposition of chiral drugs administered as racemates.

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