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RAPID AND SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF TIAPROFENIC ACID ENANTIOMERS IN HUMAN PLASMA AND URINE

REZA MEHVAR, FAKHREDDIN JAMALI* and FRANCO M. PASUTTO

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2N8 (Canada)

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SUMMARY

A sensitive stereospecific high-performance liquid chromatographic assay of tiaprofenic acid (TA) enantiomers in human plasma and urine was developed. The biological specimens are acidified, and the drug and internal standard, (±)-2-(4-benzoylphenyl)butyric acid, extracted with an isooctane–isopropanol (95:5) mixture (plasma) or chloroform (urine), followed by sequential reaction of the enantiomers with trichloroethyl chloroformate and L-leucinamide. The reactions were complete at ambient temperature in less than 3 min. The diastereoisomers of TA and internal standard were then extracted into chloroform. The organic layer was evaporated, and the reconstituted residue chromatographed at ambient temperature on a C_{18} reversed-phase column with a mobile phase consisting of 0.06 *M* monopotassium phosphate–acetonitrile–triethylamine (65:35:0.02) at a flow-rate of 1 ml/min. The TA diastereoisomers were detected at 310 nm, free of interfering peaks, with a resolution factor of 2.1. Within the examined plasma and urine enantiomeric concentration ranges of 0.2–20 and 10–100 mg/l, respectively, an excellent linear relationship was obtained between the peak-area ratios and the corresponding concentrations. The assay was reproducible and sufficiently accurate to be applied to the stereoselective pharmacokinetic analysis of TA enantiomers in plasma and urine following administration of therapeutic doses of the drug.

INTRODUCTION

Stereospecific analysis of the enantiomers of chiral arylalkanoic non-steroidal anti-inflammatory drugs (NSAIDs) has recently attracted considerable attention [1–14]. For some of these drugs, different pharmacokinetics have been described for *R* and *S* isomers [12]. In other cases, unidirectional bioinversion of *R* enantiomers to their corresponding antipodes has been primarily responsible for dissimilar plasma time courses [6,14]. These NSAIDs are usually administered as racemates. Since the *S* isomers are considered to be principally respon-

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sible for the pharmacologic activities [1], the study of individual isomers following administration of racemates is warranted.

Tiaprofenic acid (TA) is a relatively new chiral arylpropanoic NSAID which is useful in the treatment of rheumatoid arthritis, osteoarthritis, musculoskeletal disorders, soft-tissue injuries and inflammatory conditions and acute pain of varying origin [15]. A gas chromatographic (GC) method [10] was recently utilized to determine the pharmacokinetics of TA enantiomers in four arthritic patients; it was observed that the plasma and urine time courses for TA enantiomers were superimposable [11]. These results indicate that unlike ibuprofen and fenoprofen, TA does not undergo bioinversion, or this process, if present, is extremely slow. Tiaprofenic acid, however, is eliminated from the body mainly through conjugation and subsequent excretion in the urine [11,16]. Since various disease states affect these routes, they may cause stereoselectivity in the pharmacokinetics of TA enantiomers. As a consequence further studies, utilizing stereospecific assays, are warranted. The reported GC method for analysis of individual isomers of TA [11] is not ideal due to the lengthy procedure as well as a requirement for heat during sample preparation. In this article we report a very convenient, sensitive, stereospecific high-performance liquid chromatographic (HPLC) method suitable for the pharmacokinetic analysis of TA enantiomers in plasma and urine.

EXPERIMENTAL

Chemicals

Racemic TA (powder and Surgam capsules) and internal standard (I.S.), (±)-2-(4-benzoylphenyl)butyric acid, were obtained as gifts from Roussel Canada and Rhone-Poulenc Pharma (Montreal, Canada), respectively. 2,2,2-Trichloroethyl chloroformate and 4-dimethylaminopyridine (DMAP) were obtained from Aldrich (Milwaukee, WI, U.S.A.). L-Leucinamide hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and water were HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.), while triethylamine (TEA), acetic acid, isopropanol, isooctane, chloroform, hydrochloric acid and sulfuric acid were analytical grade.

Apparatus and chromatographic conditions

Samples were vortexed with an IKA-Vibrax-VXR mixer (Terochem, Edmonton, Canada) and centrifuged with a Dynac centrifuge (Becton Dickinson, Parsippany, NJ, U.S.A.). Solvents were evaporated with a Savant Speed Vac concentrator–evaporator (Emerston Instruments, Scarborough, Canada). The HPLC instrument (Waters, Mississauga, Canada) consisted of 590 pump, a variable wavelength 481 UV detector (set at 310 nm), a 710B WISP autosampler, a 10 cm × 4.6 mm analytical column containing 5 μm octadecylsilane packing material (Partisil 5 ODS-3; Whatman, Clifton, NJ, U.S.A.) and a 2-cm Uptight precolumn (Upchurch Scientific, Rexdale, Canada) packed with 37–53 μm reversed-phase material. The recorder–integrator was a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3390A.

The mobile phase, unless stated otherwise, consisted of 0.06 *M* monopotassium phosphate–acetonitrile–TEA (65:35:0.02) with a pH of 6.0, and was pumped at a flow-rate of 1 ml/min. The chromatography was carried out at ambient temperature.

Standard solutions

Trichloroethyl chloroformate (60 m*M*) and L-leucinamide (1 *M*) were prepared in acetonitrile, and 1 *M* TEA in water, respectively. A stock solution of TA was prepared by dissolving 20 mg of the powder in 15 ml of methanol and addition of distilled water to 100 ml. The I.S. solution was prepared by dissolving 20 mg of the powder in 100 ml of methanol. Blank plasma and urine samples were spiked with standard solutions of TA to give final concentrations (each enantiomer) of 0.2, 0.4, 1.0, 2.0, 4.0, 10 and 20 mg/l in plasma and 10, 20, 50 and 100 mg/l in urine.

Sample preparation

To 0.5 ml of plasma containing TA were added 0.05 ml of I.S. solution and 0.1 ml of sulfuric acid (0.6 *M*). The constituents were then extracted with 3 ml of isooctane–isopropanol (95:5), vortex-mixed for 30 s and centrifuged at 1800 *g* for 5 min. The organic layer was transferred to clean tubes and evaporated to dryness.

The TA conjugates in urine samples (0.1 ml) were hydrolyzed by addition of 0.05 ml of 1 *M* sodium hydroxide [16]. After addition of 0.2 ml of I.S. solution, the samples were then acidified (pH 5) by addition of 0.035 ml of a 10% aqueous solution of glacial acetic acid and extracted with 3 ml of chloroform in the same manner described for the plasma samples.

Residues which remained after evaporation were dissolved in 0.1 ml of a 2 mg/ml solution of DMAP (in acetonitrile) to which was added 0.1 ml of trichloroethyl chloroformate followed, 30 s later, by 0.1 ml of L-leucinamide solution. After 2 min, the reaction was stopped by addition of 0.5 ml of 0.25 *M* hydrochloric acid. The reaction mixture was vortex-mixed for 15 s, centrifuged for 2 min at 1800 *g* and extracted with 2.5 ml of chloroform. The organic layer was evaporated, and the residue was dissolved in 0.2 ml of a solution of water–acetonitrile (1:1). Aliquots of 0.01 to 0.07 ml were injected into the HPLC instrument.

Extraction yields

Plasma and urine samples ($n=5$) were spiked with TA to give final concentrations of 0.4 and 10 mg/l (plasma) and 10 and 100 mg/l (urine) for each enantiomer. The solutions were then extracted according to the procedure described above, but without addition of I.S. Exact volumes of the organic layer were taken, and the peak areas of the racemic TA after injection of the extracted samples were compared with those obtained after injection of the unextracted samples containing equivalent concentrations of the drug. The analysis of the racemic TA was carried out under the chromatographic conditions described above, but with a different mobile phase which consisted of 0.06 *M* monopotassium phosphate–acetonitrile–TEA (75:25:0.02). Under these conditions racemic TA eluted as a sharp peak with a retention time of 6.2 min.

The extraction efficiency for the amide diastereoisomers was measured by derivatization of TA (0.4 and 10 mg/l) and comparison of the areas of derivatized drug injected directly ($n=3$) into the HPLC system with those obtained after injection of extracted samples ($n=5$).

Derivatization yield

To determine the amount of unreacted TA, 0.01 mg of racemic TA was added to ten tubes. Half of the samples were then derivatized while the other half were dissolved in the same volume of solvent used in the preparation of the reagents. At this stage the solutions was basified with 0.5 ml of 1 *M* sodium hydroxide followed by the addition of 3 ml isooctane–isopropanol (95:5). The mixture was vortexed (15 s), and centrifuged (2 min), and the organic layer was discarded. To 0.5 ml of the aqueous portion was added 0.2 ml of 5 *M* hydrochloric acid, and the underivatized drug was extracted with 3 ml of the isooctane–isopropanol (95:5) mixture. Aliquots (2 ml) of the organic layer were transferred to clean glass tubes, the solvent evaporated and the residue taken up in 0.2 ml of acetonitrile–water (1:1). The derivatization yield was calculated by comparing the peak areas of the racemic TA in the derivatized and underivatized samples. The analyses were carried out using the method described for the determination of the extraction yield of racemic TA.

Accuracy and precision

TA was added to plasma ($n=6$) and concentrations of the individual isomers were determined against a standard curve. The accuracy of the method was calculated based on the difference between the mean calculated and added concentrations, while precision was evaluated by calculating the inter-day coefficients of variation.

RESULTS AND DISCUSSION

Formation of diastereoisomers of chiral NSAIDs and subsequent separation of the isomers utilizing GC or HPLC has recently become popular [2–6,8–14,17–19]. In virtually all of these cases, an amide derivative is formed by reaction of the carboxylic acid group of the NSAID with a chiral amine such as amphetamine [10,11], phenylethylamine [2,5], naphthylethylamine [18] or leucinamide [8,9,13,17,19]. Such a reaction takes place in the presence of a coupling reagent like 1,1′-carbonyldiimidazole [10,11] or through a mixed anhydride intermediate utilizing a chloroformate derivative such as ethyl chloroformate [8,9,13,17,19]. While the former reaction usually requires a relatively high temperature and/or long reaction time, the latter takes place at ambient temperature in a short period of time.

The only reported stereospecific chromatographic assay for TA is a GC method which involved precolumn derivatization of the drug with *S*-(+)-amphetamine in the presence of 1,1′-carbonyldiimidazole [11]. This method, however, suffers from a lengthy sample preparation and derivatization procedure. Furthermore, the derivatization reaction requires heating at 65°C for 1.5 h. The diastereoiso-

mers of ketoprofen [9,13], fenoprofen [17] and flurbiprofen [19] have been formed at ambient temperature in less than 3 min by utilizing L-leucinamide and ethyl chloroformate. When we applied the same derivatization method to standard solutions of TA, however, we observed several additional peaks in the chromatogram. Since these peaks were also observed in the absence of biological specimens, and as L-leucinamide does not absorb UV light at 310 nm, they may be attributed to degradation products of TA formed under the conditions used for derivatization. When the residue (after extraction) was reconstituted with a solution of TEA (50 m*M*) in acetonitrile, a purple color appeared in the solutions containing TA. Appearance of this color may be an indication of instability of TA in the reconstituting solution. This is consistent with the previously reported instability of TA in alkaline solutions [20]. When TEA was not present in the solution the color was not observed and the chromatogram consisted of two major peaks with equivalent areas. Although these peaks presumably represented the two isomers of TA, the method was not sufficiently sensitive to be applied to measurement of concentrations of the isomers after administration of therapeutic doses. This apparent insensitivity was due to the fact that less than 5% of the drug was being derivatized. Substitution of ethyl chloroformate with the more reactive trichloroethyl chloroformate increased the derivatization efficiency by several fold. Addition of DMAP to the reconstituting solution further improved the efficiency of the reaction. Using the conditions described under sample preparation, the derivatization procedure took place in less than 3 min with an overall efficiency of $63.2 \pm 2.4\%$. Prolongation of the reaction time for the formation of the mixed anhydride intermediate and the L-leucinamide derivative beyond 30 s and 2 min, respectively, did not significantly affect the overall derivatization yield.

Trichloroethyl chloroformate-mediated derivatization of the carboxylic acid group with L-leucinamide gave diastereoisomers which were easily separated by the reversed-phase system. Fig. 1 depicts chromatograms of blank and spiked (0.2 mg/l of each isomer) plasma, as well as a sample taken 24 h following oral administration of 200 mg racemic TA to a healthy volunteer. Chromatograms of a blank urine and a urine sample taken from the healthy subject are shown in Fig. 2. With high concentrations of TA an additional peak was observed to elute at a position between the internal standard diastereoisomers (Fig. 2B). This peak, however, was well resolved from other components in the chromatogram and did not interfere in the assay.

Under the stated conditions, the two isomers of TA eluted with retention times of 8.6 and 9.9 min, and were separated with a resolution factor of 2.1. Due to the unavailability of optically pure isomers, the unequivocal elution order of *R* and *S* isomers could not be assigned. However, as L-leucinamide derivatives of *R*-ketoprofen [13], fenoprofen [17] and flurbiprofen [19] eluted prior to their respective *S* isomers, the same order of elution is suggested for TA diastereoisomers.

The extraction efficiencies of TA from plasma samples were 71.2 ± 1.1 and $70.0 \pm 1.8\%$ for 0.4 and 10 mg/l enantiomeric TA concentrations, respectively. In urine, values of 102 ± 7.1 and $101 \pm 4.7\%$ were obtained for concentrations of 10 and 100 mg/ml, respectively.

The extraction efficiencies of TA diastereoisomers were 95.2 ± 5.7 and

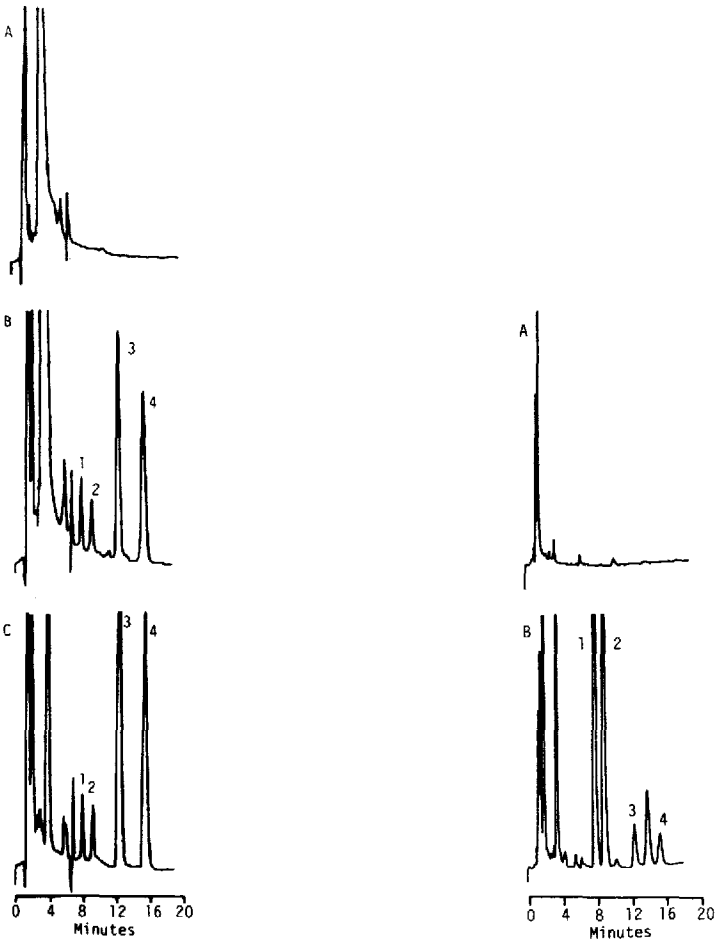

Fig. 1. Chromatograms of a blank plasma (A), a blank plasma spiked with 0.2 mg/l of each isomer (B) and a plasma sample of a subject 24 h after oral administration of a single 200-mg dose of racemic tiaprofenic acid (C). Peaks: 1 and 2 = tiaprofenic acid diastereoisomers; 3 and 4 = internal standard diastereoisomers.

Fig. 2. Chromatograms of a blank urine (A) and a urine sample collected from a healthy subject 0–2 h after oral administration of a single 200-mg dose of racemic tiaprofenic acid (B).

98.3 ± 2.3% for *R*-TA and 96.2 ± 6.8 and 101 ± 1.8% for *S*-TA at concentrations of 0.4 and 10 mg/l, respectively.

Excellent linearity was observed between the peak-area ratios (*R*- and *S*-TA/I.S.) and the corresponding plasma and urine concentrations over the examined concentration range ($r > 0.998$). In all instances the first eluting peak of I.S. (peak 3, Figs. 1 and 2) was used for quantification. A typical plasma standard curve could be described by $y = 0.5477x + 0.0052$ and $y = 0.5222x - 0.0055$, for the *R* and *S* enantiomers, respectively, where y is the peak-area ratio (*R*- or *S*-TA/I.S.) and x is the TA enantiomer concentration.

The method is accurate and reproducible as reflected by the inter-day coeffi-

TABLE I

ACCURACY AND PRECISION OF THE METHOD ($n=6$)

Theoretical concentration (mg/l)	Measured concentration (mg/l)		Error (%)		Inter-day coefficient of variation (%)	
	R-TA	*S*-TA	*R*-TA	*S*-TA	*R*-TA	*S*-TA
0.20	0.212	0.194	5.99	−3.22	13.0	11.7
0.40	0.873	0.408	−6.73	1.95	7.38	4.87
1.0	1.01	0.975	0.471	−2.46	5.89	6.37
2.0	1.92	1.89	−3.81	−5.36	9.70	10.5
4.0	3.84	3.75	−3.99	−6.25	9.35	8.69
10	9.88	9.71	−1.24	−2.90	3.99	3.92
20	20.0	20.1	0.09	0.33	7.01	5.99

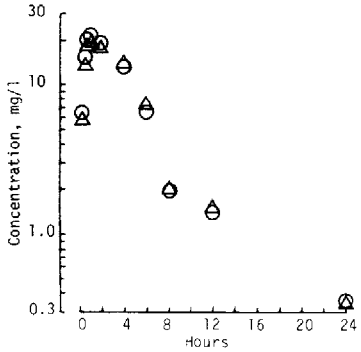

Fig. 3. Plasma concentration-time profiles of *R*-TA (○) and *S*-TA (△) in a healthy subject following a single 200-mg oral dose of racemic tiaprofenic acid.

cient of variations and the difference between added and measured concentrations (Table I). In terms of sensitivity, the lowest examined plasma concentration, 0.2 mg/l, was associated with an error of 5.99 and 3.22% and an inter-day variation of 13.0 and 11.7% for the *R* and *S* isomers, respectively. If the criterion of signal-to-noise ratio were used, a sensitivity of far better than 0.2 mg/l could be claimed (Fig. 1).

Plasma time courses of the isomers of TA are depicted in Fig. 3. Consistent with results of the previous study [11], the *R*- and *S*-TA had superimposable time courses.

In conclusion, the reported method is rapid, sensitive and convenient and is suitable for pharmacokinetic analysis of the TA enantiomers after administration of recommended therapeutic doses.

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