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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THE ENANTIOMERS OF ANTI-INFLAMMATORY 2-ARYLPROPIONATES: SUITABILITY OF THE METHOD FOR *IN VITRO* METABOLIC STUDIES

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SUMMARY

The enantiomers of 2-phenylpropionic acid, 2-(2-naphthyl)propionic acid, 2-(4-biphenyl)propionic acid and six anti-inflammatory congeners were separated by high-performance liquid chromatography via their diastereoisomeric derivatives with (*S*)-(−)-1-phenylethylamine. In agreement with a general rule, the diastereoisomers derived from the (*R*)-acids are less polar and elute first. Structural factors influencing the resolution are discussed. Good calibrations were obtained for *R*/*S* ratios and total (*R* + *S*) concentrations of flurbiprofen and naproxen added to inactivated rat liver preparations. The method is suitable for *in vitro* metabolic studies of chiral 2-arylpropionates.

INTRODUCTION

Arylpropionic acids are an important class of non-steroidal anti-inflammatory agents displaying stereoselective activity both *in vitro* (inhibition of prostaglandin synthetase) and *in vitro*, the (*S*)-enantiomers being consistently more active than the (*R*)-enantiomers[1,2]. Of considerable significance in this context is the fact that the pharmacological activity of these compounds is markedly influenced by their metabolic fate, in particular by their stereoselective biotransformation[3–5].

Up to now, stereoselective aspects of 2-arylpropionates metabolism have been investigated mainly *in vivo*. The fate of ibuprofen (II, Fig. 1) enantiomers was studied in humans dosed with the racemate; for this purpose, the drug was derivatized with (−)-1-phenylethylamine, and the diastereoisomeric amides determined by gas–liquid chromatography (GLC)[6,7]. The method is fast and efficient, but its applicability to more polar analogues may be limited by low volatility and thermal instability. The high-performance liquid chromatography (HPLC) separation of diastereoisomeric 1-phenylethylamides was applied to carprofen (VIII) extracted from human blood and urine[8]. Comparably, HPLC separation was achieved with 2-[3-(2-chlorophenoxyphenyl)]propionic acid extracted from rat plasma and derivatized with (+)-2-aminobutane[9], as well as with naproxen (IV) extracted from rabbit plasma and derivatized with (−)-1-(4-dimethylamino-1-naphthyl)ethylamine[10]. This paper reports

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an optimized procedure for the efficient and fast HPLC separation of enantiomeric pairs of a number of 2-arylpropionates as their diastereoisomeric 1-phenylethylamides. The method was quantitatively validated over the entire range of *R*/*S* ratios for 2-arylpropionates extracted from rat liver preparations.

EXPERIMENTAL

Chemicals

2-Phenylpropionic acid (I, Fig. 1) and its (*R*)-(−) and (*S*)-(+) enantiomers were bought from Alfa (Ventron, Karlsruhe, F.R.G.). The following compounds were generously donated: (±)-ibuprofen (II) by Boots (Nottingham, U.K.); naproxen (IV) and its (*S*)-(+) and (*R*)-(−) enantiomers by Syntex (Palo Alto, CA, U.S.A.); flurbiprofen (VI) and its (*S*)-(+) and (*R*)-(−) enantiomers by Boots; (±)-cicloprofen (VII) by Squibb (Princeton, NJ, U.S.A.); (±)-carprofen (VIII) by F. Hoffmann-La Roche (Basle, Switzerland); and suprofen (IX) and its (*S*)-(+) and (*R*)-(−) enantiomers by Janssen Pharmaceutica (Beerse, Belgium).

2-(2-Naphthyl)propionic acid (III) and 2-(4-biphenyl)propionic acid (V) were synthesized from the corresponding arylacetic acids (purchased from Aldrich, Beerse, Belgium) according to known methods[11–13], optimized in order to improve the yield[14]. The IR and NMR spectra confirmed the identity and purity of the compounds. The chromatographic solvents (spectroscopic quality), the reagents and reaction solvents (analytical-reagent grade) were purchased from Fluka (Buchs, Switzerland), Aldrich or Merck (Darmstadt, F.R.G.).

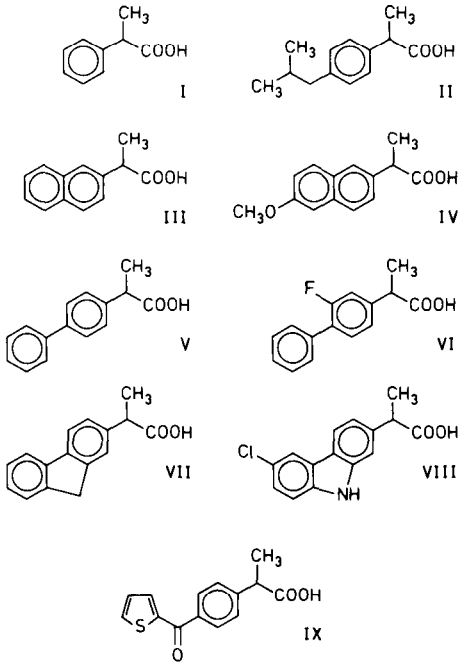

Fig. 1. 2-Arylpropionic acids investigated: I, 2-phenylpropionic acid (hydratropic acid); II, ibuprofen; III, 2-(2-naphthyl)propionic acid; IV, naproxen; V, 2-(4-biphenyl)propionic acid; VI, flurbiprofen; VII, cicloprofen; VIII, carprofen; IX, suprofen.

Derivatization

The arylpropionic acid (0.05–0.5 μmole) was dissolved in chloroform (1.5 ml); the internal standard and 1,1′-carbonyldiimidazole (0.8 ml of a freshly prepared 65 mg/ml solution in chloroform)[6] were added and the solution was agitated mechanically for 10 min. After addition of pure acetic acid (10 μl) and agitation (10 min), the coupling amine, (*S*)-(−)-1-phenylethylamine (80 μl) was added and the mixture was shaken for 25 min and centrifuged. After a period of 30 min to ensure complete reaction, 3 ml of 0.2 *N* ammonia solution and 5 ml of *n*-hexane were added and the mixture was shaken for 15 min and centrifuged. A portion (4 ml) of the organic layer was washed with 0.2 *N* hydrochloric acid (3 ml). After centrifugation, 3 ml of the organic layer were evaporated under vacuum and the residue was dissolved in 1 ml of the mobile phase.

HPLC conditions

The HPLC apparatus was a Siemens S101 chromatograph equipped with an Orlita DMP-AE 10.4 pump and a 10-μl injection loop. The detector was a Knauer UV spectrophotometer operating in the 190–600 nm range. It was connected to an HP 3390A integrator. A 250 × 6 mm O.D. (3 mm I.D.) stainless-steel column packed with LiChrosorb Si 60 (10 μm) was used. The mobile phase was isopropanol–cyclohexane (7.5:92.5 or 7:93, v/v) at a flow-rate of 2.0 ml/min. The wavelength of the detector was set at 225 nm as a good compromise for all amides. However, this wavelength was optimized to 230 and 245 nm for the determination of naproxen and flurbiprofen derivatives, respectively.

Calibration graphs for naproxen and flurbiprofen added to rat liver preparations

Rat liver 10,000 *g* supernatant preparations (2 ml, corresponding to 1 part of organ plus 3 parts of isotonic potassium chloride–buffer), were inactivated by 1 *N* hydrochloric acid (0.1 ml/ml). Aqueous solutions of the arylpropionic acids were then added to give 10^{-5}–10^{-4} *M* concentrations. After addition of an internal standard, these preparations (pH ≈ 1.5) were extracted with 5 ml of toluene. The toluene layer was evaporated to dryness under vacuum (rotatory evaporator), the residue was dissolved in 1.5 ml of chloroform and the derivatization and analysis were carried out as described above.

RESULTS AND DISCUSSION

Separation of diastereoisomeric amides

The reaction of the acids with 1,1′-carbonyldiimidazole to yield the imidazolide is fast (less than 1 min), whereas the reaction of the latter with the amine is slower and requires 30 min for completion[6]. Preliminary results showed that in previous studies the carbonylimidazole reagent had been used in sub-optimal proportions. Increasing this proportion resulted in much better yields of the derivatives and hence increased the sensitivity of the method.

The isopropanol–cyclohexane mixture proved superior to other mobile phases; its cut-off point below 210 nm permitted UV detection at short wavelengths. For the resolution of the diastereoisomeric amides of acids I–IX, the best proportions of isopropanol to cyclohexane were in the range 7:93 to 10:90. For the set of all nine

TABLE I

HPLC RESOLUTION OF THE ENANTIOMERS OF 2-ARYLPROPIONATES AFTER DERIVATIZATION WITH (*S*)-(−)-1-PHENYLETHYLAMINE

LiChrosorb Si 60 (10 μm), 250 mm column; mobile phase, isopropanol–cyclohexane (7.5:92.5, v/v); flow-rate, 2 ml/min.

No.	*Compound*	*Retention time (min)**		*Resolution factor*[15,16]
		*1st peak***	*2nd peak***	
I	2-Phenylpropionic acid	2.94	3.81	2.47
II	Ibuprofen	2.40	3.13	1.46
III	2-(2-Naphthyl)propionic acid	2.86	3.92	2.91
IV	Naproxen	3.31	4.39	2.35
V	2-(4-Biphenyl)propionic acid	2.81	3.86	2.29
VI	Flurbiprofen	3.04	4.48	3.30
VII	Cicloprofen	2.84	3.88	2.74
VIII	Carprofen	3.53	5.18	2.88
IX	Suprofen	4.92	7.38	3.94

* Mean variation ± 0.02 min.

** For compounds I, IV, VI and IX the (*R*-acid; *S*-amine) derivative corresponds to the first peak and the (*S*-acid; *S*-amine) derivative to the second peak. For compounds II, III, V, VII and VIII the same sequence is highly probable[17] but was not investigated.

acids, the best compromise appeared to be 7.5:92.5, yielding excellent resolutions and short retention times (Table I). With a 10:90 ratio the retention times were shorter (by 10–25%), but the resolution factor decreased even more (by 25–35%).

The method is thus suitable for the analytical resolution of all the 2-arylpropionates investigated. A typical chromatogram is presented in Fig. 2, which also shows how 2-naphthylacetate (as the amide) is separated from the propionate derivatives and could therefore be used as an internal standard.

Helmchen *et al.*[17] have correlated the absolute configuration of amides of structure $R_1R_2CHCONHCHR_3R_4$ with their order of elution from silica columns in liquid chromatography. In every instance tested, the diastereoisomer of configuration (*R*-acid; *S*-amine) eluted before the isomer of configuration (*S*-acid; *S*-amine). This rule was verified for compounds I, IV, VI and IX, which were available as enantiomers of known absolute configuration. In view of this consistency it can be assumed with confidence that the *S*-enantiomers of acids II, III, V, VII and VIII also yield the more polar diastereoisomer.

In Table I, the resolution factor[15,16] is defined as the distance between peaks divided by the sum of the peak widths at half-height. Quantitative analysis usually requires a resolution factor larger than 1, and it might be of interest to search for the structural factors that influence the resolution factor. One such factor is certainly the polarity of the diastereosiomers, as the resolution factor increases with the mean retention time of each pair ($r = 0.817$). Based on this partial correlation, the observed resolution of the derivatives of acid II is not as good as expected, possibly owing to its flexible isobutyl side-chain. In contrast, the resolution of the derivatives of acid VI is markedly better than expected. Conceivably, electronic delocalization and rigidity of the aryl moiety also influence the resolution of the diastereoisomers.

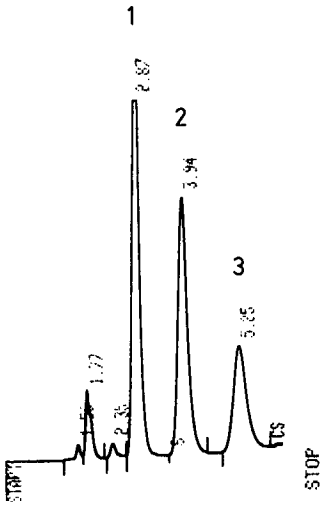

Fig. 2. Separation of the enantiomers of 2-(2-naphthyl)propionate (peaks 1 and 2) and of 2-naphthylacetate (peak 3), after derivatization with 1-phenylethylamine. Conditions: LiChrosorb Si 60 (10 μm), 250 mm column; mobile phase, isopropanol–cyclohexane (7.5:92.5); flow-rate, 2 ml/min.

Calibrations of R/S ratios and total (R + S) concentrations of flurbiprofen and naproxen added to rat liver preparations

Flurbiprofen and naproxen enantiomers were added in various *R/S* ratios and in the concentration range 10^{-5}–10^{-4} *M* to inactivated rat liver 10,000 *g* preparations. The pre-determined percentages of (*R*)- and (*S*)-enantiomers were corrected for enantiomeric contamination (optical purity ⩾95%) as determined by repeated HPLC analysis (see previous section) of each isolated isomer.

An example of the calibration of *R/S* ratios for flurbiprofen is given in Table II, showing only moderate differences between the added and found percentages. For the determination of unknown *R/S* ratios, it is certainly preferable to use calibration

TABLE II

EXAMPLE OF CALIBRATION OF *R/S* RATIOS FOR FLURBIPROFEN (VI) ADDED TO INACTIVATED RAT LIVER 10,000 *g* PREPARATIONS

*Added**			*Found*			*Δ (%)*
% R	*% S*	*R/S*	*% R*	*% S*	*R/S*	
3.0	97.0	0.0309	5.8	94.2	0.0616	2.8
9.6	90.4	0.106	4.5	95.5	0.0471	−5.1
20.6	79.4	0.259	21.4	78.6	0.272	0.8
30.2	69.8	0.433	26.0	74.0	0.351	−4.2
60.9	39.1	1.56	54.9	45.1	1.22	−6.0
79.6	20.4	3.90	74.4	25.6	2.91	−5.2
84.5	15.5	5.45	81.3	18.7	4.35	−3.2
95.0	5.0	19.0	93.2	6.8	13.7	−1.8

* These values are corrected for the optical purity of each enantiomer.

lines, as evidenced by the good linear regression (eqn. 1) calculated from the R/S ratios in Table II:

$$R/S_{found} = 0.72\ (\pm\ 0.02)\ R/S_{added} + 0.078\ (\pm\ 0.150) \quad (1)$$
$$n = 8;\ r^2 = 0.999$$

where the 95% confidence intervals are given in parentheses.

High correlation coefficients ($r^2 > 0.98$) were consistently found in experiments of this type, *e.g.*, eqn. 2 obtained with naproxen:

$$R/S_{found} = 0.87\ (\pm\ 0.07)\ R/S_{added} + 0.069\ (\pm\ 0.039) \quad (2)$$
$$n = 12;\ r^2 = 0.988$$

Experiments of this type were also repeated with the addition of an internal standard, (*R*)-(−)-ibuprofen for naproxen and (*S*)-(+)-suprofen for flurbiprofen. The internal standards were not added as the free acid, but as the phenylethylamide obtained with a high degree of diastereoisomeric purity (>99%) by preparative HPLC[14]. The ratio of isopropanol to cyclohexane in the mobile phase was chosen as 7:93. Complete separation of the investigated enantiomers and of the internal standard from minor biological contaminants was achieved, as illustrated in Fig. 3. This allowed the linearity of response to be checked in the 10^{-5}–10^{-4} *M* concentration range (total of both enantiomers). Good linearity was obtained in all instances ($r^2 \geqslant 0.99$).

In conclusion, the method presented here is suitable for the simultaneous determination of enantiomers of 2-arylpropionic acids undergoing *in vitro* metabolic studies. The method is reasonably fast and accurate and shows general applicability to a broad structural range of 2-arylpropionic acids.

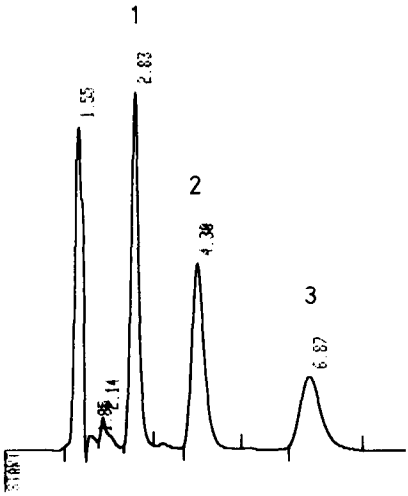

Fig. 3. Separation of the enantiomers of flurbiprofen (peaks 1 and 2) and of (+)-suprofen after extraction from an inactivated rat liver 10,000 *g* preparation and derivatization with 1-phenylethylamine. Conditions: LiChrosorb Si 60 (10 μm), 250 mm column; mobile phase, isopropanol–cyclohexane (7:93); flow-rate, 2 ml/min.

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