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# APPLICATION OF A RADIAL COMPRESSION COLUMN TO THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THE ENANTIOMERS OF SOME 2-ARYLPROPIONIC ACIDS AS THEIR DIASTEREOISOMERIC *S*-(—)-1-(NAPHTHEN-1-YL)ETHYLAMIDES

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## SUMMARY

The enantiomers of 2-phenylpropionic acid and four congeneric anti-inflammatory drugs were separated as their diastereoisomeric amides with *S*-(—)-1-(naphthen1-1-yl)ethylamine by high-performance liquid chromatography using a silica-packed radial compression cartridge. The order of elution of the diastereoisomeric amides was always *R,S* or —,*S* before *S,S* or +,*S*. The conditions for the derivatization, using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide as coupling agent, were optimized, and it was found that the addition of 1-hydroxybenzotriazole rendered the reaction quantitative. Good calibration curves were obtained for the quantitation and determination of the enantiomeric composition of 2-phenylpropionic acid in urine, and the application of the method to the study of the metabolism of this acid in vivo is described.

## INTRODUCTION

The incidence of inflammatory disorders, notably rheumatoid arthritis, is continually increasing, and thus the non-steroidal anti-inflammatory drugs (NSAIDs) are widely used. Currently, the 2-arylpropionic acid, or profen, NSAIDs are an important sub-group of this class of drug. Over recent years attention has increasingly focussed on the problems of adverse reactions to the NSAIDs, notably drugs like benoxaprofen and indoprofen.

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It is frequently the case that a knowledge of the metabolism and pharmacokinetics of compounds is essential in understanding their mechanisms of action, dose—response relationships and adverse reactions. However, conventional studies in which excreted metabolites are identified and standard pharmacokinetic parameters of the unchanged drug are defined have not shed a great deal of light on the problems of the profens (see, for example, the paper of Chatfield and Green [1] on benoxaprofen).

The anti-inflammatory action of this group of NSAIDs derives from their ability to block the conversion of arachidonic acid to prostaglandins and other mediators responsible for various features of the inflammatory process [2]. The 2-arylpropionates, as a class of drug, exhibit optical activity arising from the chiral (asymmetric) centre to which the carboxyl group is attached, and in the case of the profens, their activity appears to reside in the *S*-enantiomers. The in vitro eudismic ratios [3] [the ratio of the activity of the more active isomer, or eutomer, (here, the *S*-isomer) to that of the less active distomer (here, the *R*-isomer)] of a selection of profens are in each case much greater than 1 [4], indicating stereoselectivity of action, and in some cases are so large as to indicate that the action is stereospecific for the *S*-isomer. However, the very high degree of stereoselectivity of action which obtains in vitro is greatly obscured, indeed often completely lost, when the activities of the isomers are compared in vivo. This is due to the unique and biologically fortuitous inversion of the chiral centre of the *R*-2-arylpropionates in vivo to give rise to the *S*-enantiomer, without any other change to the drug in question.

It is therefore very important, in studies of the metabolism and pharmacokinetics of profen NSAIDs, to define the stereochemistry of the drug and any metabolites, and not simply to rely on non-stereoselective assays of the total amount of compound present [4, 5]. To meet this need, a variety of methods have been developed and reported in the literature. These are based in the main upon chromatographic resolution of diastereoisomeric derivatives formed with a chiral amine or, more rarely, a chiral alcohol (the relevant literature is reviewed in refs. 5—8). These assays generally describe the resolution of a single anti-inflammatory drug of interest. The recent availability of chiral stationary phases for liquid chromatography has prompted their evaluation for such resolutions [9]: in this case, amides formed with a non-chiral amine were resolved.

In this paper, we report on a rapid and reliable general method for the resolution of the stereoisomers of a variety of 2-arylpropionates of pharmacological interest, by high-performance liquid chromatographic (HPLC) separation using a radial compression column, of the diastereoisomeric amides formed with *S*-(—)-1-(naphthen-1-yl)ethylamine, and show the application of this to a study of the fate of the model compound, 2-phenylpropionic (hydratropic) acid, in the rat.

## EXPERIMENTAL

### *Note on nomenclature*

The enantiomers of the 2-arylpropionic acids are referred to by absolute configuration if known, as the sign of their optical rotation is pH-dependent,

e.g. the dextrorotatory free acid of pirprofen is laevorotatory as the sodium salt. The sign of optical rotation quoted in the text refers to the free acid in each case. Diastereoisomeric amides of the acids are variously referred to as *R,S*, *S,S*, +,*S* or −,*S* i.e. with the configuration or rotation of the acid given before that of the amine used.

*Compounds*

The following compounds were purchased: *S*-(−)-1-(naphthen-1-yl)ethylamine (optical purity > 99%), 1-hydroxybenzotriazole (1-HOBT), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, *RS*-2-phenylpropionic (hydratropic) acid and N-methylmorpholine from Aldrich (Gillingham, U.K.); *R*-(−)- and *S*-(+)-2-phenylpropionic acids from Lancaster Synthesis (Lancaster, U.K.). Anti-inflammatory agents were the gifts of their manufacturers, thus: *R*-, *S*- and *RS*-ibuprofen [2-(4-isobutylphenyl)propionic acid] from Boots (Nottingham, U.K.); *R*-, *S*- and *RS*-carprofen (6-chloro-α-methylcarbazole-2-acetic acid) from Hoffmann-La Roche (Nutley, NJ, U.S.A.); (±)-pirprofen (2-[3-chloro-4-(3-pyrrolin-1-yl)phenyl]propionic acid), (+)- and (−)-pirprofen sodium salts and pirprofen pyrrole (2-[3-chloro-4-(1-pyrrolyl)phenyl]-propionic acid) from Ciba-Geigy (Basel, Switzerland). HPLC solvents were purchased from Fisons (Loughborough, U.K.) or Rathburns (Edinburgh, U.K.). The structures of these various 2-arylpropionate derivatives are shown in Fig. 1.

Samples of the diastereoisomeric *S*-(−)-1-(naphthen-1-yl)ethylamides of the acids were synthesised by the mixed anhydride method using ethyl chloroformate [10, 11]. The identity and purity of the amides was confirmed by 400-MHz $^1$H nuclear magnetic resonance ($^1$H NMR), mass spectrometry (MS) and elemental analysis.

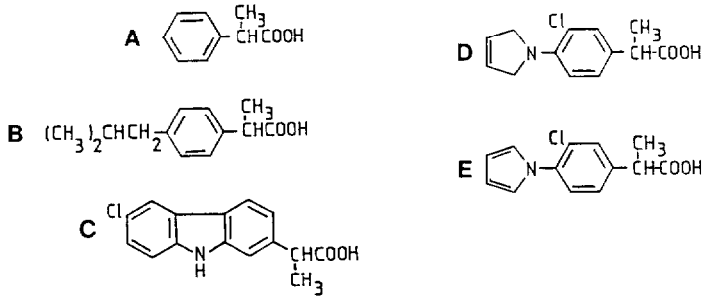


Fig. 1. Chemical structures of the acids investigated in this study. (A) 2-Phenylpropionic acid; (B) ibuprofen; (C) carprofen; (D) pirprofen; (E) pirprofen pyrrole.

*Apparatus*

HPLC analyses were performed with a Waters Assoc. (Harrow, U.K.) M45 pump and M440 UV detector equipped with a 254-nm filter, linked to a Philips (Cambridge, U.K.) PM 8251 chart recorder. The column was a Waters Radial-Pak cartridge, 100 × 5 mm I.D. containing 10-μm Porasil, housed in a Waters Z-module, and samples were introduced on to the column with a Rheodyne 7251 valve loop injector. The mobile phase was hexane—ethyl acetate (4:1, v/v), flow-rate 0.8 ml/min.

*Derivatization procedure*

The acid under investigation (50—100 μg) was dissolved in 1 ml dichloromethane, to which were added 1-hydroxybenzotriazole (100 μl of a 1 mg/ml dichloromethane solution containing 1%, w/v, pyridine), 100 μg 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (100 μl of a 1 mg/ml dichloromethane solution) and 100 μg *S*-(—)-1-(naphthen-1-yl)ethylamine (100 μl of a 1 mg/ml dichloromethane solution). The whole was mixed thoroughly and left to stand at room temperature for 1.5 h. The solvents were then removed on the rotary evaporator and the residue was taken up in 0.5 ml hexane—ethyl acetate (4:1, v/v). Aliquots of 10 μl were injected on to the HPLC column.

*Determination of the enantiomeric composition of 2-phenylpropionic acid in rat urine*

Three groups of four male Wistar rats (body weight 200 g) were given a dose of 0.8 mmol/kg *R*-(—)-, *S*-(+)- or *RS*-2-phenylpropionic acid, kept in metabolism cages, and their urine was collected. The urine samples were stored at −20° C until assayed.

Urine was adjusted to mild alkaline pH to hydrolyse the ester glucuronide of 2-phenylpropionic acid, which is its major metabolite in the rat [12]. Equal volumes (0.5 ml) of urine and 1 *M* sodium hydroxide were mixed and left at room temperature for 2 h. 1-Naphthylacetic acid (100 μg; 100 μl of a 1 mg/ml solution) was added to aliquots of this or neat urine, as internal standard, followed by 2 ml of 1 *M* hydrochloric acid. The whole was extracted with 7 ml benzene on a vortex mixer and the layers were separated by centrifugation. The benzene phase was dried (anhydrous sodium sulphate), filtered and evaporated to dryness under a stream of nitrogen. The residue was taken up in 1 ml dichloromethane, which was then treated as described above with 500 μg each of the carbodiimide and naphthylethylamine and 10 μg 1-HOBT, to form the diastereoisomeric amides of 2-phenylpropionic acid. The enantiomers were quantitated by reference to calibration curves constructed over the range 0—250 μg/ml of each isomer of 2-phenylpropionic acid, at a constant total concentration of 250 μg/ml, relating the ratio of the heights of the peaks of the derivatives of 2-phenylpropionic acid and the internal standard to the concentration of the enantiomer. These were linear over the concentration range studied, and were established freshly on each occasion the assay was used.

## RESULTS AND DISCUSSION

The HPLC system described using hexane—ethyl acetate (4:1, v/v) gave excellent separation of the diastereoisomeric amides of a variety of 2-arylpropionates. This is illustrated for 2-phenylpropionic acid and pirprofen in Fig. 2, and Table I details the chromatographic resolution of the stereoisomers of five 2-arylpropionates. In each case, the resolution factor, $R_S$, was greater than 1, indicating the analytical suitability of the separations obtained.

During the course of this work, a number of different mobile phases were

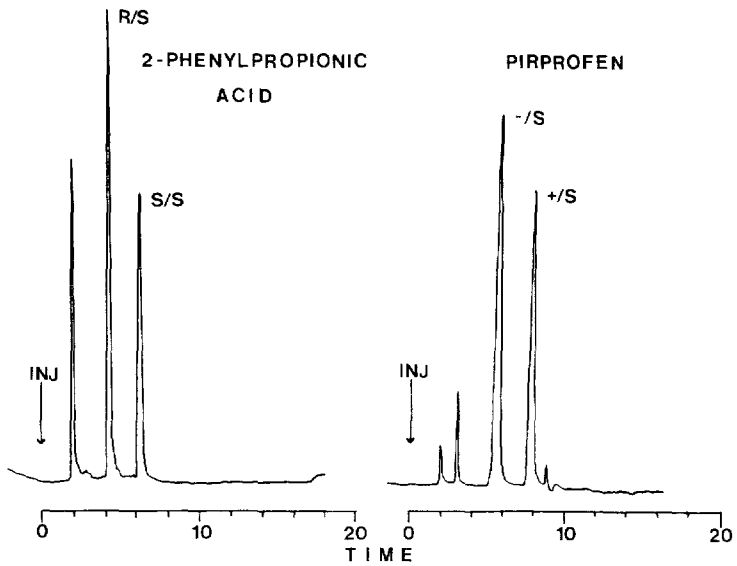


Fig. 2. HPLC resolution of the diastereoisomeric amides of *RS*-2-phenylpropionic acid and racemic pirprofen formed on reaction with *S*-(—)-1-(naphthen-1-yl)ethylamine under the conditions described in the text.

TABLE I

HPLC RESOLUTION OF ENANTIOMERS OF VARIOUS 2-ARYLPROPIONATES FOLLOWING DERIVATISATION WITH *S*-(− )-1-(NAPHTHEN-1-YL)ETHYLAMINE

HPLC system as described in the text. Parameters were calculated as follows: capacity, $k' = (t_p - t_0)/t_0$; separation factor, $\alpha = k_2'/k_1'$; resolution, $R_s = 2(t_2 - t_1)/(w_2 - w_1)$; where $t_0$ = retention time of unretained compound; $t_{1,2}$ = retention times of peaks 1 and 2; $w_{1,2}$ = widths in min of peaks 1 and 2.

| Compound | $k'$ | | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| | Peak 1* | Peak 2* | | |
| 2-Phenylpropionic acid | 1.26 | 2.32 | 1.84 | 4.69 |
| Ibuprofen | 0.84 | 1.79 | 2.13 | 5.14 |
| Carprofen | 8.84 | 15.11 | 1.11 | 5.53 |
| Pirprofen | 1.75 | 2.80 | 1.60 | 4.67 |
| Pirprofen pyrrole | 1.79 | 4.11 | 2.29 | 6.67 |

*Peaks numbered in order of elution from the column.

evaluated for the resolution of the stereoisomers, including isooctane—dichloromethane—methanol, hexane, tetrahydrofuran and dichloromethane containing small amounts (1—2%) of methanol. Only with the last of these was acceptable resolution achieved, but this was critically dependent upon methanol concentration. The use of this system was confounded by inter-batch and inter-supplier variation in the amount of methanol or ethanol used as stabiliser in dichloromethane. Additionally, resolution was lost if samples were dissolved in short-chain alcohols for injection on to the HPLC column. The effect of such alcohols upon the resolution of the stereoisomers in dichloromethane-based

systems is noteworthy, and their use cannot be recommended. It is also important to avoid possible contamination with such alcohols during work-up of samples.

It is of interest to note that in the cases of 2-phenylpropionic acid, ibuprofen and carprofen, the *R,S*-amide eluted before the *S,S*-diastereoisomer, while for pirprofen and pirprofen pyrrole, the absolute configuration of whose isomers is unknown, the amides followed the elution order −,*S* before +,*S*. The elution order from silica columns of a series of substituted amides of similar structures to the 2-arylpropionate amides examined here has been related to their absolute configuration. In each case the *R,S*-diastereoisomeric amide eluted before that of *S,S*-configuration [13]. This observation has been confirmed by examination of the elution order from a silica-packed stainless-steel HPLC column of a series of amides prepared by the reaction of certain 2-arylpropionates and *S*-1-phenylethylamine [7]. In the present study, using a radial compression column, it was found in the cases of 2-phenylpropionic acid, ibuprofen and carprofen that the *R,S*-amide eluted before its *S,S*-diastereoisomer (i.e. in Table I, peak 1 of *R,S*-configuration). The amides formed from pirprofen followed the elution order −,*S* before +,*S*. When these amides were quantitatively oxidized to the corresponding pyrrole derivatives by oxidation with 2,3-dicyano-5,6-dichlorobenzoquinone [14], they eluted in the same order, i.e. the pyrrole formed by oxidation of the −,*S*-amide elutes before the corresponding +,*S*-derivative. As this facile oxidation is most unlikely to alter the absolute configuration, the faster eluting diastereoisomer may be assumed to have the same configuration as that from (−)-pirprofen. By comparison with other 2-arylpropionates of known absolute configuration, it may be assumed that the configurations of (−)- and (+)-pirprofen, and of their pyrrole oxidation products, are *R* and *S*, respectively, based on their elution order in the HPLC. In addition, circular dichroism spectra of the free acids of the enantiomers of pirprofen were compared with those of *R*- and *S*-ibuprofen (unpublished data). Both acids exhibited strong absorption bands at 224—225 nm. *R*-Ibuprofen and (−)-pirprofen showed the same Cotton effect, and equal and opposite effects were seen with *S*-ibuprofen and (+)-pirprofen. It is thought that the sign of the Cotton effect of this band is related to the absolute configuration of α-substituted phenylacetic acids [15, 16]. These results support the configurational designation derived from the chromatographic analysis.

*Optimization of derivatization conditions*

Derivatization conditions were optimized using pirprofen as model compound, which necessitated the synthesis of its diastereoisomeric naphthylethylamides. Conditions were checked in a more limited fashion for the other acids. It is important to exclude water when making the derivatives, and this is especially critical when dealing with extracts from biological or other aqueous samples, which must be carefully dried in order to obtain the maximum yield.

In a series of preliminary experiments, the time course and effect of concentration of the carbodiimide coupling agent and of *S*-(−)-1-(naphthen-1-yl)ethylamine on the derivatization of pirprofen were examined (Fig. 3). A solution

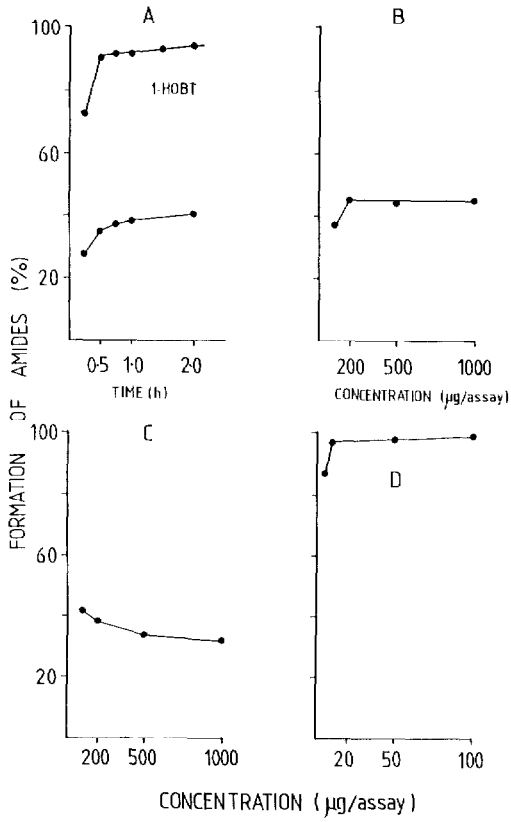


Fig. 3. Effect of time and reagent concentration on the formation of the diastereoisomeric amides formed by reaction of racemic pirprofen and *S*-(−)-1-(naphthen-1-yl)ethylamine. (A) Time course of the reaction of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (100 μg) and *S*-(−)-1-(naphthen-1-yl)ethylamine (100 μg) with racemic pirprofen (100 μg) in the presence and absence of 1-hydroxybenzotriazole (10 μg). (B) Effect of concentration of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide on the reaction of racemic pirprofen (100 μg) with *S*-(−)-1-(naphthen-1-yl)ethylamine (100 μg), reaction time 1.5 h. (C) Effect of concentration of *S*-(−)-1-(naphthen-1-yl)ethylamine on the formation of the diastereoisomeric amides of pirprofen (100 μg) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (100 μg), reaction time 1.5 h. (D) Effect of the concentration of 1-hydroxybenzotriazole on the formation of the diastereoisomeric amides of pirprofen (100 μg) and *S*-(−)-1-(naphthen-1-yl)ethylamine (100 μg) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (100 μg), reaction time 1.5 h.
Note: (i) pirprofen 100 μg ≡ 161 μg of the amide derivative; quantitation obtained by comparison of peak heights from reaction mixtures with those of synthetic compounds at equivalent concentration; (ii) peak-height ratios of −,*S*- to +,*S*-diastereoisomers were constant in all experiments showing that derivatisation was not stereoselective.

of the synthetic diastereoisomeric amides was used as a reference for the quantitation of the reaction products (e.g. 100 μg pirprofen ≡ 161 μg of the amides). It was found that the reaction, assessed by comparison of peak heights, was complete within 1.5—2 h, with a yield of ca. 40% of the desired products (Fig. 3). The concentration of carbodiimide was found to have no significant effect above 200 μg per tube, whereas increasing the quantity of amine decreased the formation of the amide, with increasing concentration

from 100 to 1000 μg per tube, from 41 to 33% of theory. The addition of the base, N-methylmorpholine, to the reaction mixture also decreased the yield to 5—10%. Upon addition of 1-HOBT, even in quantities as small as 5 μg per tube, the reaction yield increased to 87% of theory, and at concentrations of 10 μg per tube and above, the reaction was essentially quantitative. The time course of the reaction was thus reinvestigated with the addition of 10 μg 1-HOBT per tube to the reaction mixture, and was essentially unaltered, despite the increased yield (Fig. 3).

The peak-height ratio of the —,*S*- to +,*S*- diastereoisomers in all the experiments was constant, indicating that there was no stereoselective derivatization of pirprofen under the conditions employed.

*Enantiomeric composition of 2-phenylpropionic acid in rat urine*

Calibration curves of the peak-height ratios of the amides of the stereoisomers of 2-phenylpropionic acid to that of the internal standard (2-naphthylacetic acid) versus enantiomer concentration were constructed by spiking blank rat urine over the concentration range 0—250 μg/ml of the individual isomers at a constant total concentration of 250 μg/ml. The detector response was in both cases linear, with regression coefficients always > 0.99. Details of the precision and accuracy of the method are presented in Table II.

Table III shows the enantiomeric composition of 2-phenylpropionic acid in the urine of rats following the administration of *R*-(—)-, *S*-(+)- or *RS*-2-phenylpropionic acid at a dose of 0.8 mmol/kg (150 mg/kg). After the *R*-(—)-isomer, a total of 53% of the administered dose was excreted in the urine in 24 h, but only 70% of this was in the form of the unchanged *R*-(—)-acid, the remainder (30%) having undergone inversion of the chiral centre, producing *S*-(+)-2-

TABLE II

PRECISION AND ACCURACY OF THE HPLC DETERMINATION OF *S*-(+) AND *R*-(—)-2-PHENYLPROPIONIC ACID IN RAT URINE

Three replicates of each sample were prepared from 99% pure *S*-(+)- and *R*-(—)-2-phenylpropionic acids, each containing a total of 250 μg/ml 2-phenylpropionic acid.

| Sample | Isomer | Theoretical composition | Found composition | |
|---|---|---|---|---|
| | | | Mean | S.D. |
| 1 | *S*-(+) | 1 | 0.5 | 0.6 |
| | *R*-(—) | 99 | 99.3 | 4.6 |
| 2 | *S*-(+) | 25.5 | 26.0 | 1.0 |
| | *R*-(—) | 74.5 | 74.7 | 2.5 |
| 3 | *S*-(+) | 50 | 48.5 | 3.1 |
| | *R*-(—) | 50 | 50.2 | 1.1 |
| 4 | *S*-(+) | 74.5 | 75.0 | 2.0 |
| | *R*-(—) | 25.5 | 23.5 | 0.7 |
| 5 | *S*-(+) | 99 | 99.7 | 4.0 |
| | *R*-(—) | 1 | 1.7 | 2.6 |

TABLE III

URINARY EXCRETION OF 2-PHENYLPROPIONIC ACID ENANTIOMERS BY RATS GIVEN *R*-, *S*- OR *RS*-2-PHENYLPROPIONIC ACID

Rats were given 0.8 mmol/kg of the appropriate form of 2-phenylpropionic acid, their urines were collected for 24 h and assayed for the individual enantiomers of the acid as described in the text. Figures quoted are the mean ± S.D., $n = 4$.

| Form administered | Percentage of dose excreted in the 0—24 h urine as total 2-phenylpropionic acid (%) | Percentage of total acid present as | |
|---|---|---|---|
| | | *R*-Isomer | *S*-Isomer |
| *R* | 53.3 ± 5.3 | 69.6 ± 1.5 | 30.4 ± 1.6 |
| *S* | 64.6 ± 8.3 | 6.6 ± 0.3 | 93.4 ± 0.4 |
| *RS* | 58.7 ± 4.0 | 37.8 ± 0.9 | 62.2 ± 0.8 |

phenylpropionic acid. After administration of the *S*-(+)-enantiomer, the 2-phenylpropionic acid excreted (total 65% of dose) comprised 93% as the *S*-(+)-form with only 7% as *R*-(−)-2-phenylpropionic acid. When the racemic acid was given to rats, the enantiomeric composition of the 2-phenylpropionic acid in the 0—24 h urine (containing 59% of the administered dose) was 62% *S*-(+) and 38% *R*-(−).

The metabolic chiral inversion of 2-arylpropionates without other covalent change to the molecule is now well established as a feature of the disposition of this class of compound [5]. Although there are indications in the early literature that the archetypal congener, 2-phenylpropionic acid itself, undergoes this inversion, it is only in the past year that this has been confirmed in the rat [17] and the rabbit [18]. It is interesting to note that, when rats were dosed with *S*-2-phenylpropionic acid, a very small amount of the *R*-acid was excreted in the urine: the inversion of the 2-arylpropionates is usually thought of as unidirectional (*R* to *S*) but the occurrence of the reverse reaction has been suggested in the case of ibuprofen in man [19].

The analytical method reported here is presently being applied to studies of the metabolic disposition of 2-phenylpropionic acid and profen-type NSAIDs in animals and man, and the results of these studies will be reported elsewhere.

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