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ENANTIOSPECIFIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF 2-PHENYLPROPIONIC ACID, KETOPROFEN AND FENOPROFEN

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SUMMARY

A high-performance liquid chromatographic method has been developed for the quantitation of the *R*- and *S*-enantiomers of 2-phenylpropionic acid, ketoprofen and fenoprofen. The assay consists of extracting the arylpropionic acid with an internal standard and measuring the total (*R* + *S*) concentration of enantiomers by reversed-phase chromatography, derivatising the chromatographic fraction corresponding to the enantiomers to form *R*- and *S,R*-2-phenylethylamide distereoisomers which are resolved by normal-phase chromatography in order to calculate the fraction of each enantiomer. The limits of sensitivity of the assay for 2-phenylpropionic acid, ketoprofen and fenoprofen are 6, 0.2 and 2.5 mg/l, respectively.

INTRODUCTION

Many clinically important non-steroidal anti-inflammatory drugs such as ketoprofen, ibuprofen and fenoprofen are substituted 2-arylpropionic acids and exist as stereoisomers. 2-Phenylpropionic acid (2PPA), ketoprofen and fenoprofen were chosen as models with which to investigate the stereoselective disposition of this class of anti-inflammatory agents. The literature available to us contained analytical methods for the enantiomeric resolution of various related 2-arylpropionic acids (2APA) by high-performance liquid chromatography (HPLC) [1—4]; however, these were unsuitable in our hands for the analysis of 2PPA owing to lack of sensitivity and chromatographic interference from endogenous compounds in plasma. Consequently the following assay for 2PPA was developed and later modified to allow analysis of the enantiomers of ketoprofen and fenoprofen.

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EXPERIMENTAL

A schematic summary of the analytical method is shown in Fig. 1. The method for the analysis of 2PPA shall be discussed first followed by the modifications necessary for the analysis of ketoprofen and fenoprofen.

Sample preparation and chromatography of 2-phenylpropionic acid

In a 10-ml culture tube with a PTFE-lined screw cap, 0.2 ml of plasma together with 0.1 ml of a 2.5 mg/l solution of *trans*-cinnamic acid (internal standard), 0.1 ml of 2 *M* sulphuric acid and 0.2 ml of distilled water were extracted with 3 ml of dichloromethane by mixing gently for 10 min on a rotary mixer. Samples were then centrifuged at 1500 *g* for 5 min and the aqueous layer was removed. The organic layer was transferred to a 15-ml conical tube and evaporated to dryness under reduced pressure (Buchler vortex evaporator, Fort Lee, NJ, U.S.A.) at 45°C for 30 min. Samples were

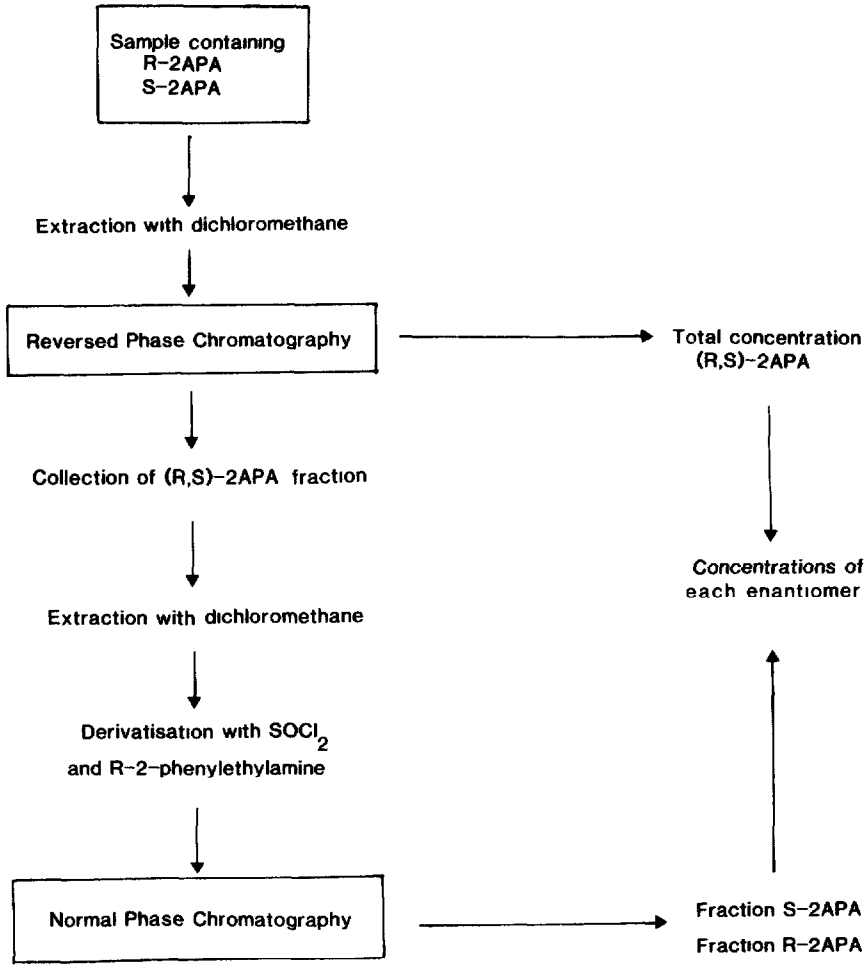

Fig. 1. Schematic outline of the analysis of 2-arylpropionic acid (2APA) non-steroidal anti-inflammatory drugs.

TABLE I

COMPARISON OF REVERSED-PHASE CHROMATOGRAPHIC CONDITIONS AND CALIBRATION RESULTS FOR 2PPA, KETOPROFEN AND FENOPROFEN

	2PPA	Ketoprofen		Fenoprofen	
Internal standard	*trans*-Cinnamic acid	Naproxen		Ketoprofen	
Mobile phase	Methanol—0.05 *M* phosphate buffer, pH 7 (12:88)	Methanol—0.005 *M* phosphate buffer, pH 3 (55:45)		Methanol—0.05 *M* phosphate buffer, pH 7 (50:50)	
Retention time (min)					
Compound	4.00	7.00		5.70	
Internal standard	5.90	9.30		3.75	
Calibration curve					
Range (mg/l)	6—300	0.2—100	1—100	2.5—100	5—100
n	14	3	7	9	8
Mean C.V. (%)	6.5	9.2	6.5	5.1	7.1
Limit of sensitivity (mg/l)	6.0	0.2		2.5	

redissolved in 0.1 ml of mobile phase and the total volume was injected into the reversed-phase chromatographic column.

Reversed-phase chromatography. Chromatography was carried out on a 250 mm × 4 mm I.D., 10-μm LiChrosorb RP-18 column (Merck, Darmstadt, F.R.G.) fitted with an RP-18 precolumn (Millipore-Waters, Milford, MA, U.S.A.). The mobile phase was as described in Table I with a flow-rate of 2.0 ml/min. Detection of 2PPA was carried out using a fixed-wavelength UV detector (Altex Model 153, Altex, Berkeley, CA., U.S.A.) at a wavelength of 254 nm.

The reversed-phase chromatographic fraction corresponding to (*R,S*)-2PPA was collected in a 15-ml culture tube with a PTFE-lined screw cap and to it 0.1 ml of 2 *M* sulphuric acid was added and the (*R,S*)-2PPA was extracted into 6.0 ml of dichloromethane by mixing gently for 10 min on a rotary mixer. Samples were centrifuged at 1500 *g* for 5 min and the aqueous layer was removed. The organic layer was transferred to a fresh 10-ml tube and evaporated to dryness under reduced pressure as previously described, making sure that there was no trace of water remaining in the tubes. To each tube 0.1 ml of dichloromethane containing 0.001 ml of thionyl chloride was added and each tube was tightly sealed, mixed and heated for 1 h at 70°C. The dichloromethane—thionyl chloride solution was made fresh each day using thionyl chloride redistilled less than four weeks previously. The samples were allowed to cool to room temperature and 0.5 ml of dichloromethane containing 0.005 ml of *R*-2-phenylethylamine (Sigma, St. Louis, MO, U.S.A.) were added. This solution was also prepared each day. Each sample was resealed and mixed for 30 s. After 20 min 3 ml of dichloromethane and 1 ml of 2 *M* sulphuric acid were added and the samples were

mixed for 10 min on a rotary mixer, centrifuged and the aqueous layer was removed. Another 1 ml of acid was added and the procedure repeated. The organic layer was transferred to a 15-ml conical tube and evaporated to dryness as described previously. The samples were redissolved in 0.05 ml of dichloromethane and the total volume was injected into the normal-phase chromatographic column.

Normal-phase chromatography. Chromatography was carried out on a 5-μm LiChrosorb Si-60 column, 250 mm × 4 mm I.D. (Merck) fitted with a Silica precolumn (Millipore-Waters). The mobile phase was as described in Table II with a flow-rate of 2.0 ml/min. Detection of the *R*- and *S*-2PPA derivatives was carried out using a variable-wavelength detector (Waters Model 45) at a wavelength of 254 nm.

TABLE II

COMPARISON OF NORMAL-PHASE CHROMATOGRAPHIC CONDITIONS AND CALIBRATION RESULTS FOR 2PPA, KETOPROFEN AND FENOPROFEN

	2PPA	Ketoprofen	Fenoprofen
Mobile phase	Acetonitrile—dichloromethane (2:98)	Acetonitrile—dichloromethane (5:95)	Acetonitrile—dichloromethane (4:96)
Wavelength (nm)	254	254	272
Retention time (min)			
R	6.60	3.00	4.50
S	7.60	3.67	5.4
Calibration curve*			
enantiomer fraction	0.10—0.91	0.02—0.98	0.08—0.92
n	8	5	4
b (mean ± S.D.)	0.790 ± 0.098	0.977 ± 0.032	0.939 ± 0.036
d (mean ± S.D.)	0.790 ± 0.098	0.976 ± 0.032	0.939 ± 0.036
a + *b* + *c* (mean ± S.D.)	1.00025 ± 0.00047	0.9998 ± 0.0004	1.000 ± 0.000
r^2 (mean ± S.D.)	0.993 ± 0.008	0.999 ± 0.001	0.999 ± 0.001

*a, b and c as in eqns. 1 and 2.

Calibration

Reversed-phase chromatography. Calibration of the reversed-phase chromatography was carried out using plasma standards of known concentrations of (*R*,*S*)-2PPA (Chemical Dynamics, South Plainfield, NJ, U.S.A.) from 6 to 300 mg/l. A normalised peak-height ratio was determined for each plasma standard by dividing the peak-height ratio of (*R*,*S*)-2PPA to internal standard by the corresponding (*R*,*S*)-2PPA concentration. The mean normalised peak-height ratio was used to calculate the amount of (*R*,*S*)-2PPA in unknown samples and the coefficient of variation (C.V.) was used to establish the reproducibility of the method over the calibration range [5].

Normal-phase chromatography. Calibration of the normal-phase chromato-

graphy was carried out using plasma standards of total (*R*,*S*)-2PPA concentration of 100 mg/l with known fractions (FKn) of *S*-2PPA and *R*-2PPA, in the range 0.15—0.85 for each enantiomer. The standards were taken through the complete analytical procedure and the observed peak-height fractions (FOb) for each enantiomer were measured, where FOb is the peak height for one enantiomer divided by the sum of the peak heights of both enantiomers.

Regression analysis of FOb against FKn was carried out to give equations of the form:

$$\text{FOb} = a + b\text{FKn for } S\text{-2PPA} \quad (1)$$

$$\text{FOb} = c + d\text{FKn for } R\text{-2PPA} \quad (2)$$

For any unknown sample the actual fraction of each enantiomer, equivalent to FKn of eqns. 1 and 2, can be calculated from the observed fraction using eqns. 1 and 2. To simplify quantitation of unknown samples only the actual fraction of *S*-enantiomer (FS) was calculated using the regression equation, the actual fraction of *R* enantiomer (FR) was calculated as:

$$\text{FR} = 1 - \text{FS} \quad (3)$$

This relationship, however, is only true if for eqns. 1 and 2 $b = d$ and $a + b + c = 1$ (see Appendix).

The r^2 of the regression equations was used to establish the reproducibility of the method over the calibration range.

Modifications for ketoprofen analysis

Minor modifications were made to the above analytical procedures to allow quantitative measurement of the enantiomers of ketoprofen. Naproxen was chosen as the internal standard and was prepared as a 20 mg/l solution in 0.01 *M* phosphate buffer at pH 6. The volume of plasma assayed was not altered, 0.5 ml of the internal standard solution was used and 0.3 ml of 0.01 *M* phosphate buffer at pH 6 were added rather than distilled water. The pH was then lowered to pH 2 by the addition of 1 ml of 1 *M* phosphate buffer at pH 2 and the extraction was carried out in 3.0 ml of dichloromethane as described. The mobile phase for the reversed-phase chromatography was modified as shown in Table I. Before derivatisation of the ketoprofen enantiomers could be carried out, the samples were concentrated at 40°C under reduced pressure for 1 h (Speed VacConcentrator, Servant Instrument, Hicksville, NY, U.S.A.). This was done in order to remove the large amount of methanol present in the mobile phase. The samples were then transferred to a 10-ml culture tube and the pH adjusted to 2 by addition of 1.0 ml of 1 *M* phosphate buffer at pH 2, 3 ml of dichloromethane were added and the derivatisation carried out as described for 2PPA. The mobile phase for the normal-phase chromatography was modified as shown in Table II.

Calibration of the reversed-phase chromatography was carried out as described for 2PPA over a range of concentrations of ketoprofen (May and Baker Australia, Melbourne, Australia) from 0.2 to 50 mg/l and 1 to 100 mg/l. Calibration of the normal-phase chromatography was also carried out as de-

scribed for 2PPA over a range of known enantiomer fractions of 0.02 to 0.98 for each enantiomer.

Modifications for fenoprofen analysis

The 2PPA assay was also easily modified to allow quantitation of the fenoprofen enantiomers. The extraction procedure was identical to that of 2PPA except that the internal standard chosen was ketoprofen, and 0.1 ml of a 2.5 mg/l solution was used. The mobile phase for the reversed-phase chromatography was modified as shown in Table I. The derivatisation procedure is also as previously described except the amount of thionyl chloride used was decreased to 0.0002 ml in 0.1 ml of dichloromethane. This was done to reduce interference in the normal-phase chromatography. The mobile phase for the normal-phase chromatography was modified as shown in Table II.

Detection of the *R*- and *S*-fenoprofen-2-phenylethylamide derivatives was carried out at a wavelength of 272 nm. Calibration of the reversed-phase chromatography was carried out using concentrations of fenoprofen (Lilly Industries, Sydney, Australia) from 2.5 to 100 mg/l and 5 to 100 mg/l. Calibration of the normal-phase chromatography was also carried out as described for 2PPA over a range of known enantiomer fractions of 0.08 to 0.92 for each enantiomer.

RESULTS AND DISCUSSION

Reversed-phase chromatography

Table I shows the retention times and calibration results obtained for the reversed-phase analysis of 2PPA, ketoprofen and fenoprofen. An example of the reversed-phase chromatograms for 2PPA is shown in Fig. 2. There were no peaks in plasma from healthy rabbits that interfered with either the (*R,S*)-2PPA or the internal standard. Similar results were obtained with both ketoprofen and fenoprofen.

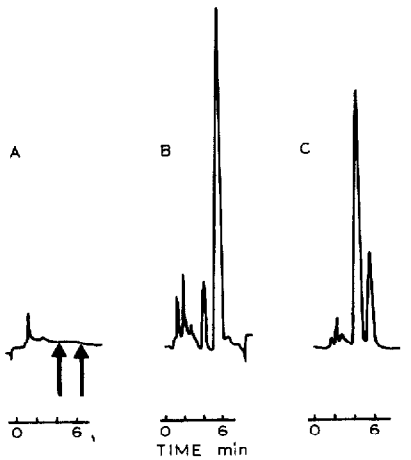

Fig. 2. Reversed-phase chromatograms for 2PPA obtained from (A) blank plasma, 0.04 a.u.f.s.; (B) 20 mg/l 2PPA plasma standard, 0.04 a.u.f.s.; and (C) 300 mg/l 2PPA plasma standard, 0.08 a.u.f.s. Arrows indicate the retention times for (*R,S*)-2PPA (4.0 min) and internal standard (5.90 min).

Derivatisation

The derivatisation procedure in this method was based on that for indoprofen by Tosolini et al. [6], but was significantly modified in order to make it suitable for use with our three compounds. For 2PPA dichloromethane was found to be a more suitable solvent than ethyl acetate, producing better extraction recoveries for both enantiomers and taking less time to evaporate to dryness. Excess thionyl chloride was not evaporated at the end of the reaction with 2PPA since it was found that this caused the loss of a significant fraction of 2PPA. This may reflect the smaller molecular weight and greater volatility of 2PPA compared to indoprofen. The amount of thionyl chloride used for the formation of the acid chloride was also decreased from 0.025 to 0.001 ml in order to reduce the amount of interference in the normal-phase chromatography but this did not result in loss of sensitivity. The acid wash at the end of the derivatisation procedure ensures the removal of any excess *R*-2-phenylethylamine before chromatography.

Normal-phase chromatography

Table II shows the retention times and calibration results obtained for the normal-phase analysis of 2PPA, ketoprofen and fenoprofen. Examples of the normal-phase chromatograms for 2PPA, ketoprofen and fenoprofen are shown in Fig. 3. For each compound, the two distereoisomers are well resolved and there are no peaks in blank plasma that interfere with either of the 2-phenylethylamide derivatives. The results of a typical normal-phase calibration curve for 2PPA are shown in Fig. 4. The chromatograms and calibration curves for normal-phase analysis indicate that the analysis is stereoselective for the *R*-enantiomer. This selectivity could have a number of causes

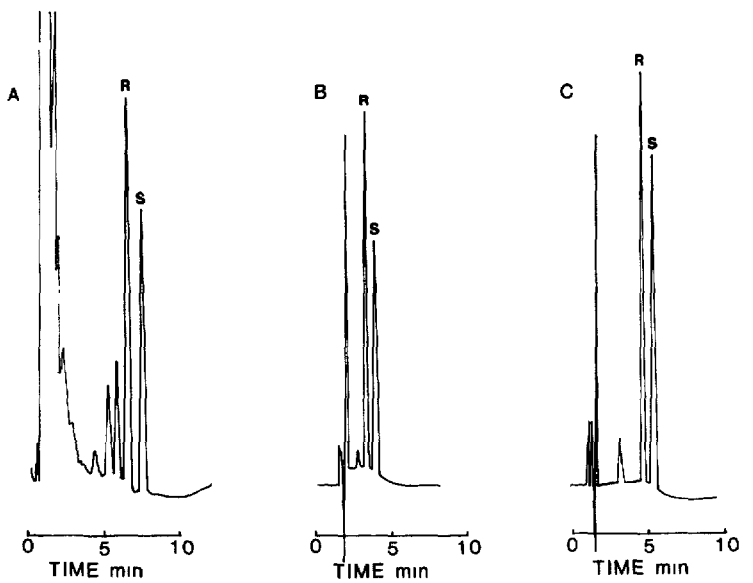

Fig. 3. Normal-phase chromatograms obtained for racemic standards of (A) (*R,S*)-2PPA, (B) (*R,S*)-ketoprofen and (C) (*R,S*)-fenoprofen. (See Table II for chromatographic conditions and retention times.)

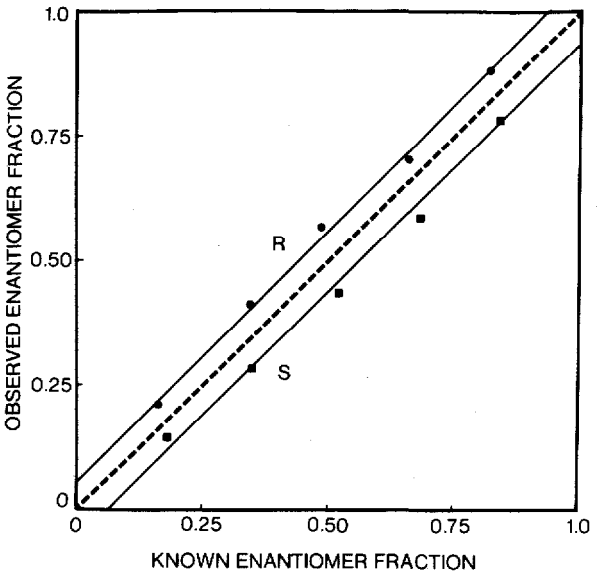

Fig. 4. Results of a typical calibration curve for 2PPA in which FKn (x) is plotted against FOb (y) for both *R*- (•) and *S*- (▪) enantiomers.

including different rates or extent of reaction with *R*-2-phenylethylamine, selective loss of the *S*-distereoisomeric amide or differences in the spectral characteristics of the two distereoisomeric amides. It is therefore necessary to *account for this enantioselectivity when measuring enantiomer fractions in* unknown samples by using an appropriate calibration procedure in which the response of the assay to samples of known enantiomeric composition is measured.

During the development of this method, resolution of *R*- and *S*-2PPA was attempted by using only the derivatisation and normal-phase chromatography, as has been found successful with some other arylpropionic acids such as indoprofen and benoxaprofen [2, 6]. Using this technique, it was possible to resolve the *R*- and *S*-enantiomers of 2PPA, however, in plasma standards endogenous compounds were present which interfered with the two enantiomer peaks under a variety of chromatographic, extraction and derivatisation conditions. It was therefore necessary to introduce the reversed-phase chromatography as a means of sample isolation before resolution of the *R*- and *S*-enantiomers. Stoltenborg et al. [3] experienced similar problems with carprofen and adopted thin-layer chromatography for this purpose. The problem of interfering peaks was greatest for 2PPA which has a lower molar extinction coefficient than either ketoprofen or fenoprofen for which interfering peaks were less troublesome.

Applications

The analytical method has been applied to a series of animal studies using rabbits. Fig. 5 shows the plasma concentration versus time profiles for *R*- and *S*-2PPA resulting from the administration of 10 mg/kg/h of racemic 2PPA to a rabbit.

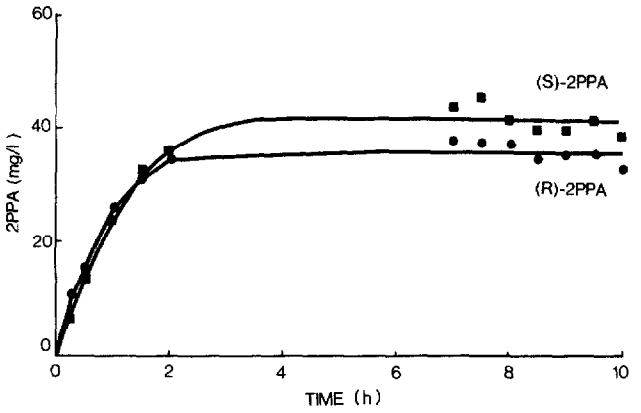

Fig. 5. Plasma concentration versus time profiles for *R*-2PPA (•) and *S*-2PPA (▪) resulting from the administration of 10 mg/kg/h of racemic 2PPA to a rabbit.

APPENDIX

For *S*-2PPA let $(FOb)_S = a + b(FKn)_S$

For *R*-2PPA let $(FOb)_R = c + d(FKn)_R$

let $(FOb)_R = 1 - (FOb)_S$

$$\text{then } (FKn)_R = \frac{1}{d} - \frac{1}{a} - \frac{b(FKn)_S}{d} - \frac{c}{d}$$

$$\text{if } b = d \text{ then } (FKn)_R = \frac{1 - a - c}{d} - (FKn)_S$$

if $a + c + d = 1$, then $(FKn)_R = 1 - (FKn)_S$

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