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Indirect enantiomeric separation of 2-arylpropionic acids and structurally related compounds by reversed phase HPLC

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Abstract

A reversed-phase high-performance liquid chromatographic method, using an organic modifier-phosphate buffered mobile phase, for the determination of the enantiomeric composition of 2-arylpropionic acids and other structurally related compounds in microbial media is described. The method is based on the resolution of diastereoisomeric amides formed from the reaction of the arylpropionic acid with either (−)-(*S*)-α-methylbenzylamine or (−)-(*S*)-1-(naphthen-1-yl)ethylamine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl and 1-hydroxybenzotriazole and incorporating an internal standard. The addition of sodium pentanesulphonate to the mobile phase as an ion-pairing agent was necessary to remove unreacted amine to avoid rapid column deterioration. The method provides an efficient, rapid and reproducible means of monitoring the microbial chiral inversion of 2-arylpropionic acids and other structurally related molecules. © 1997 Elsevier Science B.V.

Keywords: Reversed phase HPLC; Indirect enantiomeric separation; Microbial chiral inversion; 2-Arylpropionic acid non-steroidal anti-inflammatory drugs

1. Introduction

Approximately 7% of the worlds population are affected by inflammatory disease states such as arthritis. One of the most important group of agents used in the treatment of such diseases are the non-steroidal anti-inflammatory drugs (NSAIDs), with a world market value estimated to be in excess of $10 billion per year [1]. The 2-arylpropionic acids (2-APAs, the 'profens') are an important subgroup within this class of agents, and include several of the most commonly used drugs such as ibuprofen and ketoprofen. These agents are chiral and the majority are marketed as racemates even though stereoselectivity is observed in both their disposition and activity. The main pharmacological activity of these agents, inhibition of cyclooxygenase, resides in the enantiomers of the (+)-(*S*)-absolute configuration, with the (*R*)-enantiomer being either inactive or weakly active in vitro [2]. However, in vivo many of these agents undergo metabolic chiral inversion in mammals from the (*R*)-enantiomers to their active (*S*)-antipodes, the rate and extent of inversion being dependent on both the structure of the

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drug and the species under investigation [3–5]. Recently the potential of microbial systems to mediate the chiral inversion of 'profen' NSAIDs has been examined, using 2-phenylpropionic acid (2-PPA) as a model substrate [6–13]. These investigations have led to the isolation of two fungi, *Verticillium lecanii* and *Cordyceps militaris*, capable of mediating the chiral inversion of both ibuprofen and 2-PPA.

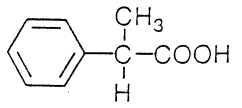
Investigation of the chiral inversion of these molecules requires a stereoselective analytical technique to differentiate between the two isomeric forms and allow determination of the enantiomeric composition. Chiral chromatography is the most commonly used technique and both direct and indirect separations have been used extensively in the investigation of the metabolic chiral inversion of the profens in mammalian systems [3,5]. Examples include derivatization with L-leucinamide via the mixed anhydride intermediate in the reversed phase HPLC assay of ketoprofen enantiomers in human plasma and urine [14]; the direct separation of ibuprofen enantiomers in biological fluids with a β-cyclodextrin chiral HPLC column [15]; derivatization with (*S*)-1-(4-dimethylamino-1-naphthyl)-ethylamine in the normal phase HPLC assay of naproxen enantiomers in serum [16]; combination of derivatization with (*R*)-1-methylbenzylamine and the use of a (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine chiral HPLC column to effect resolution of pirprofen enantiomers from those of its pyrrole metabolite [17]; derivatization with (*S*)-amphetamine in the GC assay of tiaprofenic acid [18] and combination of derivatization with non-chiral benzylamine and the use of a tris(4-methylbenzoate)cellulose chiral column to effect resolution of eight 2-APAs [19,20].

Previous investigations of the microbial chiral inversion of 2-APAs [7] have used the indirect isocratic normal phase HPLC assay developed for investigations of ibuprofen enantiomers in human plasma [21,22]. This involved toluene extraction from the microbial media followed by derivatization with (−)-(*S*)-1-(naphthen-1-yl)ethylamine to form a pair of diastereoisomeric amides, which were analysed using a silica column and a hexane/ethylacetate mobile phase. Although this system produced adequate resolution of 2-PPA and ibuprofen enantiomers, it is not likely to adapt well to the analysis of more polar members of the 2-APAs and it is not a convenient system for the routine analysis of large numbers of samples as the technique has several disadvantages. The non-aqueous solvents used in the mobile phase have high disposal costs, 1-hydroxybenzotriazole, one of the derivatization reagents, has a relatively long retention time of 45 min, there are large differences in peak heights but not peak areas between equal concentrations of both enantiomers and the derivatization reaction itself takes 2 h to complete. Although chiral columns offer an alternative to indirect enantiomeric separation the cost and lifetime of such columns presently precludes their use in many laboratories. This paper reports the development of a series of alternative indirect reversed phase HPLC systems for the efficient, rapid and reproducible resolution of the enantiomers of the 2-APAs and structurally related compounds. The chemical structures of the compounds under investigation are shown in Fig. 1.

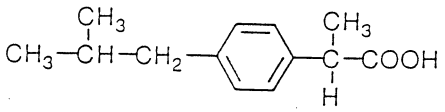
2. Experimental

2.1. Materials

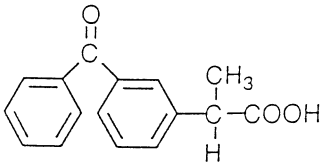
(*R*,*S*)-, (*R*)- and (*S*)-2-PPA, phenylacetic acid (PAA), *p*-toluic acid (*p*-TOL), (*S*)-naproxen (NAP), (*R*,*S*)-mandelic acid (MA), (*R*,*S*)-, (*R*)- and (*S*)-2-phenylbutyric acid (2-PBA), (*R*,*S*)-, (*R*)- and (*S*)-α-methoxyphenylacetic acid (α-MPAA), (*R*,*S*)-2-phenoxypropionic acid (2-PXPA), 1-hydroxybenzotriazole, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl, (−)-(*S*)-α-methylbenzylamine, (−)-(*S*)-1-(naphthen-1-yl)-ethylamine, sodium pentane sulphonate, HPLC grade acetonitrile, HPLC grade pyridine and HPLC grade dimethylformamide (DMF) were purchased from Aldrich Chemical Company, Gillingham, Dorset. (*R*,*S*)-fenoprofen (FEN), (*R*,*S*)-indoprofen (IND), (*R*,*S*)-suprofen (SUP) and (*R*,*S*)-etodolac (ETOD) were purchased from Sigma Chemical Company, Poole, Dorset. (*R*,*S*)-, (*R*)- and (*S*)-ibuprofen (IBU) and (*R*,*S*)-, (*R*)- and (*S*)-flurbiprofen (FLUR) were generous gifts



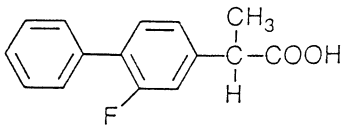
2-Phenylpropionic acid



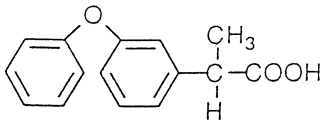
Ibuprofen
2-(4-Isobutylphenyl)propionic acid



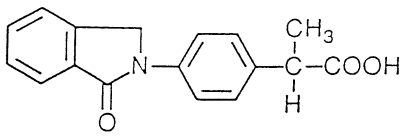
Ketoprofen
2-(3-Benzylphenyl)propionic acid



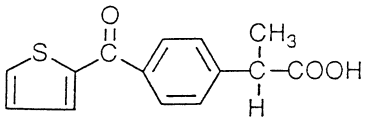
Flurbiprofen
2-(2-Fluoro-4-biphenyl)propionic acid



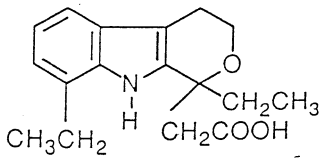
Fenoprofen
2-(3-Phenoxyphenyl)propionic acid



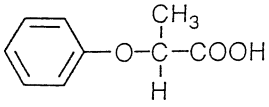
Indoprofen
2-[4-(1-Oxo-2-isoindolinyl)
phenylpropionic acid



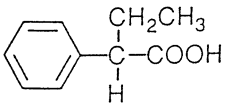
Suprofen
α-Methyl-4-(2-thienylcarbonyl)
phenylpropionic acid



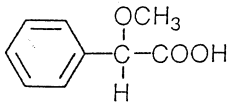
Etodolac
1,8-Diethyl-1,3,4,9-tetrahydropyrano
[3,4-b]indole-1-acetic acid



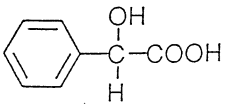
2-Phenoxypropionic acid



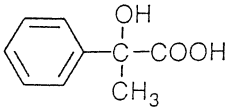
2-Phenylbutyric acid



α-Methoxyphenylacetic acid



Mandelic acid
2-Hydroxy-2-phenylacetic acid



Atrolactic acid
2-Hydroxy-2-phenylpropionic acid

Fig. 1. The chemical structures of the molecules under investigation.

of the BASF Pharma, Nottingham. (*R*,*S*)-, (*R*)- and (*S*)-ketoprofen (KET) was a generous gift of Chiroscience, Cambridge. HPLC grade dichloromethane, HPLC grade toluene, HPLC grade methanol and HPLC grade ortho phosphoric acid were purchased from Surechem, Needham Market, Suffolk. Sodium dihydrogen phosphate, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from BDH Chemicals, Poole, Dorset. HPLC solvents were degassed by ultrasonication for 10 min in a Decon FS200 ultrasonic bath prior to use. Water for use in the HPLC system was double distilled and passed through a 0.2 μm filter (Purite, Thame, Oxfordshire).

2.2. Instrumentation

HPLC analysis was carried out using a PU4015 pump, Pye-Unicam, Cambridge; an ISS-100 autosampler, Perkin-Elmer, Beaconsfield, Buckinghamshire; a Spectraflow 757 variable wavelength UV detector set at 254 nm, Kratos, Urmston, Manchester; and a SP4100 computing integrator set to measure peak area, Spectra-Physics, San Jose, California, USA. A constant flow rate of 1 ml min^{-1} was maintained, producing an operating pressure in the range 120–150 bar at room temperature (20–22°C). The mobile phases employed consisted of either CH_3CN or MeOH and 0.075 M NaH_2PO_4 mixed in varied ratios containing sodium pentanesulphonate (0.005 M) and sufficient H_3PO_4 to produce a pH* of 2.8 (Table 1). The stationary phase was a Techsphere ODS column (particle size 5 μm; 250 × 5 mm id) with a 1 cm guard column, HPLC Technology, Macclesfield, Cheshire.

2.3. Sample preparation

Incubation mixtures of cell suspensions in Sørensen's phosphate buffer [23] containing drug substrate were centrifuged at 2000 × *g* for 4 min to remove the microorganisms and the supernatant liquid stored at −20°C prior to analysis. The appropriate internal standard (200 μl of a solution in DMF) was added to 2 ml aliquots of the supernatant fluid in 10 ml stoppered glass centrifuge tubes. The samples were acidified to pH 1 by the addition of HCl (5M; 200 μl) and the whole extracted with toluene (2 × 3 ml). The combined extracts were evaporated to dryness and the residue taken up in CH_2Cl_2 (1 ml). The samples were derivatized by the addition of 1-hydroxybenzotriazole (20 μl; 10 mg ml^{-1} in CH_2Cl_2 containing pyridine, 1%); 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (300 μl; 10 mg ml^{-1} in CH_2Cl_2) and either (−)-(*S*)-α-methylbenzylamine (300 μl; 10 mg ml^{-1} in CH_2Cl_2) or (−)-(*S*)-1-(naphthen-1-yl)ethylamine (300 μl; 10 mg ml^{-1} in CH_2Cl_2) and allowed to stand until derivatization was complete. After this time the solvent was removed by evaporation, the residue reconstituted in the HPLC mobile phase (0.5 ml) and 10 μl injected onto the column.

2.4. Validation

Seven standard samples of each substrate in the range 50–500 μg ml^{-1} were prepared in 2 ml volumes in 10 ml stoppered glass centrifuge tubes by appropriate dilution of stock solutions (10 mg ml^{-1} in DMF) with Sørensen's phosphate buffer [23]. The appropriate internal standard (200 μl of a solution in DMF) was then added to each sample, and the samples were then acidified, extracted and derivatized as described above. The peak area ratios of the (*S*)-amide derivatives of each substrate enantiomer to the internal standard were used to construct standard linear analytical response curves calculated by a least-squares linear regression analysis of peak area ratios versus concentration.

The within day precision of injection was investigated by comparison of peak areas following six replicate injections of extracted samples of 250 μg ml^{-1} of each substrate on the same day. The reproducibility of the whole sample preparation method was investigated by comparison of peak area ratios of the analyte to internal standard following six replicate extractions of samples of 250 μg ml^{-1} of each substrate carried out on different days.

The extraction efficiency was determined by the derivatization and analysis as described above of six replicate 1 ml solutions of 500 μg ml^{-1} of each

Table 1
Chromatographic data

Analyte	Mobile phase	Derivatization reagent	Internal standard	k_{IS}	k'_1	k'_2	α	R
2-PPA	CH_3CN: buffer 45:55 + 0.09% v/v H_3PO_4	Phenyl	PAA 200 μg ml^{-1}	4.25	6.25	7.10	1.10	1.92
IBU	CH_3CN: buffer 60:40 + 24% v/v H_3PO_4	Phenyl	NAP 1 μg ml^{-1}	3.05	6.63	7.11	1.07	1.43
KET	CH_3CN: buffer 50:50 + 0.15% v/v H_3PO_4	Phenyl	PAA 4 mg ml^{-1}	2.50	6.60	7.55	1.14	2.77
FLUR	CH_3CN: buffer 60:40 + 0.24% v/v H_3PO_4	Phenyl	(*S*)-NAP 200 μg ml^{-1}	3.15	4.75	5.35	1.13	2.24
FEN	CH_3CN: buffer 55:45 + 0.21% v/v H_3PO_4	Phenyl	(*S*)-NAP 100 μg ml^{-1}	3.42	4.95	5.42	1.09	1.69
IND	CH_3CN: buffer 50:50 + 0.15% v/v H_3PO_4	Phenyl	(*S*)-NAP 100 μg ml^{-1}	7.45	4.60	5.10	1.11	1.49
SUP	CH_3CN: buffer 50:50 + 0.15% v/v H_3PO_4	Phenyl	(*S*)-NAP 200 μg ml^{-1}	7.45	5.65	6.40	1.13	2.17
ETOD	CH_3CN: buffer 65:35 + 0.24% v/v H_3PO_4	Phenyl	(*R*)-IBU 100 μg ml^{-1}	5.21	5.67	6.08	1.07	1.42
2-PXPA	CH_3CN: buffer 50:50 + 0.15% v/v H_3PO_4	Naphthyl	PAA 200 μg ml^{-1}	5.45	8.85	9.35	1.06	0.95
2-PBA	CH_3CN: buffer 53:47 + 0.18% v/v H_3PO_4	Naphthyl	PAA 200 μg ml^{-1}	3.85	7.00	7.45	1.06	1.42
α-MPAA	MeOH: buffer 70:30 + 0.18% v/v H_3PO_4	Naphthyl	PAA 200 μg ml^{-1}	2.79	3.67	4.58	1.25	3.22
MA	MeOH: buffer 65:35 + 0.15% v/v H_3PO_4	Naphthyl	PAA 200 μg ml^{-1}	4.63	3.17	3.54	1.12	1.69
ATLA	MeOH: buffer 65:35 + 0.15% v/v H_3PO_4	Naphthyl	*p*-Toluic acid 200 μg ml^{-1}	7.04	5.13	5.75	1.12	2.27

t_0 Time of first baseline disturbance by the solvent front.
t_{IS} Retention time of the internal standard.
t_1 Retention time of the first eluted analyte enantiomer.
t_2 Retention time of the second eluted analyte enantiomer.
k'_1 Capacity factor for the first eluted enantiomer: $(t_1\text{-}t_0)/t_0$.
k'_2 Capacity factor for the second eluted enantiomer: $(t_2\text{-}t_0)/t_0$.
α Separation factor: k'_2/k'_1.
R Resolution factor: $R = 1.18(t_2\text{-}t_1)/(w_1 + w_2)$. Where w is the peak width at half height.

substrate plus the appropriate internal standard prepared in CH_2Cl_2. These solutions thus contained the same quantity of analyte and internal standard as the 2 ml samples subjected to analysis above. The resultant average peak areas were then compared with the average peak areas for corresponding extracted samples for calculation of the extraction efficiency.

The limit of detection, under standard experimental conditions, was determined for each substrate by the preparation, extraction, derivatization and analysis as described above of a progression of samples of decreasing concentration to determine the lowest concentration that could be detected with the peak threshold setting set at three times the background noise on the computing integrator.

The time course of the derivatization reaction was investigated by the preparation and derivatization of 14×1 ml samples of 500 μg ml^{-1} of each substrate plus appropriate internal standard in CH_2Cl_2. The samples were sequentially rapidly rotary evaporated to dryness after 5, 10, 15, 20, 25, 30, 60, 90 and 120 min and subjected to

analysis as described above. The standard time course for the derivatization of each substrate was then set at 5 min after there was no further observed increase in peak area of the analyte enantiomers or the internal standard.

The elution order of the diastereoisomeric (*S*)-methylbenzylamides of 2-PPA, 2-PBA, α-MPAA, ibuprofen, ketoprofen and flurbiprofen were determined by the preparation, extraction, derivatization and HPLC analysis of the pure enantiomers of these compounds under the standard experimental conditions described above.

3. Results

Analysis of a sample prepared by the extraction of uninoculated microbial media and the addition of the derivatization reagents demonstrated no interfering peaks due to the constituents of the microbiological media or the derivatization reagents. Typical chromatograms after addition of analyte and internal standard for (*R*)-, (*S*)- and (*R*,*S*)-2-PPA are shown in Figs. 2–4, and the chromatographic data for all the compounds investigated are presented in Table 1.

Using the pure enantiomers of 2-PPA, 2-PBA, α-MPAA, ibuprofen, ketoprofen and flurbiprofen, it was found that no appearance of the other enantiomer was observed during the analysis of either the (*R*)- or (*S*)-enantiomer, indicating that neither enantiomer has a tendency to racemize under the experimental conditions used and are enantiomerically stable throughout the analytical procedure. The results indicated that in all the examples, the first eluted peak corresponds to diastereomeric amide of configuration [*S*-acid: *S*-amine] and the second eluted peak represents the amide of [*R*-acid: *S*-amine] configuration. This indicates that the (*S*)-enantiomer yields the diastereoisomer that interacts least with the non-polar stationary phase, and is in agreement with previously reported data [22].

The validation data for the 13 compounds investigated are presented in Table 2 and Table 3.

4. Discussion

The results presented in Table 2 and Table 3 demonstrate that the majority of the assay systems developed were efficient, rapid, reproducible and exhibited good resolution of the enantiomeric forms, and all the assays exhibited sufficient resolution to quantify any chiral inversion of stereoselective metabolism of the substrate. In general, the extraction efficiency into toluene was higher with the less polar analytes, the within run and between run coefficients of variation were smaller for the analyte enantiomers with the highest resolution factors (*R*) and the limits of detection were

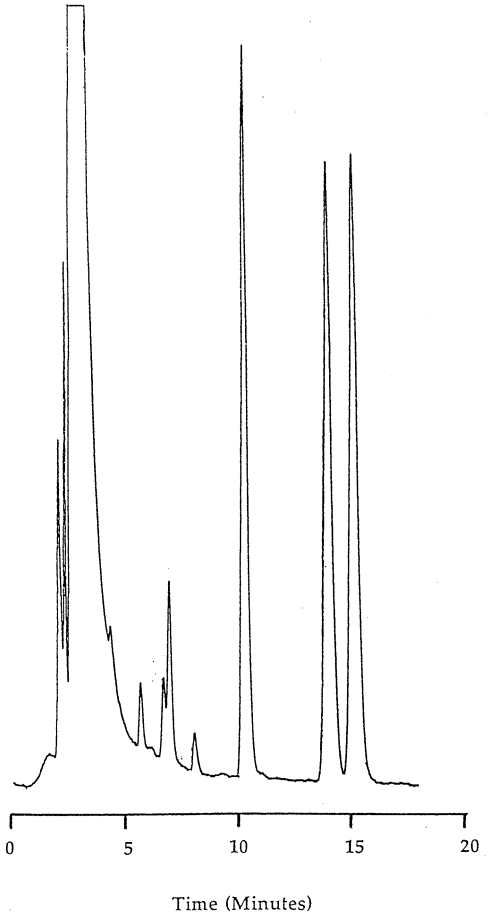


Fig. 2. A chromatogram of a sample of 250 μg ml^{-1} (*R*,*S*)-2-phenylpropionic acid and 200 μg ml^{-1} phenylacetic acid internal standard ($t_{IS} = 10.0$, $t_1 = 13.7$ and $t_2 = 14.8$ min).

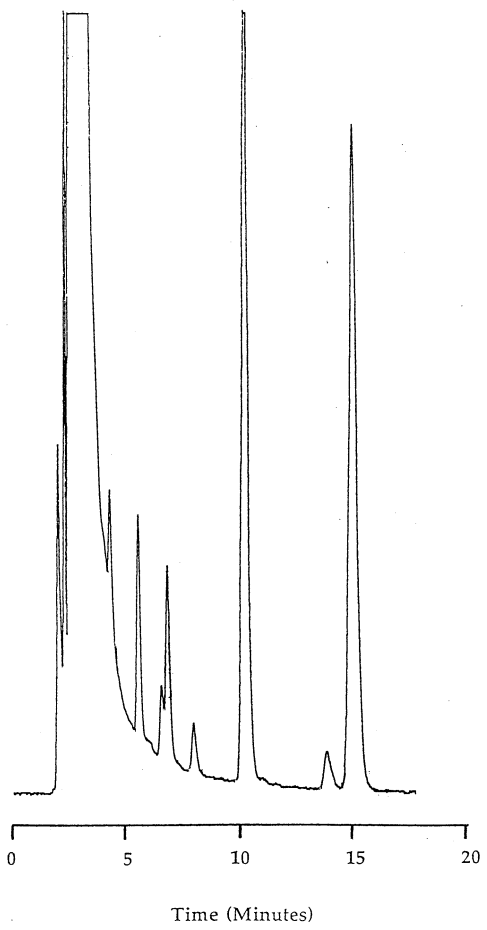


Fig. 3. A chromatogram of a sample of 125 μg ml^{-1} (*R*)-2-phenylpropionic acid and 100 μg ml^{-1} phenylacetic acid internal standard.

lowest for the analytes with the highest UV absorbance at 254 nm. The detection limits for the analytes under investigation were relatively poor in comparison to some other assays in the literature [15–17], but all are at least a factor of 10 to 15 times smaller than the substrate concentrations used experimentally and so are adequate for the investigation of the microbial chiral inversion of these molecules.

A relatively high internal standard concentration of 4 mg ml^{-1} PAA was required in the assay for ketoprofen to produce an internal standard peak of approximately the same size as the analyte peaks, and consequently the concentration of the 3 derivatization reagents had to be increased by a factor of 4–40 mg ml^{-1} to ensure complete derivatization. Etodolac has a very different chemical structure to the 2-APAs, with the chiral centre part of a tetrahydropyrano ring system fused to an indole moiety. However, the molecule does contain an acetic acid group attached to the chiral centre, and so was found to derivatize with (−)-(*S*)-α-methylbenzylamine and resolve adequately for the determination of any chiral inversion. 2-Phenoxypropionic acid contains an oxygen atom between the chiral centre and the phenyl ring which alters the priority of that group according to the Cahn-Ingold-Prelog sequence rules.

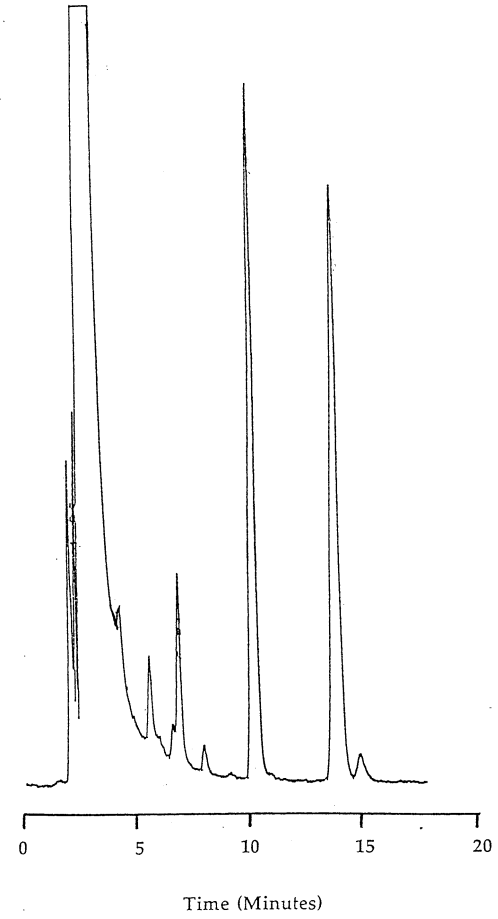


Fig. 4. A chromatogram of a sample of 125 μg ml^{-1} (*S*)-2-phenylpropionic acid and 100 μg ml^{-1} phenylacetic acid internal standards.

Table 2
Linear regression analysis

Substrate	Linear regression equation	
(*S*)-2-PPA	$y = 0.00420\ (\pm 0.000169)x + 0.106\ (\pm 0.0534)$	$r^2 = 0.998$
(*R*)-2-PPA	$y = 0.00442\ (\pm 0.000156)x + 0.117\ (\pm 0.0493)$	$r^2 = 0.998$
(*S*)-IBU	$y = 0.00900\ (\pm 0.000527)x - 0.0350\ (\pm 0.0782)$	$r^2 = 0.997$
(*R*)-IBU	$y = 0.00975\ (\pm 0.000883)x - 0.0569\ (\pm 0.131)$	$r^2 = 0.997$
(*S*)-KET	$y = 0.0166\ (\pm 0.000983)x + 0.133\ (\pm 0.146)$	$r^2 = 0.997$
(*R*)-KET	$y = 0.0171\ (\pm 0.000967)x + 0.138\ (\pm 0.143)$	$r^2 = 0.998$
(*S*)-FLUR	$y = 0.0152\ (\pm 0.000590)x - 0.00443\ (\pm 0.0875)$	$r^2 = 0.999$
(*R*)-FLUR	$y = 0.0154\ (\pm 0.000556)x + 0.0244\ (\pm 0.0823)$	$r^2 = 0.999$
(*S*)-FEN	$y = 0.00240\ (\pm 0.000111)x + 0.000240\ (\pm 0.0165)$	$r^2 = 0.998$
(*R*)-FEN	$y = 0.00237\ (\pm 0.0000953)x - 0.00902\ (\pm 0.0141)$	$r^2 = 0.998$
(*S*)-IND	$y = 0.00539\ (\pm 0.000203)x - 0.0327\ (\pm 0.0300)$	$r^2 = 0.999$
(*R*)-IND	$y = 0.00559\ (\pm 0.000242)x - 0.0322\ (\pm 0.0358)$	$r^2 = 0.998$
(*S*)-SUP	$y = 0.00702\ (\pm 0.000444)x - 0.0392\ (\pm 0.0658)$	$r^2 = 0.997$
(*R*)-SUP	$y = 0.00735\ (\pm 0.000470)x - 0.0459\ (\pm 0.0696)$	$r^2 = 0.997$
(*S*)-ETOD	$y = 0.00729\ (\pm 0.000493)x - 0.0691\ (\pm 0.0730)$	$r^2 = 0.998$
(*R*)-ETOD	$y = 0.00736\ (\pm 0.000419)x - 0.0814\ (\pm 0.0621)$	$r^2 = 0.998$
(*R*)-2-PXPA	$y = 0.00511\ (\pm 0.000187)x - 0.00383\ (\pm 0.0277)$	$r^2 = 0.999$
(*S*)-2-PXPA	$y = 0.00565\ (\pm 0.000178)x - 0.0179\ (\pm 0.0264)$	$r^2 = 0.999$
(*S*)-2-PBA	$y = 0.00469\ (\pm 0.000261)x - 0.0361\ (\pm 0.0387)$	$r^2 = 0.998$
(*R*)-2-PBA	$y = 0.00483\ (\pm 0.000316)x - 0.0255\ (\pm 0.0468)$	$r^2 = 0.998$
(*S*)-α-MPAA	$y = 0.00400\ (\pm 0.000186)x + 0.00386\ (\pm 0.0275)$	$r^2 = 0.998$
(*R*)-α-MPAA	$y = 0.00387\ (\pm 0.000264)x + 0.00425\ (\pm 0.0392)$	$r^2 = 0.997$
(*S*)-MA	$y = 0.00482\ (\pm 0.000343)x + 0.0148\ (\pm 0.0508)$	$r^2 = 0.996$
(*R*)-MA	$y = 0.00480\ (\pm 0.000342)x - 0.0322\ (\pm 0.0507)$	$r^2 = 0.996$
(*S*)-ATLA	$y = 0.00314\ (\pm 0.000127)x + 0.00368\ (\pm 0.0188)$	$r^2 = 0.998$
(*R*)-ATLA	$y = 0.00302\ (\pm 0.000142)x - 0.000566\ (\pm 0.0211)$	$r^2 = 0.998$

This means that (*R*)-2-phenoxypropionic acid and (*S*)-2-PPA have opposite absolute (*R*)- and (*S*)-configurations but the same spatial arrangements of groups around the chiral centre. As the pure enantiomers of 2-phenoxypropionic acid were not available for analysis, the elution order was thus assumed to be the same as that found for the other 2-APAs investigated. Accounting for the reversal of absolute configuration, this results in the first eluted peak corresponding to the diasteriomeric amide of configuration [*R*-acid: *S*-amine] and the second eluted peak representing the amide of [*S*-acid: *S*-amine configuration]. Mandelic acid was found to be too polar in nature to extract into an organic solvent such as toluene, chloroform and ethyl acetate, and so the samples were freeze dried at −40°C under vacuum for 12 h to remove all traces of water, reconstituted in 1 ml of CH_2Cl_2, derivatized and subjected to analysis as described above. Thus, extraction efficiency and reproducibility of extraction investigations were omitted from the validation of the assay for mandelic acid.

The time course investigations of the derivatization reactions involving the use of (−)-(*S*)-1-(naphthen-1-yl)ethylamine showed derivatization to be complete after only 30 min. This was much shorter than the 2 h reaction time reported previously for 2-PPA, ibuprofen and ketoprofen derivatization [24]. However, in this investigation (−)-(*S*)-1-(naphthen-1-yl)ethylamine was used with different substrates and different concentrations of derivatization reagents. The peak area ratios of the (*S*)-amide derivatives of both enantiomers of each substrate were constant throughout the time course investigations, indicating that there was no stereoselective derivatization under the conditions employed.

The phenyl containing derivatization agent was found to produce diastereomeric amides with much lower UV absorbtivity coefficients than those produced by the corresponding naphthyl amine, thus decreasing the sensitivity. This was overcome by increasing the analyte concentration on the column from that used previously [21] by increasing the sample size and the derivatization solutions concentration by a factor of ten from 0.2 ml and 1 mg ml^{-1} to 2 ml and 10 mg ml^{-1} respectively.

Table 3
Validation data

Substrate	Relative standard deviations (RSD)		Extraction efficiency	Derivatization time course	Detection limit[a]
	of Injection	of Method			
(*S*)-2-PPA	0.91%	1.65%	95.5% ± 6.3%	15 min	10 μg ml^{-1}
(*R*)-2-PPA	1.55%	1.06%	96.6% ± 6.3%	15 min	10 μg ml^{-1}
PAA IS	0.74%		95.2% ± 7.9%	15 min	
(*S*)-IBU	1.58%	2.93%	93.2% ± 5.7%	25 min	10 μg ml^{-1}
(*R*)-IBU	0.99%	3.49%	92.0% ± 7.2%	25 min	10 μg ml^{-1}
(*S*)-NAP IS	0.73%		95.7% ± 3.9%	15 min	
(*S*)-KET	1.85%	5.47%	92.0% ± 6.9%	20 min	1 μg ml^{-1}
(*R*)-KET	1.53%	4.22%	93.5% ± 6.2%	20 min	1 μg ml^{-1}
PAA IS	0.83%		90.2% ± 3.2%	15 min	
(*S*)-FLUR	1.00%	2.69%	85.8% ± 4.8%	15 min	0.5 μg ml^{-1}
(*R*)-FLUR	1.08%	2.00%	87.2% ± 4.1%	15 min	0.5 μg ml^{-1}
(*S*)-NAP IS	0.95%		89.8% ± 5.3%	15 min	
(*S*)-FEN	1.03%	3.40%	92.1% ± 3.7%	15 min	5 μg ml^{-1}
(*R*)-FEN	1.24%	4.24%	92.0% ± 4.2%	15 min	5 μg ml^{-1}
(*S*)-NAP IS	1.06%		93.7% ± 4.2%	15 min	
(*S*)-IND	1.37%	1.33%	90.9% ± 3.1%	15 min	0.5 μg ml^{-1}
(*R*)-IND	1.00%	3.35%	90.3% ± 6.1%	15 min	0.5 μg ml^{-1}
(*S*)-NAP IS	0.92%		91.6% ± 3.1%	15 min	
(*S*)-SUP	1.96%	3.23%	88.0% ± 4.9%	15 min	0.5 μg ml^{-1}
(*R*)-SUP	1.51%	3.26%	87.7% ± 5.1%	15 min	0.5 μg ml^{-1}
(*S*)-NAP IS	1.91%		87.7% ± 6.3%	15 min	
(*S*)-ETOD	4.71%	4.16%	91.7% ± 5.2%	15 min	1 μg ml^{-1}
(*R*)-ETOD	6.00%	5.53%	91.6% ± 6.6%	15 min	1 μg ml^{-1}
(*R*)-IBU IS	4.28%		96.8% ± 4.8%	15 min	
(*R*)-2-PXPA	2.07%	5.13%	87.8% ± 7.5%	30 min	5 μg ml^{-1}
(*S*)-2-PXPA	2.17%	5.24%	88.3% ± 7.6%	30 min	5 μg ml^{-1}
PAA IS	0.98%		89.3% ± 7.3%	30 min	
(*S*)-2-PBA	2.02%	1.39%	88.1% ± 5.2%	30 min	5 μg ml^{-1}
(*R*)-2-PBA	1.66%	2.09%	88.3% ± 5.4%	30 min	5 μg ml^{-1}
PAA IS	1.11%		93.3% ± 3.8%	30 min	
(*S*)-α-MPAA	1.27%	3.48%	87.1% ± 4.2%	30 min	2.5 μg ml^{-1}
(*R*)-α-MPAA	1.24%	3.06%	87.6% ± 4.0%	30 min	2.5 μg ml^{-1}
PAA IS	1.20%		86.1% ± 4.1%	30 min	
(*S*)-MA	1.59%			30 min	2.5 μg ml^{-1}
(*R*)-MA	1.56%			30 min	2.5 μg ml^{-1}
PAA IS	1.21%			30 min	
(*S*)-ATLA	1.31%	2.55%	86.9% ± 4.2%	30 min	2.5 μg ml^{-1}
(*R*)-ATLA	1.61%	2.84%	85.3% ± 4.0%	30 min	2.5 μg ml^{-1}
p-TOL IS	1.22%		91.7% ± 5.8%	30 min	

[a] Limit of detection in microbial media determined as the concentration that produced a peak greater than three times the background noise.

Initially, adequate separation was achieved with simple unbuffered mobile phases of different proportions of organic modifier and water. However, as more samples were run a gradual deterioration in the resolving power of the column was observed. Investigations involving HPLC analysis of the individual components in the derivatization reaction showed the derivatization amines (−)-(*S*)-α-methylbenzylamine and (−)-(*S*)-1-(naphthen-1-yl)-ethylamine to be binding to the column. The pH of the mobile phase was found to be approximately 8, at which the amines would be extensively ionized and thus be able to interact with free Si-OH groups on the column. The amines are basic in nature (pK_a approximately 10), and are thus likely to raise the local pH if bound to the column. This may lead to hydrolysis of the Si-O-C18 stationary phase linkage, accounting for the deterioration in the resolving power of the column. The pH of the mobile phase was reduced to 2.8 with phosphoric acid to protect the column, and stabilized with a phosphate buffer to stop any local pH rises due to the underivatized amine. Addition of sodium pentanesulphonate ensured the swift removal of the protonated amine from the column as an ion-pair complex.

5. Conclusion

In summary, these assays provide an efficient, rapid and reproducible method for the analysis of the enantiomers of a range of 2-APAs and structurally related compounds on extraction from microbial media which will aid the further investigations of the phenomenon of microbial mediated chiral inversion.

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