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Chiral discrimination of multiple profens as diastereomeric (*R*)-(+)-1-phenylethylamides by achiral dual-column gas chromatography

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Abstract

Profens were converted into diastereomeric (*R*)-(+)-1-phenylethylamides using ethyl chloroformate and triethylamine in dichloromethane. Gas chromatographic analysis on dual-columns with different polarities provided complete enantioresolution of eight profens, facilitating chiral discrimination based on matching with retention index sets characteristic of each enantiomer. The present method was linear ($r \geq 0.9992$) with good precision (0.8–6.0%) and accuracy (−9.3 to 0.003%), allowing detection of trace (*R*)-profens in optical purity test on four (*S*)-profen mixture in a single run. And the method allowed simultaneous enantiomeric screening for ibuprofen enantiomers and their chiral metabolites excreted in urine following administration of racemic ibuprofen.

Keywords: Enantiomer separation; Profens; (*R*)-(+)-1-Phenylethylamides

1. Introduction

The antiinflammatory activities of chiral profens, non-steroidal antiinflammatory drugs of 2-arylpropionic acid type, are mainly ascribed to (*S*)-enantiomers [1,2]. They are, however, mostly marketed as racemic mixtures, though some of their enantiomers exhibit different toxicological and pharmacological properties [3,4]. Hence, the production of active profens in enantiomerically pure forms, their optical purity control and stereoselective pharmacokinetic study have become important tasks in the chiral drug development [5,6].

The enantioseparation of profens requires the use of enantioselective analytical methods. Among the diverse methods employing high-resolution capillary electrophoresis (CE), high-performance liquid chromatography (HPLC) or gas chromatography (GC), the chiral CE permits faster method development with easier reversal of enantiomer migration order for the direct enantioseparation without derivatization [7–9]. However, CE has precision problem in the migration times with runs compared to HPLC and GC. Many chiral stationary phases available for direct enantioseparation of profens by HPLC require prior derivatization [10–12]. In contrast, chiral mobile phase additives provide direct enantioseparation of profens as diastereomeric ion-pairs without derivatization [13,14]. However, it is not easy to find universal chiral column or chiral additive able to separate multiple profens in a single analysis [6]. Therefore, over the last decade, the majority of selective, sensitive and versatile HPLC methods have been based on indirect separation of profen enantiomers as diastereomeric derivatives on conventional reversed-phase columns [15–23]. However, the responses of diastereomers with UV or fluorescence detection are not necessarily identical but this is not a problem when using GC with flame ionization detection as pointed out elsewhere [6,24]. The indirect GC methods employing an achiral packed column [25–27] or capillary columns with incomparably higher resolving power and long-term durability [24,23–30] were mostly used in earlier years but rarely in recent years, though they appear to be more preferred over the HPLC methods when measuring profen enantiomers at low concentrations in biological matrices

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[25,28]. If indirect separation as diastereomeric derivatives is to be used, high-resolution GC combined with mass spectrometry (MS) offers rapid and robust analyses with positive peak identification suitable for metabolic studies [26,29,30].

Prior to GC and GC–MS analysis, profens are converted to volatile diastereomeric derivatives. For this purpose, diastereomeric amide formation has been preferentially employed because of the chemical stability and rigidity of amide bond. Among the chiral resolving reagents used for chiral amidation such as L-leucinamide [16,18,19], amphetamine [28], (*R*)-(+)-1-phenylethylamine (1-PEA) or its antipode [24–27,29,30], 1-PEA with ethyl chloroformate (ECF) activation provides more volatile diastereomeric amides suitable for GC analysis at room temperature within a few min. Moreover, the amidation reaction with 1-PEA was found not to be stereospecific to any significant extent [24], and the stereochemical conversion of profens [18,31,32] could be minimized by using excess amount (≥17 mM) of ECF [18]. Polar acetonitrile as the reaction solvent was found to induce more racemization than toluene [32]. Therefore, the rapid ECF/1-PEA procedure required to be optimized prior to GC analysis.

The chirality of each separated profen enantiomer is mainly determined by cochromatography with enantiomerically pure (*S*) or (*R*) standards, necessitating at least a second run with real samples. In our previous studies on the enantiomeric separation of chiral acids as *O*-trifluoroacetylated (*S*)-(+)-3-methyl-2-butyl esters [33] and as *O*-trifluoroacetylated (−)-menthyl esters [34], the achiral dual-columns with different polarities were found to be useful in achieving a complete resolution of 18 and 30 enantiomeric pairs in one analytical run, respectively. Moreover, retention index (*I*) matching with the reference values allowed chemical identification and chiral discrimination of each enantiomer, thus solving the tedious problem of conventional cochromatographic procedure. Therefore, the overall analysis time was considerably shortened.

The present study was undertaken to combine indirect GC enantioseparation with simple GC-*I* matching for the rapid chiral profiling and screening analysis of multiple profens as diastereomeric (*R*)-(+)-1-phenlyethylamide derivatives in a single run. The optimal amounts of dichloromethane as the reaction solvent and of triethylamine (TEA) as the proton scavenger in the activation with ECF and amidation reaction with (*R*)-(+)-1-PEA were examined for suppressing artifact formation and racemization. The resulting amides were subjected to GC analysis on thermally stable achiral dual-columns with different polarities for the chemical identification and accurate chiral discrimination. This method was intended to complement our recent direct chiral CE method for the chiral profens [35] as the confirmation tool for wider range of applications in the chiral purity analysis of acidic drugs and their metabolic inversion study.

2. Experimental

2.1. Materials

The following seven racemic profen standards and four enantiomerically pure profen standards were obtained from Sigma–Aldrich (St. Louis, MO, USA) and other vendors: ibuprofen, fenoprofen, flurbiprofen, suprofen, indoprofen, ketoprofen, carprofen, (*S*)-(+)-ibuprofen (99%), (*S*)-(+)-flurbiprofen (98%), (*S*)-(+)-naproxen (99%) and (*S*)-(+)-ketoprofen (98.9%). (*R*)-(−)-Ibuprofen and racemic naproxen were supplied by Dr. Nariyasu Mano (Graduate School of Pharmaceutical Science, Tohoku University, Sendai, Japan). (*R*)-(+)-1-Phenylethylamine (1-PEA) (99.5%), triethylamine (TEA), ethyl chloroformate (ECF) and L-alanine were purchased from Sigma–Aldrich and *n*-hydrocarbon standards (C_{22}–C_{44}, even numbers only) from Polyscience (Niles, IL, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Diethyl ether, acetonitrile, ethyl acetate, toluene, dichloromethane and isooctane of spectroanalyzed grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical-reagent grade and used as received.

2.2. Profen and reagent standard solutions

Each standard stock solution of profens was made up at 10 μg/μl in acetonitrile as their free acid forms. The working solutions at varied concentrations were then prepared by diluting each stock solution with acetonitrile. The internal standard (IS) stock solutions was prepared by dissolving two *n*-hydrocarbons (C_{26} and C_{34}) each at 10.0 μg/μl in toluene and was used to prepare IS working solution at 1.0 μg/μl in toluene. (*R*)-(+)-1-PEA solution was prepared at 0.5 M in methanol. TEA and ECF solutions were prepared in acetonitrile at 50.0 and 60.0 mM, respectively. A mixed hydrocarbon solution containing 11 *n*-hydrocarbons (C_{22}–C_{40} and C_{44}, even numbers only), each at 1.0 μg/μl in isooctane, was used as the IS solution for *I* measurement. Calibration samples were prepared at different amount ranges from 0.1 to 50.0 μg depending on profens by mixing appropriate aliquots of each working solution. All standard solutions prepared were stored at 4 °C.

2.3. Gas chromatography and gas chromatography–mass spectrometry

The GC analyses were performed with an Agilent 6890 gas chromatograph, equipped with electronic pneumatic control system, a split/splitless inlet system, an automatic liquid sampler, two flame ionization detectors (FIDs) and GC Chemstation (Agilent Technologies, Atlanta, GA, USA). The injector was installed with a dual-column system made of DB-5 MS (SE-54 bonded) and DB-17 MS (OV-17 bonded) fused-silica capillary columns (15 m × 0.25 mm

i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The injector and detector temperatures were 260 and 290 °C, respectively. Samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 0.7 min. The flow rate of helium as carrier gas was initially set at 1.0 ml/min (23 min) and then programmed to 6 ml/min at 2 ml/min^2 in ramp flow mode. The oven temperature was initially 150 °C (1 min) and programmed at 30 °C/min to 240 °C and finally at 4 °C/min to 290 °C (24 min). A standard solution of *n*-hydrocarbons (C_{22}–C_{40} and C_{44}, even numbers only) in toluene was co-injected with samples to compute temperature-programmed *I* values according to the following equation: $I = 100z + 100(y - z)\{[t_{R(x)} - t_{R(z)}]/[t_{R(y)} - t_{R(z)}]\}$, where $t_{R(x)}$, $t_{R(z)}$, and $t_{R(y)}$ were the retention times of enantiomer *x*, the hydrocarbon with *z* carbon number eluting before *x* and the hydrocarbon with *y* carbon number eluting after *x*, respectively, while *z* and *y* were the carbon numbers of the hydrocarbon with $t_{R(z)}$ and $t_{R(y)}$, respectively.

A database of reference *I* library was constructed based on *I* sets of eight profen enantiomeric pairs measured on the dual-columns. The GC analyses for the method optimization and validation were conducted employing a single DB-5 MS column (15 m × 0.25 mm i.d., 0.25 μm film thickness) under the identical temperature condition as above. The inlet pressure of helium was set to 6.5 kPa. All GC analyses were performed in triplicate.

GC–MS analyses were performed with an Agilent 6890 gas chromatograph interfaced to an Agilent 5973 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (SE-54 bonded phase; 25 m × 0.20 mm i.d., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of injector, interface and ion source were 260, 300 and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 ml/min with constant flow mode. Samples were introduced in the split-injection mode (10:1) and the oven temperature was maintained at 100 °C (2 min) and programmed to 260 °C at 3 °C/min and finally to 300 °C (10 min) at a rate of 20 °C/min. The mass range scanned was 50–650 u at a rate of 0.99 scan/s.

2.4. Preparation of *N*-ethoxycarbonyl-L-alanine

Two-phase EOC reaction of L-alanine (10 mg) in 1.0 ml of alkaline solution (pH ≥ 12) was conducted in one-step by vortex mixing (10 min) with ECF (20 μl) present in dichloromethane phase (1.0 ml). The reaction mixture was adjusted to pH ≥ 12 with 5.0 M sodium hydroxide, followed by washing with diethyl ether (3 ml). The aqueous phase was acidified (pH ≤ 2.0) with 10.0% sulfuric acid and saturated with sodium chloride with subsequent extraction with diethyl ether (3 ml × 2). The combined extracts were evaporated to dryness under a gentle stream of nitrogen. For the structure confirmation by GC–MS, an aliquot (2 μg) of *N*-EOC-L-alanine was reacted (60 °C, 1 h) with MTBSTFA (20 μl) in toluene (30 μl) to form *tert*-butyldimethylsilyl (TBDMS) ester.

2.5. Diastereomeric (*R*)-(+)-1-phenylethylamide formation

An aliquot of standard solutions containing eight racemic profens at varied amounts for method linearity test or four (*S*)-enantiomers (2.0 μg each) for optical purity test was added with IS solution (*n*-C_{26} and *n*-C_{34}, each at 1.0 μg) and evaporated to dryness (under gentle nitrogen stream). The residue dissolved in dichloromethane (200 μl) was sonicated (1 min) after addition of 0.6 μmol of TEA (50 mM, 12 μl) and 2.4 μmol of ECF (60 mM, 40 μl). Subsequently, 0.5 M (*R*)-(+)-1-PEA (20 μl) was added and sonicated (2 min). After acidification (pH ≤ 2) with 0.1 M hydrochloric acid (200 μl), the amide derivatives were extracted with diethyl ether (600 μl) and ethyl acetate (600 μl) in sequence. The combined extracts were evaporated to dryness (under gentle nitrogen stream) and the residue was reconstituted in a mixture (20 μl) of toluene and ethyl acetate (1:1) for the direct GC and GC–MS analysis. For the reaction optimization tests employing ibuprofen (5 μg) as a target compound, the amounts of dichloromethane and TEA were varied from 0 to 400 μl and from 0.15 to 0.60 μmol, respectively, under the constant amount of ECF (2.4 μmol).

For the optical purity measurement of (*R*)-(+)-1-PEA, an aliquot (2 μg) of *N*-EOC-L-alanine prepared in the preceding section was subjected to amidation under the optimal conditions as described for profens above.

2.6. Method validation for assay of profen enantiomers

The quantitative calculation of each profen enantiomer was based on the peak area ratios relative to that of IS: IS_1 (*n*-C_{26}) was used for the first seven profen enantiomeric pairs while IS_2 (*n*-C_{34}) was used for the late eluting carprofen and indoprofen. Linearity was tested by least-squares regression analysis on the peak area ratios against increasing amount ratios of racemic profens. For the linearity test in quantitative assay for (*S*)-profens, four standard samples were prepared by mixing increasing amounts (0.0, 2.0, 3.0, or 8.0 μg each) of four (*S*)-enantiomers of ibuprofen, flurbiprofen, naproxen and ketoprofen at constant amount (1.0 μg) of IS_1. The amounts of mixed (*S*)-profens were corrected for each optical purity measured in the preceding section. The limit of detection of each (*S*)-profen was estimated based on the lowest concentration giving a signal taken as the sum of the mean blank signal plus three times the standard deviation of the blank signal obtained via three blank measurements. Precision expressed as percentage of relative standard deviation (% R.S.D.) and accuracy as percentage of relative error (% RE) of the method were determined in triplicate. Each calibration sample was added with IS_1 and IS_2 (each at 1.0 μg) and then subjected to amidation according to the preceding procedures.

2.7. Sample preparation for profiling analysis of urinary metabolites of racemic ibuprofen

Urine sample was collected on the first day following the administration of the racemic ibuprofen (600 mg) to a healthy volunteer. An aliquot (50 μl) of the urine sample after dilution with distilled water (1 ml) was adjusted to pH ≥ 12 (with 5.0 M sodium hydroxide) and washed with diethyl ether (3 ml). The aqueous phase was acidified (pH ≤ 2.0) with conc. sulfuric acid and saturated with sodium chloride, followed by extraction with diethyl ether (3 ml × 2). The extract was added with IS solutions (*n*-C_{26} and *n*-C_{34}, each at 1.0 μg) and evaporated to dryness (under gentle nitrogen stream). The residue was then subjected to amidation with subsequent analysis by GC and GC–MS as described in the preceding section.

3. Results and discussion

3.1. N-Ethoxycarbonyl-L-alanine and optical purity of (R)-(+)-1-phenylethylamine

The chemical structure of *N*-EOC-L-alanine prepared for testing the optical purity of (*R*)-(+)-1-PEA was readily confirmed as *O*-TBDMS ester by its characteristic mass spectral pattern (Fig. 1A). Like the previous *N*-isobutoxycarbonyl/*O*-TBDMS derivative [36], the molecular ion peak at *m*/*z* 275 was unobserved and $[M-57]^+$ ion constituted the base peak. The $[M-57]^+$, $[M-131]^+$ and $[M-159]^+$ fragment ions were formed by the loss of $C(CH_3)_3$, OTBDMS, and COOTBDMS from the molecular ion, respectively. The peaks at $[M-85]^+$ and $[M-103]^+$ were assumed to be formed by the loss of $CHCH_3$ and $HOCH_2CH_3$ of EOC group from $[M-57]^+$ ion, respectively. The $[M-146]^+$ ion was most likely formed by the consecutive elimination of OTBDMS and CH_3 from the molecular ion.

The optical purity of (*R*)-(+)-1-PEA measured in triplicate was 99.9% with high precision (better than ±0.1% R.S.D.) when the freshly prepared *N*-EOC-L-alanine as chiral reagent was 100% pure. This value was higher than its labeled purity (99.5%). The characteristic mass spectrum of *N*-EOC-L-alanine/(*R*)-(+)-1-phenylethyamide derivative allowed positive confirmation of its chemical structure (Fig. 1B). The molecular ion peak at *m*/*z* 264 was weak and the base peak at *m*/*z* 116 corresponding to $CH_3CH_2OCONHCHCH_3$ was formed by the bond cleavage next to the newly formed amide bond in the alanine side. The second abundant peak at *m*/*z* 105 was due to the methyltropylium ion formed by benzylic cleavage in the PEA side while the third most abundant peak at *m*/*z* 120 was formed by amide bond cleavage. The prominent fragment ion at *m*/*z* 88 corresponds to CH_3CH_2OCONH.

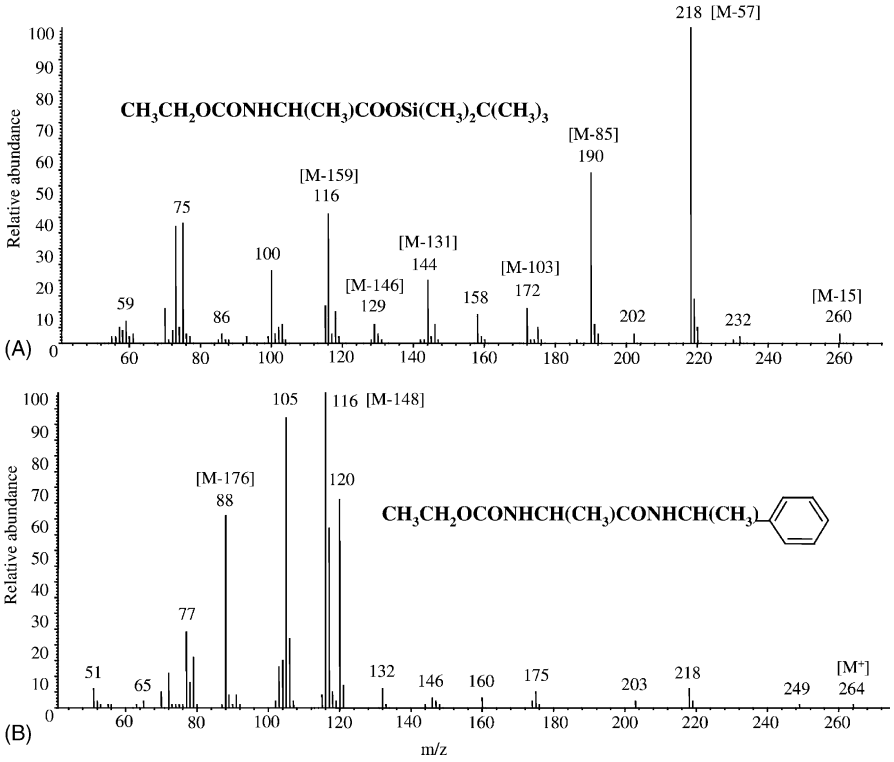

Fig. 1. Electron-impact mass spectra of *N*-EOC-L-alanine/*O*-TBDMS derivative (A) and *N*-EOC-L-alanine/(*R*)-(+)-1-phenylethylamide derivative (B) obtained in the scanning mode at a rate of 0.99 scan/s with a mass range of 50–650 u.

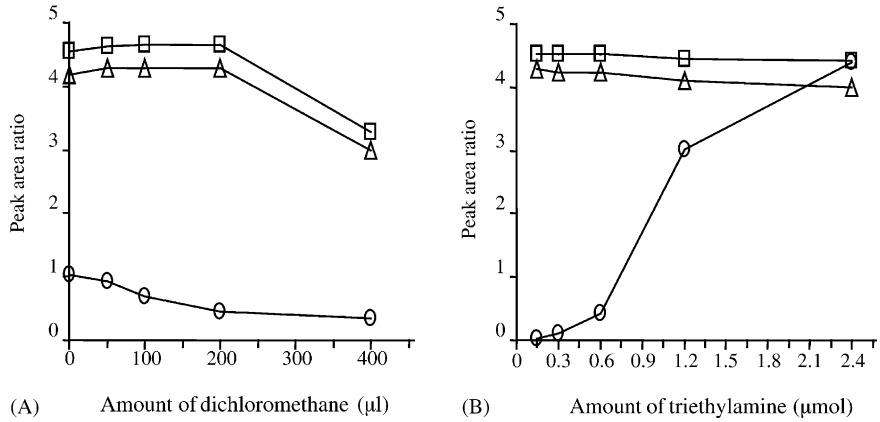

Fig. 2. Effects of dichloromethane amount: (A) under 2.4 μmol of ECF in the presence of 0.6 μmol of TEA, and effect of TEA amount and (B) under 2.4 μmol of ECF in 200 μl of dichloromethane on responses of artifact (○), (*R*)-ibuprofen (□) and (*S*)-ibuprofen (△).

3.2. Optimal condition for (R)-(+)-1-phenylethylamide formation

In this study, (*R*)-(+)-1-PEA was selected as the chiral reagent since all bioactive (*S*)-profens as their (*R*)-(+)-1-phenylethylamide derivatives emerged after the corresponding (*R*)-enantiomers on both nonpolar DB-5 and intermediately polar DB-17 columns. This is especially desirable when peak tailing, fronting or overlapping is observed for the (*S*)-profens. Based on the previous finding that the excess amount of ECF suppressed possible racemization during derivatization reaction [18], and acetonitrile as the reaction solvent induced racemization [32], the amount of ECF in the presence of TEA (as proton scavenger) in dichloromethane (as the optimal solvent) was set at 2.4 μmol throughout the experiments. Pilot studies showed that the amounts of dichloromethane and TEA in reaction media affected the formation of an artifact peak that could not be identified from its mass spectral pattern. It was eluted right in front of (*R*)-ibuprofen on DB-5 column but after (*S*)-ibuprofen on DB-17 column, thus interfering with the accurate quantitation of ibuprofen enantiomers only. Therefore, the reaction conditions were examined employing racemic ibuprofen as a target compound. When the amount of dichloromethane was varied from 0 to 400 μl under the fixed amounts of ECF (2.4 μmol) and TEA (0.6 μmol), the artifact response was gradually decreased while the responses of (*R*)- and (*S*)-ibuprofens maintained the constant levels up to 200 μl. But they were considerably decreased at 400 μl (Fig. 2A), probably due to the dilution effect of solvent on the amidation reaction. When the amount of TEA was increased from 0.15 to

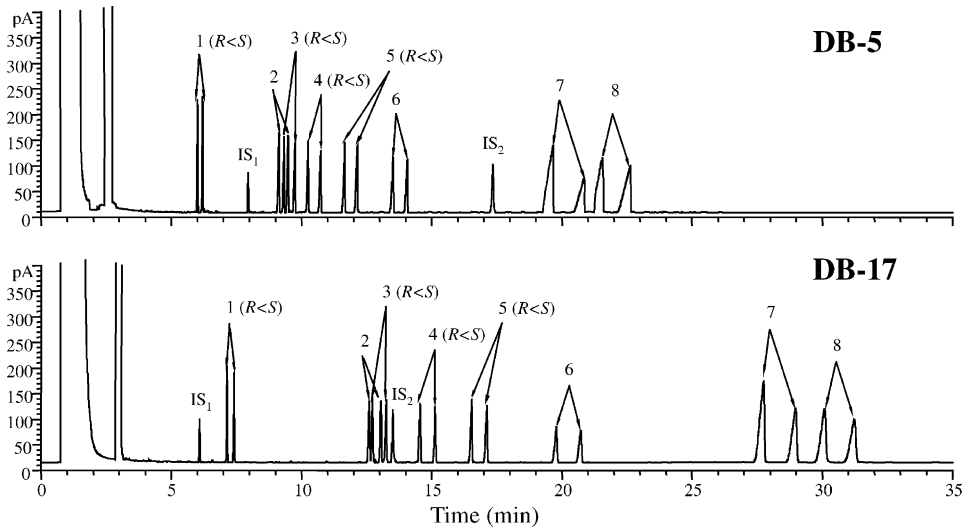

Fig. 3. Dual-enantiomeric profiles of eight racemic profen standards as their diastereomeric (*R*)-(+)-1-phenylethylamide derivatives separated on DB-5 MS and DB-17 MS fused-silica capillary columns (both 15 m × 0.25 mm i.d., 0.25 μm film thickness) dual-column system. The helium flow rate was initially set at 1.0 ml/min (23 min) and then programmed to 6 ml/min at 2 ml/min^2 in ramp flow mode. The oven temperature was initially 150 °C (1 min) and programmed at 30 °C/min to 240 °C and finally at 4 °C/min to 290 °C (24 min). Samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 0.7 min. Peaks: (1) ibuprofen; (2) fenoprofen; (3) flurbiprofen; (4) naproxen; (5) ketoprofen; (6) suprofen; (7) carprofen; (8) indoprofen.

Table 1
Gas chromatographic data of profen enantiomers as diastereomeric (*R*)-(+)-1-phenylethylamides

No.	Profen	Separation factor α[a]		Resolution actor R[b]		GC I data set[c]	
		DB-5	DB-17	DB-5	DB-17	DB-5	DB-17
1	(*R*)-Ibuprofen	1.032	1.038	4.2	4.7	2337.7	2668.0
	(*S*)-Ibuprofen					2371.4	2800.0
2	Fenoprofen	1.039	1.036	4.4	4.4	2723.2	3318.2
	Fenoprofen					2758.9	3357.7
3	(*R*)-Flurbiprofen	1.044	1.042	4.8	5.6	2743.4	3329.0
	(*S*)-Flurbiprofen					2785.0	3374.9
4	(*R*)-Naproxen	1.047	1.040	5.6	5.5	2832.3	3485.1
	(*S*)-Naproxen					2874.2	3533.9
5	(*R*)-Ketoprofen	1.041	1.035	4.2	4.7	2954.3	3645.5
	(*S*)-Ketoprofen					3000.0	3688.6
6	Suprofen	1.040	1.047	4.4	5.1	3107.0	3861.7
	Suprofen					3150.8	3910.2
7	Carprofen	1.060	1.044	2.5	3.6	3524.0	4400.0
	Carprofen					3600.0	>4400.0
8	Indoprofen	1.049	1.038	2.5	2.9	3627.9	>4400.0
	Indoprofen					3683.9	>4400.0

[a] Separation factor α was the ratio of retention times of two peaks.
[b] Resolution factor R was calculated according to $2(t_{R2} - t_{R1})/[1.702(w_{h1} + w_{h2})]$ where t_{R2} and t_{R1} are retention times, and w_{h1} and w_{h2} are peak widths at half height.
[c] Retention index (I) measured in triplicate on DB-5 MS and DB-17 MS (both 15 m × 0.25 mm i.d., 0.25 μm film thickness) dual-columns programmed from 150 °C (1 min) to 240 °C at 30 °C/min, then to 290 °C (20 min) at 4 °C/min. The helium flow rate was programmed from 1.0 ml/min (23 min) to 6 ml/min at 2 ml/min^2 in ramp flow mode. Samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 0.7 min.

2.4 μmol under the optimal amounts of ECF (2.4 μmol) and dichloromethane (200 μl), the artifact response was abruptly increased from 0.6 μmol, while the responses of (*R*)- and (*S*)-ibuprofens were relatively constant but they were gradually reduced from 1.2 μmol (Fig. 2B). Therefore, 200 μl of dichloromethane, 0.6 μmol of TEA and 2.4 μmol of ECF were selected as the optimal reaction conditions throughout this experiment. Upon the simultaneous amidation reaction under the optimal conditions, all carboxylic acid groups of eight racemic profens were converted within 3 min to (*R*)-(+)-1-phenylethylamide groups with no evidence of racemization.

3.3. Enantiomeric profiling analysis of profens as (R)-(+)-1-phenylethylamide derivatives

Under the present temperature-programmed GC condition in ramp flow mode, simultaneous enantioseparation of eight racemic profens as diastereomeric (*R*)-(+)-1-phenylethylamide derivatives on achiral nonpolar DB-5 and intermediately polar DB-17 columns was completed within 32 min (Fig. 3). Complete resolution of each enantiomeric pair was achieved on both columns with resolution factors in the range of 2.5–5.6 (Table 1). A single peak was observed for each enantiomer with good peak shape except that the late eluting two pairs showed peak broadening due to the slow elution caused by their low volatility. It was noticed that the enantiomer elution orders on the two columns were identical but retention times were different. Therefore, the temperature- and flow-programmed I values measured on the dual-columns were characteristic of each resolved enantiomer (Table 1) and the chemical identification and chiral discrimination of profens were possible by simple I matching with the reference values.

When the present achiral dual-column profiling analysis combined with diastereomeric amidation was applied to the optical purity tests, impure (*R*)-profens present at trace levels were detected as well-separated peaks in front of their respective huge (*S*)-profen peaks (Fig. 4). The calculated optical purities of (*S*)-ibuprofen, (*S*)-naproxen, (*S*)-flurbiprofen and (*S*)-ketoprofen were 99.7, 99.1, 99.7 and 99.7%, respectively, each with good precision better than ±0.1% R.S.D. The measured purities were higher than their respective labeled purities of 99, 98, 99 and 98.9%. The present method is expected to be useful for the simultaneous determination of optical purities of multiple profens in a single run.

3.4. Method validation for simultaneous chiral assay of profens

When the detector responses (expressed as peak area ratios) of the resolved enantiomers were plotted versus increasing amounts (0.1–5.0, 1.0–10.0 or 0.1–50.0 μg) of the

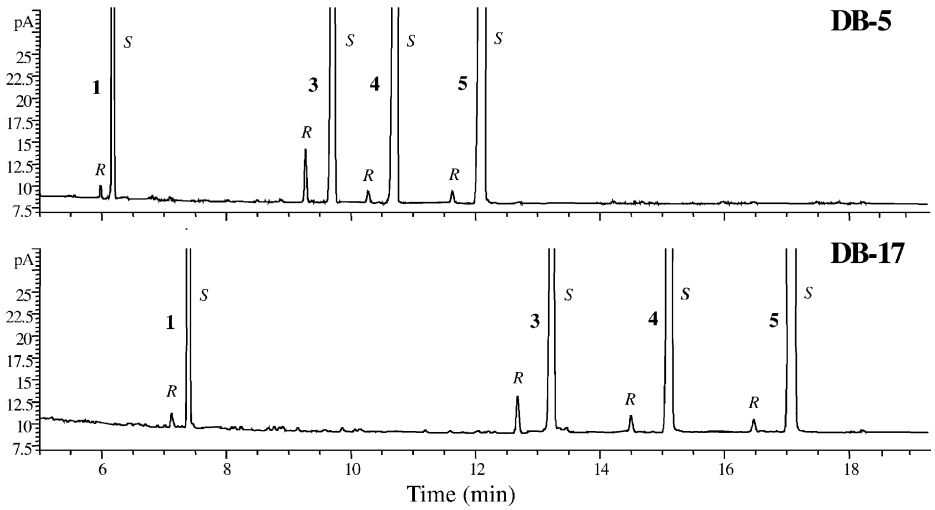

Fig. 4. Dual-enantiomeric profiles of four optically pure (*S*)-profen standards as their diastereomeric (*R*)-(+)-1-phenylethylamide derivatives. Peaks: (1) ibuprofen; (3) flurbiprofen; (4) naproxen; (5) ketoprofen. GC conditions as in Fig. 3.

racemic profens (expressed as weight ratios), good linear relationships were obtained with regression coefficients better than 0.9996 for both enantiomers (Table 2). The excellent overall linearity proved suitability of the present method for quantitative assay of profen enantiomers. When the present method was tested with calibration samples of four (*S*)-profens (each in the range of 0.0–8.0 μg), each response was linear ($r \geq 0.9992$) (Table 3). The LODs were ranged from 0.0001 to 0.003 ng. The ranges of precision (% R.S.D.) and accuracy (% RE) of the overall procedure at three amounts for each (*S*)-enantiomer varied from 0.8 to 6.0 and from −9.3 to 0.003, respectively. These values of the present method indicated that the levels of pure (*S*)-enantiomers could be measured with acceptable precision and accuracy. From the storage stability test (data were not shown), the derivatives of all enantiomers were found to be stable for at least 10 days when refrigerated securely capped in vials.

Table 2
Linearity for responses of profen enantiomers as diastereomeric (*R*)-(+)-1-phenylethylamide derivatives

No.	Profen	Calibration range (μg)[a]	Regression line		
			m[b]	b[c]	r[d]
1	(*R*)-Ibuprofen	0.1–5.0	0.808 ± 0.009	−0.03 ± 0.02	0.9998
	(*S*)-Ibuprofen		0.805 ± 0.008	−0.04 ± 0.02	0.9999
2	Fenoprofen	0.1–5.0	0.598 ± 0.008	−0.05 ± 0.02	0.9998
	Fenoprofen		0.566 ± 0.009	−0.05 ± 0.02	0.9997
3	(*R*)-Flurbiprofen	0.1–5.0	0.549 ± 0.009	−0.06 ± 0.03	0.9997
	(*S*)-Flurbiprofen		0.502 ± 0.009	−0.05 ± 0.02	0.9996
4	(*R*)-Naproxen	0.1–5.0	0.2270 ± 0.0009	−0.089 ± 0.002	0.9999
	(*S*)-Naproxen		0.21918 ± 0.00001	−0.09011 ± 0.00002	0.9999
5	(*R*)-Ketoprofen	0.1–5.0	0.37 ± 0.01	−0.04 ± 0.03	0.9996
	(*S*)-Ketoprofen		0.332 ± 0.009	−0.04 ± 0.03	0.9996
6	Suprofen	0.1–5.0	0.182 ± 0.003	−0.056 ± 0.009	0.9996
	Suprofen		0.154 ± 0.003	−0.047 ± 0.008	0.9996
7	Carprofen	1.0–10.0	0.0371 ± 0.0002	−0.021 ± 0.001	0.9999
	Carprofen		0.0326 ± 0.0004	−0.016 ± 0.002	0.9999
8	Indoprofen	1.0–50.0	0.0310 ± 0.0002	−0.016 ± 0.002	0.9999
	Indoprofen		0.0273 ± 0.0004	−0.009 ± 0.005	0.9997

All quantitative calculations were based on peak area ratios relative to that of IS (1.0 μg) measured on DB-5 MS column in triplicate.
[a] Calibration range of racemic profens.
[b] *m*, slope (mean ± standard deviation).
[c] *b*, intercept (mean ± standard deviation).
[d] *r*, correlation coefficient.

Table 3
Linearity, limit of detection, precision and accuracy for the assay of four (*S*)-profens as diastereomeric (*R*)-(+)-phenylethylamide derivatives

Profen	Calibration range (μg)[a]	Linearity r[b]	LOD (ng)[c]	Amount added (μg)	Precision (% R.S.D.)	Accuracy (% RE)
(*S*)-Ibuprofen	0.0–8.0	0.9992	0.001	2.0	4.1	3.9
				3.0	4.0	5.1
				8.0	2.0	−1.0
(*S*)-Flurbiprofen	0.0–8.0	0.9995	0.003	2.0	3.4	2.5
				3.0	3.8	4.1
				8.0	0.8	−0.7
(*S*)-Naproxen	0.0–8.0	0.9992	0.0001	2.0	4.1	−9.3
				3.0	5.0	4.2
				8.0	0.8	−0.04
(*S*)-Ketoprofen	0.0–8.0	0.9998	0.0005	2.0	6.0	−3.0
				3.0	3.6	−1.0
				8.0	1.7	0.003

[a] Calibration range of (*S*)-profens.
[b] r, correlation coefficient.
[c] LOD, limit of detection calculated according to (3 standard deviation of blank response/slope) × 1000. All quantitative calculations were based on peak area ratios relative to that of IS (1.0 μg) measured on DB-5 MS column.

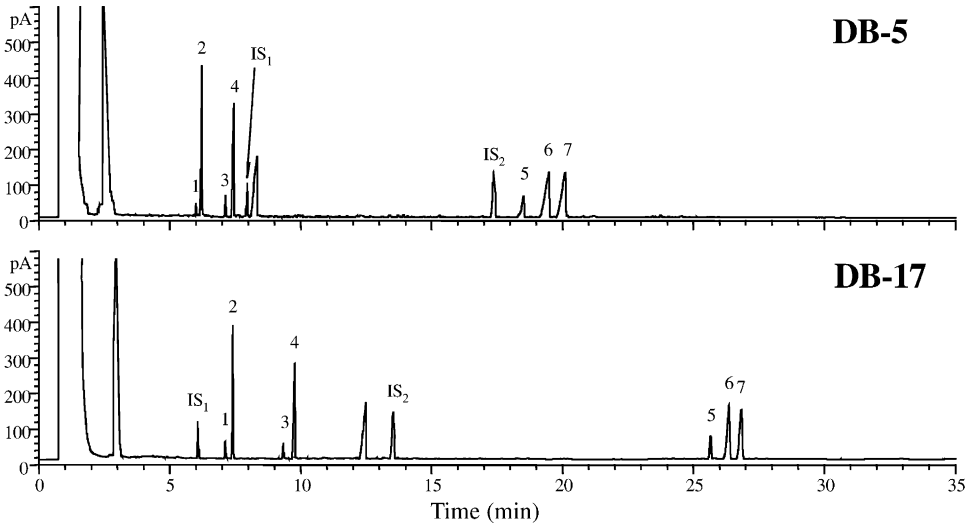

Fig. 5. Dual-enantiomeric profiles of urinary metabolites of racemic ibuprofen as diastereomeric (*R*)-(+)-1-phenylethylamide derivatives. Peaks: (1) (*R*)-ibuprofen; (2) (*S*)-ibuprofen; (3) (*R*)-hydroxyibuprofen; (4) (*S*)-hydroxyibuprofen; (5) (*RR*)-carboxyibuprofen; (6) mixture of (*RS*)-and (*SR*)-carboxyibuprofens; (7) (*SS*)-carboxyibuprofen. GC conditions as in Fig. 3.

3.5. Enantiomeric profiling analysis of urinary metabolites of racemic ibuprofen

When applied to urine sample after the oral administration of racemic ibuprofen, dual-profiles composed of ibuprofen and its two major stereoisomeric metabolites were obtained in one analytical run from minimal volume (50 μl) of urine (Fig. 5). The peaks corresponding to ibuprofens, hydroxyibuprofen and carboxyibuprofen were positively identified upon GC–MS analysis. And the chiral discrimination of ibuprofen enantiomers was achieved by simple *I* matching with the reference values. The chiralities of two hydroxyibuprofen peaks and three peaks of carboxyibuprofen were assigned based on the enantiomer elution orders reported previously [26]. In good agreement with the previous findings [15,26,29], (*S*)-ibuprofen (peak 2) was found in massive amount relative to trace (*R*)-ibuprofen (peak 1). (*S*)-Hydroxyibuprofen (peak 4) was detected as the abundant metabolite compared to trace (*R*)-hydroxyibuprofen (peak 3). And carboxyibuprofen was detected as three peaks corresponding to minor (*RR*)- (peak 5), mixture (peak 6) of (*RS*)- and (*SR*)-, and major (*SS*)- (peak 7) configurations.

4. Conclusions

The present diastereomeric amidation by ECF/(*R*)-(+)-1-PEA in the presence of TEA in dichloromethane combined with dual-column GC analysis was suitable for the simultaneous enantioseparation of eight profens in a single run. The

dual-*I* set measured on nonpolar DB-5 column and on intermediately polar DB-17 column was characteristic of each enantiomer. Therefore, crosschecking *I* set for each enantiomer with those of authentic profen standards enhanced the confidence in peak identification and chiral discrimination. Further optimization of the present chiral profiling and screening system is under way to include other chiral profens and similar acidic drugs for their effective quality control and stereospecific metabolism study.

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