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Fluorescence derivatization reagent for resolution of carboxylic acid enantiomers by high-performance liquid chromatography

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ABSTRACT

A novel chiral fluorescence derivatization reagent, (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole (APMB), was synthesized from 4-(6-methoxy-2-benzoxazolyl)acetophenone in several steps. Enantiomeric carboxylic acids were readily condensed with the chiral reagent in the presence of 2,2′-dipyridyl disulphide and triphenylphosphine. The diastereomeric amides formed were separated on a normal- and a reversed-phase column and were sensitively detected fluorimetrically, at 375 nm with excitation at 320 nm in normal-phase chromatography and at 380 nm with excitation at 320 nm in reversed-phase chromatography. The detection limit of (−)-APMB derivative of 2-phenylpropionic acid was 10 fmol at a signal-to-noise ratio of 3.

INTRODUCTION

The stereoisomers of racemic drugs are often readily distinguished by biological systems, and may have different pharmacokinetic properties and different pharmacological or toxicological effects. The development of racemic drugs raises issues of acceptable manufacturing control of synthesis and impurities, adequate pharmacological and toxicological assessment, proper characterization of metabolism and distribution and appropriate clinical evaluation. Therefore, stereospecific analysis becomes a key technique in chiral drug development.

High-performance liquid chromatography (HPLC) has been most widely used for chiral separations. A chiral separation can be performed in either a direct mode, using chiral stationary phases or chiral mobile phases, or an indirect mode, using a chiral derivatization reagent [1]. A chiral derivatization reagent that has an appropriate chromophore or fluorophore is a useful tool for trace analysis of biological specimens with respect to selectivity, sensitivity and versatility. Therefore, many chiral derivatization reagents have been developed for the resolution of enantiomeric drugs by HPLC [2–22].

This paper deals with the synthesis of (−)-2-[4-(1-aminoethyl)phenyl]-6-methoxybenzoxazole (APMB) as a novel chiral derivatization reagent and its applicability to the resolution of enantiomeric carboxylic acids, which include non-steroidal anti-inflammatory drugs (NSAIDs), by HPLC.

EXPERIMENTAL

Materials and chemicals

All chemicals for synthesis were of guaranteed-reagent grade and all organic solvents for chromatographic purposes were of special grade

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for HPLC, obtained from Wako (Osaka, Japan). 4-Isobutyl-α-methylphenylacetic acid (ibuprofen) and 6-methoxy-α-methyl-2-naphthaleneacetic acid (naproxen) were purchased from Funakoshi (Tokyo, Japan), 2-phenylpropionic acid from Norse Laboratories (Newburyport, CA, USA) and 2-fluoro-α-methyl-4-biphenylacetic acid (flurbiprofen) from Sigma (St. Louis, MO, USA). Water was purified with a Milli-Q water purification unit (Millipore, Bedford, MA, USA).

Instruments

A Hitachi (Tokyo, Japan) Model 650-40 spectrofluorimeter was used for the measurement of fluorescence spectra. The HPLC system consisted of a Shimadzu (Kyoto, Japan) Model LC-5A HPLC pump equipped with a Reodyne (Cotati, CA, USA) Model 7125 sample injector and a Shimadzu Model RF-550 fluorescence HPLC monitor; the system was linked to a Shimadzu Model C-R6A chromatographic integrator.

Synthesis of chiral derivatization reagent

The following methods were used for analysis of the synthetic products. Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured using a Model JNM-GSX400 spectrometer (JEOL, Tokyo, Japan) at 400 MHz, chemical shift values being expressed in ppm downfield from tetramethylsilane as an internal standard. A Perkin-Elmer (Norwalk, CT, USA) Model 1750 infrared spectrometer was used for infrared spectral measurements. Mass spectra were measured with a Model DX-300 mass spectrometer (JEOL) in the electron impact mode.

2-[4-(1-Hydroxyiminoethyl)phenyl]-6-methoxybenzoxazole (2). To a solution of 4-(6-methoxy-2-benzoxazolyl) acetophenone (**1**) (10.1 g), which was prepared by condensation of ethyl 4-acetylbenzimidate hydrochloride and 2-amino-5-methoxyphenol [21], in 95% ethanol (500 ml), was added hydroxylamine hydrochloride (7.0 g) and sodium acetate (8.2 g). The mixture was refluxed for 1 h, then poured into ice–water. The resulting precipitate was filtered and recrystallized from 90% ethanol to give faint reddish needles of **2**; 6.3 g, yield 57%, m.p. 212°C. Elemental analysis: calculated for $C_{16}H_{14}N_2O_3$, C 68.08, H 5.00, N 9.92; found, C 68.16, H 5.20, N 9.94%. ^{1}H NMR ([$^2H_6$]dimethyl sulphoxide; DMSO-d_6), ppm: 2.21 (3H, s, CH_3), 3.86 (3H, s, OCH_3), 7.02 (1H, dd, $J = 2.4$, 8.8 Hz, benzoxazole-5H), 7.42 (1H, d, $J = 2.4$ Hz, benzoxazole-7), 7.69 (1H, d, $J = 8.8$ Hz, benzoxazole-4), 7.88 and 8.14 (each 2H, each d, $J = 8.5$ Hz, phenyl), 11.48 (1H, s, oxime-OH). Mass spectrum: m/z 282 (M^+, base peak). IR (KBr): 1638, 1609, 1489, 1411, 1323, 1143, 1113, 998, 925 cm^{-1}.

(±)-2-[4-(1-Aminoethyl)phenyl]-6-methoxybenzoxazole (3a). To a solution of **2** (4.7 g) in methanol (300 ml) was added 10% Pd–C (3.0 g) and ammonium formate (10.5 g) [23] and the mixture was refluxed for 30 min. After the removal of Pd–C by filtration, the resulting solution was evaporated *in vacuo*. The residue was dissolved in 5% HCl (100 ml) and washed with ethyl acetate (100 ml). The pH of the aqueous layer was adjusted to 13–14 with 10% NaOH. Ethyl acetate (200 ml) was added to the alkaline solution to extract the amine. The ethyl acetate layer was then washed with water (100 ml), dried over anhydrous sodium sulphate and evaporated *in vacuo* to give racemic APMB (**3a**), 3.6 g.

(−)-2-[4-(1-Aminoethyl)phenyl]-6-methoxybenzoxazole (3b). To a solution of **3a** (3.6 g) in ethanol (50 ml) was added (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetic acid (3.5 g) and the mixture was allowed to stand overnight at 5°C. The resulting precipitate was collected by filtration and fractionally crystallized from ethanol four times. The free amine that dissociated with 5% NaOH was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulphate and evaporated *in vacuo*. Recrystallization of the crude product from ethanol gave faint pale yellow crystals of **3b**; 0.5 g, yield 14%, m.p. 74°C. Elemental analysis: calculated for $C_{16}H_{16}N_2O_2$, C 71.62, H 6.01, N 10.44; found, C 70.91, H 5.85, N 10.34%. ^{1}H NMR (DMSO-d_6), ppm: 1.33 (3H, d, $J = 6.6$ Hz, CH_3), 3.32 (2H, bs, NH_2), 3.85 (3H, s, OCH_3), 4.08 (1H, q, $J = 6.6$ Hz,

$CHCH_3$), 7.00 (1H, dd, $J = 2.2$, 8.7 Hz, benzoxazole-5), 7.41 (1H, d, $J = 2.2$ Hz, benzoxazole-7), 7.59 (2H, d, $J = 8.2$ Hz, phenyl), 7.66 (1H, d, $J = 8.7$ Hz, benzoxazole-4), 8.07 (2H, d, $J = 8.2$ Hz, phenyl). Mass spectrum: m/z 268 (M^+), 253 (base peak). IR (KBr): 3436, 1619, 1488, 1319, 1145, 837, 815 cm^{-1}. $[\alpha]_D = -14.2°$ ($c = 1.0$, methanol). The optical purity of (−)-APMB was checked on a TSK gel Enantio P1 chiral stationary phase column (Tosoh, Tokyo, Japan) with *n*-hexane–1,2-dichloroethane–2-propanol (6:3:1, v/v/v) as the mobile phase, after derivatization with 3,5-dinitrobenzoyl chloride.

Preparation of (S)-(+)-2-phenylpropionic acid derivative as fluorescence reference

The authentic (−)-APMB derivative of (*S*)-(+)-2-phenylpropionic acid was synthesized on a preparative scale in order to evaluate its reactivity and fluorescence properties. To a solution of (*S*)-(+)-phenylpropionic acid (0.4 mmol) and 2,2′-dipyridyl disulphide (0.4 mmol) in 20 ml of dichloromethane were added (−)-APMB (0.4 mmol) and triphenylphosphine (0.4 mmol). The resulting solution was allowed to stand at room temperature for 1 h, followed by washing with 5% HCl, 5% $NaHCO_3$ and water. After drying with anhydrous sodium sulphate, the resulting solution was evaporated *in vacuo*. The residue was purified on a silica gel column with *n*-hexane–ethyl acetate (1:1, v/v) as eluent. The main fraction was evaporated *in vacuo* to give the product; 0.11 g, yield 69%, m.p. 179°C. Elemental analysis: calculated for $C_{25}H_{24}N_2O_3$, C 74.98, H 6.04, N 7.00; found, C 74.92, H 6.32, N 6.97%. ^1H NMR (C^2HCl_3), ppm: 1.42 (3H, d, $J = 7.1$ Hz, CH_3), 1.52 (3H, d, $J = 6.9$ Hz, CH_3), 3.60 (1H, q, $J = 7.1$ Hz, $COCHCH_3$), 3.89 (3H, s, OCH_3), 5.14 (1H, m, $J = 7.1$, 7.8 Hz, CH_3CHNH), 5.57 (1H, d, $J = 7.8$ Hz, NHCO), 6.96 (1H, dd, $J = 2.3$, 8.8 Hz, benzoxazole-5), 7.11 (1H, d, $J = 2.3$ Hz, benzoxazole-7), 7.20 (2H, d, $J = 8.3$ Hz, phenyl), 7.2–7.4 (5H, m, phenyl), 8.07 (2H, d, $J = 8.3$ Hz, phenyl). Mass spectrum: m/z 400 (M^+), 252 (base peak). IR (KBr): 3279, 1640, 1533, 1489, 1348, 1224, 1150, 843, 702 cm^{-1}.

Analytical derivatization of carboxylic acids

In a 4-ml amber-coloured screw-capped vial were placed 100 μl of a dichloromethane solution of carboxylic acids (1–200 nmol/ml), 100 μl of a dichloromethane solution of (−)-APMB (0.8 μmol/ml), 100 μl each of a dichloromethane solution of 2,2′-dipyridyl disulphide (1.6 μmol/ml) and triphenylphosphine (1.6 μmol/ml). After being mixed, the reaction solution was allowed to stand for 20 min at room temperature. The solvent was evaporated to dryness with a stream of nitrogen and the residue was dissolved in 400 μl of mobile phase to make a sample solution and a 10-μl aliquot of the sample solution was injected into the HPLC system.

Chromatographic conditions

In the normal-phase mode, resolution of the resulting diastereomers was accomplished using a TSK gel silica-60 column (5-μm particle size, 25 × 0.46 cm I.D.) (Tosoh) with *n*-hexane–ethyl acetate–2-propanol–acetic acid (900:50:50:1, v/v) as the mobile phase. The mobile phase was pumped isocratically at 1 ml/min. The excitation and emission wavelengths were adjusted to 320 and 375 nm, respectively.

In the reversed-phase mode, with a TSK gel ODS-80TM column (5-μm particle size, 15 × 0.46 cm I.D.) (Tosoh) and acetonitrile–water–acetic acid (600:400:1, v/v/v), good separations were achieved at ambient temperature and a flow rate of 1 ml/min. The excitation and emission wavelengths were adjusted to 320 and 380 nm, respectively.

RESULTS AND DISCUSSION

Synthesis of fluorescence derivatization reagent

Some fluorescence derivatization reagents have been reported for the resolution of carboxylic acid enantiomers [2–11]. However, few chiral derivatization reagents have strong fluorescence to permit the more sensitive detection of trace amounts of carboxylic acid enantiomers. It has already been demonstrated that a series of 2-phenylbenzoxazole derivatives possess strong fluorescence, and hence some of them were applicable as sensitive fluorescence probes for

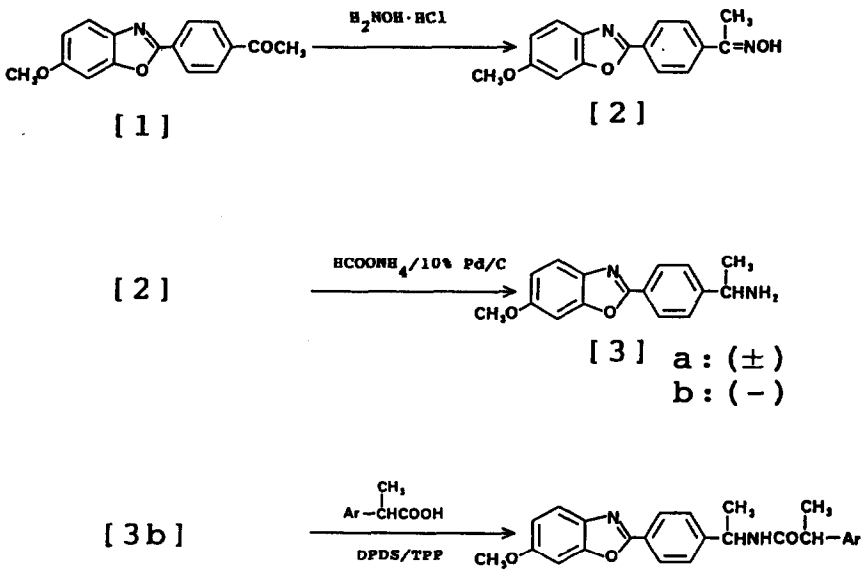

Fig. 1. Synthesis scheme for the chiral fluorescence derivatization reagent, (−)-APMB (**3b**), and derivatization of 2-arylpropionic acid with (−)-APMB. DPDS = 2,2′-dipyridyl disulphide; TPP = triphenylphosphine.

the trace analysis of organic compounds [10, 24–26]. Our attention became directed toward substituted 2-phenylbenzoxazoles, especially with electron-donating substituents, as new fluorescent probes for the sensitive determination of trace amounts of drugs by HPLC. For this purpose we synthesized a novel fluorescence chiral derivatization reagent, APMB, which has ethylamine as a reactive functional group toward carboxylic acid. The synthetic route to (−)-APMB is shown in Fig. 1. Reaction of 4-(6-methoxy-2-benzoxazolyl)acetophenone with hydroxylamine provided the hydroxyimino derivative, which on treatment with ammonium formate and 10% Pd–C was easily converted into the ethylamine. Optical resolution of (±)-APMB was accomplished by repeated fractional crystallization of the (*S*)-(−)-α-methoxy-α-trifluoromethylphenylacetic acid salt from ethanol, to give (−)-APMB. The optical purity of this reagent was 99.90%, as judged by chromatographic separation using TSK gel Enantio P1 with *n*-hexane–1,2-dichloroethane–2-propanol (6:3:1, v/v/v) as the mobile phase after derivatization with 3,5-dinitrobenzoyl chloride, as shown in Fig. 2.

(−)-APMB was stable at room temperature for at least 3 months with protection from humidity and light, and the dichloromethane solution was also stable for at least 1 week when stored in a refrigerator.

Fluorescence properties of (−)-APMB derivative

As shown in Table I, the excitation and emission spectra of the (−)-APMB amide of (S)-(+)-2-phenylpropionic acid were measured in water and various solvents, which have been widely used as components of mobile phases in

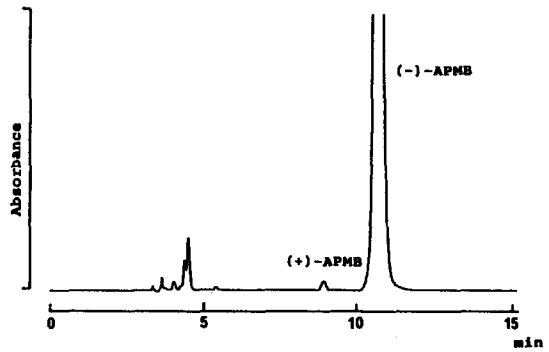

Fig. 2. Chromatograms of 3,5-dinitrobenzoyl derivative of the chiral fluorescence derivatization reagent (−)-APMB (**3b**). HPLC conditions: column, TSK gel Enantio P1; mobile phase, *n*-hexane–1,2-dichloroethane–2-propanol (6:3:1, v/v/v); flow-rate, 1.0 ml/min; detection, UV at 325 nm; column temperature, 40°C.

TABLE I

FLUORESCENCE SPECTRAL PROPERTIES OF (−)-APMB AMIDE OF (*S*)-(+)-2-PHENYLPROPIONIC ACID IN VARIOUS SOLVENTS

Solvent	Fluorescence		
	λ_{ex} (max.) (nm)	λ_{em} (max.) (nm)	RFI[a]
n-Hexane	316	374	78
Toluene	320	366	73
Dichloromethane	318	374	94
Ethyl acetate	317	368	94
Tetrahydrofuran	318	370	91
Ethanol	316	377	99
Methanol	318	378	99
Acetone	334	373	42
Acetonitrile	317	374	100
Water	316	394	78

[a] RFI = Relative fluorescence intensity (acetonitrile = 100).

HPLC. The fluorescence intensity was not affected by the polarity of the solvent.

The effects of water concentration and pH on the fluorescence intensity were also investigated. The fluorescence intensity was almost a maximum and was constant at water concentrations of 0–90% in aqueous acetonitrile. The most intense fluorescence was constant between pH 3 and 12.

These results suggest that the polarity of the solvent, water concentration and pH have no influence on the fluorescence intensity, which will allow a wide choice of mobile phases in normal- or reversed-phase HPLC.

Derivatization of carboxylic acids with (−)-APMB

Various derivatization reactions of carboxylic acids with amines have been developed in the area of peptide synthesis. Of these methods, Mukaiyama *et al.* [27] reported that oxidation–reduction condensation using 2,2′-dipyridyl disulphide and triphenylphosphine produced peptides in high yields with high optical purity under mild reaction conditions. The fluorescence intensity–time profile of the reaction of (*S*)-(+)-2-phenylpropionic acid with (−)-APMB was investigated in the presence of 2,2′-dipyridyl disulphide and triphenylphoshine in dichloromethane at room temperature. Under these conditions, the rate of formation of the fluorescent amide was rapid and the derivatization reaction was completed almost quantitatively by evaporation with a stream of nitrogen within 10 min, as shown in Fig. 3.

No racemization of the product or derivatization reagent occurred, even when reaction time was prolonged to 2 h.

Chromatographic separation

In general, normal-phase chromatography is more suitable than reversed-phase chromatography for the separation of diastereomeric amides, because it is sufficiently substantiated that the hydrogen bonding between an amide group and the stationary phase is important for the efficient resolution of diastereomers [7,28,29]. In this study, we attempted to separate the diastereomers formed from (−)-APMB and 2-arylpropionic acids by normal- and reversed-phase chromatography. Table II gives the capacity factors (k'), separation factors (α) and resolutions (R_s) for the diastereomeric amides derived from 2-arylpropionic acids with (−)-APMB on normal- and reversed-phase columns.

Fig. 4 shows typical chromatograms of a reaction mixture of 2-arylpropionic acids with (−)-APMB obtained by normal- and reversed-phase HPLC. The diastereomers were found to be

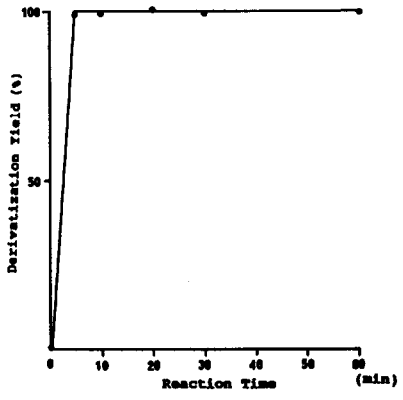

Fig. 3. Time course for the derivatization of (*S*)-(+)-2-phenylpropionic acid with (−)-APMB: 10 nmol/ml of (*S*)-(+)-2-phenylpropionic acid was treated with 0.8 μmol/ml of (−)-APMB in the presence of DPDS and TPP at room temperature.

TABLE II

HPLC SEPARATIONS OF DIASTEREOMERIC AMIDES DERIVED FROM 2-ARYLPROPIONIC ACIDS WITH (−)-APMB

2-Arylpropionic acid	Enantiomer	NP-HPLC			RP-HPLC		
		k'	α	R_s	k'	α	R_s
2-Phenylpropionic acid	*R*	2.00	1.95	10.2	3.07	1.11	1.27
	S	3.89			2.76		
Ibuprofen	*R*	1.42	2.17	10.7	12.16	1.21	3.80
	S	3.08			10.03		
Naproxen	*R*	2.55	1.84	10.1	5.57	1.19	2.72
	S	4.61			4.67		
Flurbiprofen	*R*	1.75	2.42	9.8	8.97	1.24	4.58
	S	4.24			7.22		

readily resolvable on both normal and reversed stationary phases. No exceptions were observed in the elution order of each pair of enantiomers. In normal-phase chromatography, (*S*)-arylpropionic acids were eluted before the corresponding (*R*)-enantiomers. On the other hand, the elution order of (*R*)- and (*S*)-arylpropionic acids was reversed on the reversed-phase column.

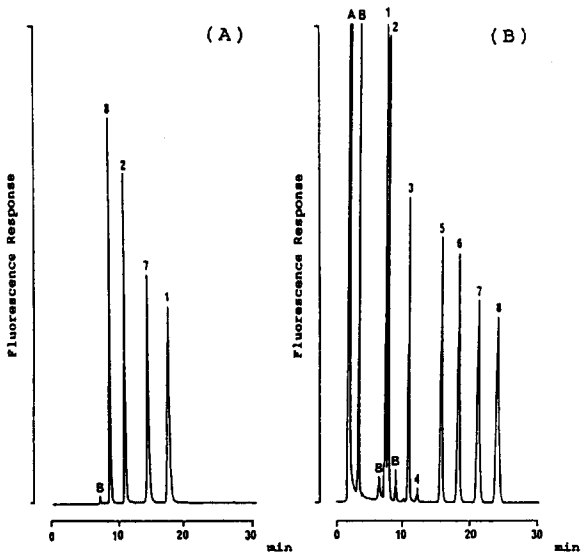

Fig. 4. Chromatograms of (−)-APMB derivatives of 2-arylpropionic acids. (A) Normal-phase HPLC; (B) reversed-phase HPLC. Peaks: 1 = (*S*)-2-phenylpropionic acid; 2 = (*R*)-2-phenylpropionic acid; 3 = (*S*)-naproxen; 4 = (*R*)-naproxen; 5 = (*S*)-flurbiprofen; 6 = (*R*)-flurbiprofen; 7 = (*S*)-ibuprofen; 8 = (*R*)-ibuprofen; A = (−)-APMB; B = degradation peak of the reagent.

Excess of the derivatization reagent was eluted with the solvent front and small degradation peaks of the reagent were observed in the reversed-phase chromatogram. In contrast, under the normal-phase chromatographic conditions employed, excess of (−)-APMB was strongly retained in the column and not eluted. Therefore, a simple clean-up procedure, such as acidic liquid–liquid extraction to remove the excess of reagent, may be necessary to avoid interference of the excess of reagent in some applications.

The amides obtained were highly responsive to a fluorescence detector; the detection limits of the authentic derivative obtained from the reaction of (*S*)-(+)-2-phenylpropionic acid with (−)-APMB on the normal- and reversed-phase columns were 10 fmol at a signal-to-noise ratio of 3. This result shows that the sensitivity of (−)-APMB is better than those of 1-(1-naphthyl)-ethylamine [8], 1-(4-dimethylamino-1-naphthyl)-ethylamine [4] and 1-(1-anthryl)- and 1-(2-anthryl)ethylamine [7].

A calibration graph of peak area *versus* concentration of (*S*)-(+)-phenylpropionic acid labelled with (−)-APMB was plotted. A good linear detector response (linear regression coefficient = 0.999) was observed in the range 0.5–200 pmol injected on-column.

Multiple derivatization ($n = 5$) of (±)-flurbi-

TABLE III

REPRODUCIBILITY OF THE PROPOSED METHOD

Carboxylic acid	Relative standard deviation (%)[a]		
	1.25 pmol	5 pmol	10 pmol
(*S*)-Flurbiprofen	5.62	2.13	0.65
(*R*)-Flurbiprofen	6.38	3.14	0.51
(*S*)-Ibuprofen	5.28	2.28	1.04
(*R*)-Ibuprofen	4.00	2.81	0.75

[a] $n = 5$.

profen and (±)-ibuprofen followed by HPLC gave peak areas with relative standard deviations of 0.5–6.8% in the range 1.25–25 pmol of the racemic acids analysed, as shown in Table III.

The newly synthesized reagent is of great use for the separation of enantiomeric carboxylic acids by HPLC. The proposed method is expected to be suitable for the resolution of enantiomeric carboxylic acids, and its high sensitivity may provide much more precise information on the determination of enantiomeric carboxylic acids, including NSAIDs that have a structure containing 2-arylpropionic acid, in biological fluids.

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