Journal of Chromatography A, 662 (1994) 87–93
Elsevier Science B.V., Amsterdam

CHROM. 25 674

Resolution and sensitive detection of carboxylic acid enantiomers using fluorescent chiral derivatization reagents by high-performance liquid chromatography

Kazuo Iwaki*, Toyohiko Bunrin, Yuuko Kameda and Mitsuru Yamazaki

School of Pharmacy, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa-shi, Ishikawa 920-11 (Japan)

(First received July 27th, 1993; revised manuscript received October 22nd, 1993)

ABSTRACT

Chiral derivatization reagents possessing a dansyl (N-dimethylaminoaphthalene-5-sulphonyl) moiety as fluorophore were developed for the separation and sensitive detection of carboxylic acid enantiomers by high-performance liquid chromatography (HPLC). Synthesis of *d*- and *l*-1-(4-dansylaminophenyl)ethylamine (DAPEA) from easily obtained starting materials by simple four-step reactions gave satisfactory yields. The reagents reacted with carboxylic acid enantiomers such as anti-inflammatory drugs in the presence of condensing agents (2,2′-dipyridyl disulphide and triphenylphosphine) at room temperature for 2.5 h to give corresponding diastereomeric amide derivatives quantitatively. The fluorescence characteristics of the amide formed from DAPEA with 2-phenylpropionic acid enantiomer (DAPE-PPA) were hardly affected by the pH and water content of the mobile phase in the range commonly used for reversed-phase HPLC. The diastereomeric pairs formed from five anti-inflammatory drugs and phenylpropionic acid with DAPEA were efficiently separated by reversed-phase HPLC. The detection limit (signal-to-noise ratio = 3) of DAPE-PPA, when the resulting derivatization reaction mixture was directly injected on to the column, was 170 fmol per injection.

INTRODUCTION

The chiral derivatization method is one of the methods used for the separation of racemic compounds by high-performance liquid chromatography (HPLC). This method requires derivatization steps for the formation of diastereomers from target enantiomers with a chiral reagent prior to separation. This causes the method to be tedious compared with the related chiral stationary phase and chiral mobile phase methods, which can give the direct resolution of enantiomers. However, when the enantiomers have reactive functional groups and no useful chromophores to be detected, this disadvantage becomes a great advantage with respect to the sensitivity and selectivity for the detection of enantiomers, because this diastereomeric method is able to form diastereomers and introduce the appropriate chromophore at the same time. Therefore, numerous chiral derivatization reagents have been developed for the separation of racemic amino and carboxylic acid compounds [1–7].

The dansyl (N-dimethylaminonaphthalene-5-sulphonyl) residue, possessing both fluorescent and chemiluminescent moieties, is a very useful chromophore. For this reason, it has not only been used for the labelling of amino compounds as dansyl chloride, but also recently applied to chromophores of many labelling reagents for trace analysis by HPLC *e.g.*, 4-dansylaminophenyl isothiocyanate [8], dansylsemipiperazine [9], monodansylcadaverine [10] and N-bromoacetyl-N′-dansylpiperazine [11].

* Corresponding author.

0021-9673/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(93)E1122-G

This paper deals with the synthesis of chiral derivatization reagents having a dansyl residue to give fluorescent diastereomers by reaction with carboxylic acid enantiomers. Reaction conditions for the derivatization of arylpropionic acids were optimized, and the fluorescence characteristics of the resulting diastereomeric amide compound were investigated. The separation of the diastereomers by reversed-phase HPLC is also demonstrated.

EXPERIMENTAL

Reagents

Dansyl chloride (Dns-Cl), enantiomeric 1-(4-nitrophenyl)ethylamine hydrochloride, diethyl phosphorocyanidate (DEPC), 2,2′-dipyridyl disulfide (DPDS) and triphenylphosphine (TPP) were obtained from Tokyo Kasei (Tokyo, Japan), racemic ibuprofen, sodium acetate and 5% palladium–carbon (Pd–C) from Wako (Osaka, Japan), enantiomeric ibuprofen and *d*-naproxen from Funakoshi (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively, and di-*tert.*-butyl dicarbonate [$(Boc)_2O$], isomeric 2-phenylpropionic acid (PPA) and N,N′-dicyclohexylcarbodiimide (DCC) from Nacalai Tesque (Kyoto, Japan). Racemic and isomeric pranoprofen, racemic and isomeric flurbiprofen and racemic phenoprofen were donated by Yoshitomi Pharmaceutical (Osaka, Japan), Kaken Pharmaceutical (Tokyo, Japan) and Yamanouchi Pharmaceutical (Tokyo, Japan), respectively, and *l*-naproxen was kindly supplied by Dr. Toshimasa Toyo'oka (National Institute of Hygienic Science, Tokyo, Japan). Acetonitrile was of HPLC grade. Water was purified by distillation, followed by final clean-up through a Milli-Q Labo system (Nihon Millipore, Tokyo, Japan). All other reagents were of analytical-reagent grade.

Apparatus

All melting-points (m.p.) were taken on a Yanagimoto (Tokyo, Japan) micro-m.p. apparatus and are uncorrected. Proton nuclear magnetic resonance (NMR) spectra were measured on a Jeol (Tokyo, Japan) PMX60SI instrument at 60 MHz using tetramethylsilane as an internal standard; abbreviations used are *s* for singlet, d for doublet, t for triplet, q for quartet and m for multiplet. Mass spectrometry (MS) was carried out on a Jeol DX-300 mass spectrometer with 70-eV electron-impact ionization (EI). Optical rotations were recorded on a Jasco (Tokyo, Japan) DIP-370 digital polarimeter equipped with a 100×3.5 mm I.D. cylindrical cell. Fluorescence characteristics were measured on a Hitachi (Tokyo, Japan) F-1200 spectrofluorimeter fitted with a 1-cm silica cell unit.

The HPLC system consisted of an L-6200 delivery system (Hitachi), a Model 7125 loop injector (Rheodyne, Cotati, CA, USA), an ODS-80TM (5 μm) (Tosoh, Tokyo, Japan) prepacked column (150×4.6 mm I.D.) and an L-1200 spectrofluorimeter (Hitachi) fitted with a 12-μl flow cell unit. The column was operated at room temperature. The detector excitation and emission wavelengths were set at 338 and 535 nm, respectively. Results were recorded on a D-2500 chromato-integrator (Hitachi). The flow-rate of the mobile phase was maintained at 1.0 ml/min.

Synthesis of the fluorescent chiral derivatization reagents (Fig. 1)

N - tert. - Butoxycarbonyl - 1 - (4 - nitrophenyl) - ethylamine (II). To a stirred acetonitrile solution (20 ml) of optically active 1-(4-nitrophenyl)ethylamine hydrochloride (**I**) (2.0 g, 10 mmol) and triethylamine (1.1 g, 11 mmol) in ice-bath was added dropwise $(Boc)_2O$ (2.2 g, 10 mmol). The mixture was stirred at room temperature for 1 h and then evaporated *in vacuo*. The residue was dissolved in ethyl acetate (50 ml). This solution was washed with 10% aqueous citric acid and water, dried over anhydrous sodium sulphate and evaporated *in vacuo* gave **II** (2.4 g, 90%) as white crystals, m.p. 85–87°C for the *R*-configuration and 86–89°C for the *S*-configuration. EI-MS, m/z 266 (M^+). NMR (ppm) in C^2HCl_3, 8.38 (2H, d, $J = 9$ Hz, Ar–H), 7.35 (2H, d, $J = 9$ Hz, Ar–H), 4.82 (1H, q, $J = 7$ Hz, CH), 1.53 (3H, d, $J = 7$ Hz, CH_3), 1.40 [9H, s, $C(CH_3)_3$].

N-Boc-1-(4-aminophenyl)ethylamine (III). To a solution of **II** (2.0 g, 6.6 mmol) in methanol (40 ml) was added 0.2 g of Pd–C. The suspen-

sion was stirred for 3 h while hydrogen gas was passed through at room temperature. After the Pd–C had been filtered off, evaporation of the filtrate *in vacuo* gave **III** (1.4 g, 79%) as a colourless oil. This was identified only by MS. EI-MS, m/z 236 (M^+).

N - Boc - 1 - (4 - dansylaminophenyl)ethylamine (IV). Isomeric **III** (1.4 g, 5.9 mmol) was dissolved in the mixture of acetonitrile (10 ml) and 0.1 *M* sodium hydrogencarbonate (pH 9.0, 50 ml). The solution was stirred during dropwise addition of dansyl chloride (1.9 g, 7.1 mmol) dissolved in acetonitrile (30 ml). The pH of the mixture was kept at 9.0 with 1.0 *M* sodium hydroxide solution. The resulting mixture was stirred for 20 min at room temperature and further for 2 h at 45°C, cooled to room temperature and extracted with ethyl acetate (3 × 30 ml). The extracts were combined, dried over anhydrous sodium sulphate, and evaporated *in vacuo*. Crystallization of the residue from benzene–hexane gave **IV** (2.2 g, 81%) as pale yellow crystals, m.p. 97–100°C for the *R*-configuration and 97–101°C for the *S*-configuration. EI-MS, m/z 469 (M^+). NMR (ppm) in C^2HCl_3, 6.8–8.8 (10H, m, Ar–H), 4.85 (1H, q, $J = 7$ Hz, CH), 2.93 [6H, s, $N(CH_3)_2$], 1.37 [9H, s, $C(CH_3)_3$], 1.20 (3H, d, CH_3).

1 - (4 - Dansylaminophenyl)ethylamine (DAPEA, V). To a solution of **IV** (1.5 g, 3.2 mmol) in methanol (10 ml) was added concentrated HCl (2ml). The mixture was stirred at room temperature for 30 min and evaporated *in vacuo*. The residue was dissolved in water (30 ml), the pH was adjusted to 8.0 with sodium hydrogencarbonate and the solution was extracted with ethyl acetate (3 × 30 ml). The extracts were combined, dried over anhydrous sodium sulphate and evaporated *in vacuo*. Recrystallization of the resulting powder from ethanol gave DAPEA (**V**) (1.0 g, 85%) as pale yellow crystals, m.p. 159–161°C for the *R*-configuration and 157–160°C for the *S*-configuration. EI-MS, m/z 369 (M^+). NMR (ppm) in $C^2H_3O^2H$, 6.9–8.6 (10H, m, Ar–H), 3.90 (1H, q, $J = 7$ Hz, CH), 2.80 [6H, s, $N(CH_3)_2$], 1.27 (3H, d, $J = 7$ Hz, CH_3). $[\alpha]_D$, 11.0° for the *R*-configuration (28°C) and −11.1° for the *S*-configuration (28°C), $c = 0.2$ in acetonitrile. Calculated for $C_{20}H_{23}N_3O_2S$, C 65.02, H 6.27, N 11.37; found for *R*-configuration, C 65.01, H 6.21, N 11.23%, and for *S*-configuration, C 65.13, H 6.30, N 11.27%.

Synthesis of the diastereomeric amide compound (R)-1-(4-dansylaminophenyl)-N-[(S)-2-phenylpropionyl]ethylamine (DAPE-PPA)

To a stirred solution of *d*-PPA (0.5 g, 3.3 mmol) and *d*-DAPEA (1.2 g, 3.3 mmol) in acetonitrile (30 ml) was added DPDS (1.1 g, 5.0 mmol) and TPP (1.3 g, 5.0 mmol). The mixture was stirred for 3 h at room temperature, evaporated *in vacuo* and the residue dissolved in ethyl acetate (50 ml). This solution was washed with saturated aqueous sodium hydrogencarbonate and water, dried over anhydrous sodium sulphate and evaporated *in vacuo*. The oily residue was purified by column chromatography. Elution with benzene–acetone gave DAPE-PPA (1.0 g, 60%) as a pale yellow oil. EI-MS, m/z 500 [$(M - H)^+$]. NMR (ppm) in C^2HCl_3, 6.9–8.7 (15H, m, Ar–H), 4.98 (1H, q, $J = 7$ Hz, 1–H), 3.53 (1H, q, $J = 7$ Hz, 2″–H), 2.85 [6H, s, $N(CH_3)_2$], 1.43 (3H, d, $J = 7$ Hz, 1–CH_3), 1.20 (3H, d, $J = 7$ Hz, 2″–CH_3). Calculated for $C_{29}H_{31}N_3O_3S$, C 69.44, H 6.23, N 8.38; found, C 69.52, H 6.21, N 8.37%.

Measurement of fluorescence characteristics of DAPE-PPA in aqueous medium

A solution of DAPE-PPA in acetonitrile (0.01 mg/ml) was diluted with a ninefold volume of 50 m*M* sodium acetate–acetonitrile (50:50–10:90, v/v) or of 50 m*M* acetate buffer (pH 3–9)–acetonitrile (50:50, v/v). The maximum wavelength and relative fluorescence intensity for each acetonitrile concentration or pH value were measured with the use of a 1-cm quartz cell.

Derivatization procedures for carboxylic acid enantiomers with DAPEA

In a brown micro test-tube, to 100 μl of sample solution in acetonitrile were added 100 μl of 2 m*M* DAPEA solution in acetonitrile, 100 μl of 3 m*M* DPDS solution in acetonitrile and 100 μl of 3 m*M* TPP solution in acetonitrile, successively. The tube was vortex mixed and allowed to stand for 3 h at room temperature. An aliquot

(5 μl) of the resulting mixture was injected on to the HPLC column.

RESULTS AND DISCUSSION

Synthesis of the fluorescent chiral amine

1-Substituted ethylamines with a bulky residue such as phenyl, naphthyl or dimethylaminonaphthyl have mainly been used as chiral derivatization agents for carboxylic acid enantiomers. Because these agents have the chiral centre directly bonded with both the bulky residue and the reactive functional residue for the carboxyl group, the resulting diastereomers derived from the reagent with enantiomers can form an advantageous rigid conformation for separation by conventional HPLC. 1-(4-Nitrophenyl)ethylamine (**I**) is a 1-substituted ethylamine that can be easily obtained as an optically active compound, and the presence of the nitro residue, easily modified to a primary amino group, is of great advantage for the introduction of a dansyl residue. Optically active DAPEA as a 1-substituted ethylamine having a more useful fluorophore was synthesized from optically active **I**.

The synthesis of DAPEA in satisfactory yields was accomplished by very simple four-step reactions as shown in Fig. 1. In order to determine the optical purity of two chiral DAPEAs, the amide compounds, which were derivatized from each DAPEA with N-carbobenzoxy-L-phenylalanine (optical purity ≥99.9%) (Peptide Institute, Osaka, Japan), were injected into the HPLC system. Calculated from the peak areas their optical purity was >99.5%.

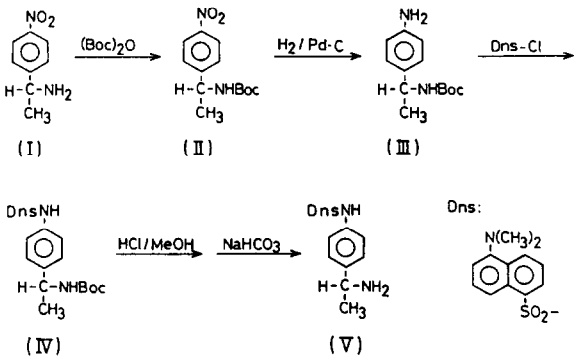

Fig. 1. Reaction course for synthesis of DAPEA from 1-(4-nitrophenyl)ethylamine.

Fluorescence characteristics of DAPE-PPA

In order to examine the fluorescence properties of the amide compound derived from a carboxylic acid with DAPEA in aqueous media commonly used in reversed-phase HPLC, the maximum excitation and emission wavelengths and the fluorescence intensities of DAPE-PPA were measured in solutions with various acetonitrile concentrations and pH values. Acetonitrile concentrations in the range 50–90% (v/v) did not affect the excitation wavelength. With increase in acetonitrile content, the emission wavelength was slightly shifted and the intensity was increased by about 30% (Table I). These variations hardly influence the practical detection of the derivatives by HPLC. In the pH range 3–9, all three factors were almost constant (Table II). The diastereomer of DAPE-PPA (synthesized from *d*-DAPEA with *l*-PPA) indicated almost same fluorescence characteristics. The results suggest that the mobile phase used for the detection of the derivatives is not limited in the above range. In the subsequent HPLC studies described below, a neutral pH (6.5) was selected to maintain the column lifetime.

Optimization of derivatization reaction

Fig. 2 shows the derivatization reaction of the arylpropionic acids with DAPEA. Three reagents, DCC [12], DEPC [13] and DPDS–TPP

TABLE I

COMPARISON OF WAVELENGTH MAXIMA AND FLUORESCENCE INTENSITIES IN WATER–ACETONITRILE OF THE AMIDE DERIVED FROM *d*-PPA AND *d*-DAPEA

Acetonitrile (% v/v)	Wavelength (nm)		Relative fluorescence intensity (%)
	λ_{ex}	λ_{em}	
50	335	542	71.5
60	339	536	79.3
70	339	539	86.9
80	339	531	95.2
90	339	526	100.0

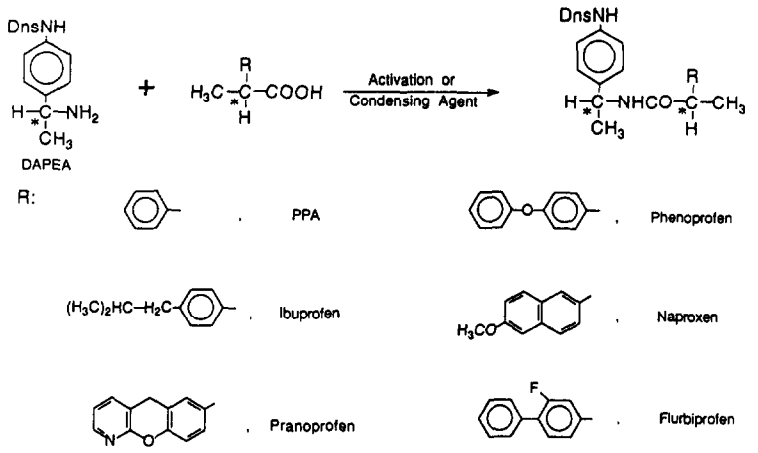

Fig. 2. Derivatization reaction of the arylproprionic acids with DAPEA in the presence of the activation or condensing agents.

[14], as activation or condensing agents for the reaction, were tested. After *d*-PPA (0.1 mg/ml) had reacted with 2 m*M* *d*-DAPEA for 20 min at room temperature in the presence of 5 m*M* of each reagent, the peak height of DAPE-PPA produced from the each reaction was compared with that of authentic DAPE-PPA. DCC and DEPC gave yields of only ≤10% and were discarded. In contrast, DPDS–TPP gave yields of ≥80% under very mild reaction conditions. Therefore, this system was extremely useful and was adopted for the derivatization.

In order to optimize the concentrations of the agents, the peak heights of DAPE-PPA derived from *d*-PPA (0.1 mg/ml) with *d*-DAPEA (1–5 m*M*) using various concentrations of the agents (1–5 m*M*, reaction time 20 min) were measured. When the concentration of DAPEA was ≥2 m*M* and those of DPDS and TPP were each ≥3 m*M*, the maximum peak height was obtained (Fig. 3) and the concentrations specified under Experimental were selected. Under these conditions, the time course of the derivatization was investigated. The reaction proceeded quantitatively for 2.5 h at room temperature, and subsequently DAPE-PPA was stable for at least 24 h in the resulting reaction mixture. The same studies were also carried out with *l*-PPA to compare the reactivity and similar results were obtained; the details are therefore omitted.

Hence the derivatization conditions as given under Experimental were established. The derivatization of *d*-PPA in the range 0.02–10 μg/

TABLE II

COMPARISON OF WAVELENGTH MAXIMA AND FLUORESCENCE INTENSITIES AT VARIOUS pH VALUES OF THE AMIDE DERIVED FROM *d*-PPA AND *d*-DAPEA

pH	Wavelength (nm)		Relative fluorescence intensity
	λ_{ex}	λ_{em}	
9	341	536	96.6
8	339	533	96.3
7	337	536	96.5
6	337	532	99.0
5	338	532	99.0
4	338	529	99.9
3	338	530	100.0

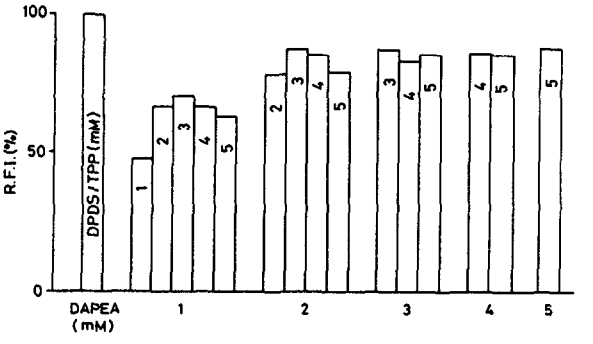

Fig. 3. Effect of reagent concentrations on the formation of the diastereomeric amide. Sample concentration, 125 ng per injection. R.F.I. = Relative fluorescence intensity.

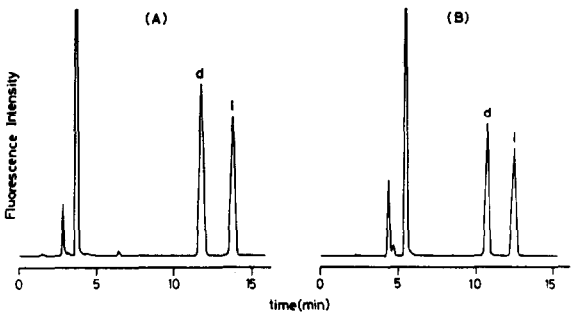

Fig. 4. Chromatographic profiles of the diastereomers derived from *l*-DAPEA with (A) ibuprofen and (B) pranoprofen. Mobile phase, 50 m*M* sodium acetate (pH 6.5)–acetonitrile [(A) 30:70 and (B) 45:55]. Each peak corresponds to 125 ng of enantiomeric ibuprofen and pranoprofen.

ml showed sufficient linearity ($r = 0.9994$), the reproducibility (relative standard deviation) was 2.2% (1.0 μg/ml PPA, $n = 7$) and the detection limit (signal-to-noise ratio = 3) by direct injection of the reaction mixture was 170 fmol per injection (derivatized to 12.5 ng/ml *d*-PPA).

Separation of diastereomers derived from carboxylic acid enantiomers with DAPEA

Separation of the diastereomers derived from six arylpropionic acid enantiomers with DAPEA using the proposed derivatization method was investigated by reversed-phase HPLC. Fig. 4 shows the chromatographic profiles of the diastereomers derivatized from ibuprofen and pranoprofen with *l*-DAPEA, and separation data for the six arylpropionic acids are given in Table III. All of the enantiomeric pairs were excellently separated in 10–15 min without any special techniques for elution. In this work, as optically active compounds of flurbiprofen and phenoprofen could not be obtained, elution orders of enantiomeric pairs were not identified. However, the elution orders probably the same as for the other four compounds. Because the order may depend on the absolute configuration around the asymmetric carbon of the arylpropionic acids (*d*-isomers of the all the arylpropionic acids in Table III are of *S*-configuration and the *l*-isomers are of *R*-configuration). *l*-DAPEA derivatives of

TABLE III

HPLC SEPARATION OF DIASTEREOMERS DERIVED FROM *l*- OR *d*-DAPEA WITH 2-ARYLPROPIONIC ACID DERIVATIVES

$t_0 = 1.4$ min; k', α and R_s refer to the capacity factor, separation factor and resolution, respectively, for a pair of diastereomers. Mobile phase, 50 m*M* sodium acetate (adjusted to pH 6.5)–acetonitrile; flow-rate, 1.0 ml/min; amount injected, 250 ng of each racemic carboxylic acid.

Carboxylic acid	Enantiomer	*l*-DAPEA			*d*-DAPEA			Acetonitrile concentration in eluent (% v/v)
		k'	α	R_s	k'	α	R_s	
PPA	*d*	8.45	1.15	3.53	9.74	1.14	3.45	55
	l	9.69			8.52			
Ibuprofen	*d*	7.44	1.20	4.53	8.74	1.20	4.48	70
	l	8.90			7.29			
Flurbiprofen[a]	*d*	8.71	1.21	4.85	10.57	1.21	4.85	65
	l	10.57			8.71			
Pranoprofen	*d*	6.76	1.18	3.81	8.07	1.19	3.95	55
	l	8.01			6.80			
Phenoprofen[a]	*d*	8.29	1.18	4.18	9.75	1.18	4.20	65
	l	9.76			8.27			
Naproxen	*d*	4.52	1.21	4.01	5.53	1.21	4.28	60
	l	5.45			4.57			

[a] Elution order is estimated.

d-isomers were eluted faster than those of *l*-isomers and *d*-DAPEA derivatives were eluted in the reverse order. However, the configuration of DAPEA did not affect the retention and resolution values. This suggests that the elution order is freely changed for the determination of one enantiomer in a large excess of the other enantiomer.

CONCLUSIONS

The convenient synthesis of fluorescent chiral derivatization agents, *d*- and *l*-DAPEA, was achieved by conventional four-step reactions. The derivatization procedures for carboxylic acid enantiomers using these agents for chromatographic separation and sensitive detection by reversed-phase HPLC, which have applicability in the analysis of biological samples, were achieved. This suggests that the proposed method may serve for the determination of arylpropionic acids in biological fluids for pharmacokinetic studies. In addition, an increase in detectability can be expected, as the chemiluminescence yield of the dansyl moiety by the peroxyoxalate chemiluminescence reaction is extremely high. In order to confirm these expectations, the determination of arylpropionic acids in biological fluids by HPLC with chemiluminescence detection is under study.

ACKNOWLEDGEMENTS

We thank Dr. Toshimasa Toyo'oka of the National Institute of Hygienic Sciences (Tokyo, Japan) for kindly donating *l*-naproxen. This work was supported in part by the Special Research Fund of Hokuriku University.

REFERENCES

1 J. Goto, N. Goto, A. Hikichi, T. Nishimaki and T. Nambara, *Anal. Chim. Acta*, 120 (1980) 187.
2 N. Nimura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
3 J. Goto, M. Ito, S. Katsuki, N. Saito and T. Nambara, *J. Liq. Chromatogr.*, 9 (1986) 683.
4 N. Nimura and T. Kinoshita, *J. Chromatogr.*, 352 (1986) 169.
5 S. Einarsson, B. Josefsson, P. Moller and D. Sanchez, *Anal. Chem.*, 59 (1987) 1191.
6 K. Iwaki, S. Yoshida, N. Nimura, T. Kinoshita, K. Takeda and H. Ogura, *Chromatographia*, 23 (1987) 899.
7 T. Toyo'oka, M. Ishibashi and T. Terao, *Analyst*, 117 (1992) 727.
8 S.-W. Jin, G.-X. Chen, Z. Palacz and B. Wittmann-Liebold, *FEBS Lett.*, 198 (1986) 150.
9 I. Yanagisawa, M. Yamane and T. Urayama, *J. Chromatogr.*, 345 (1985) 229.
10 Y.M. Lee, H. Nakamura and T. Nakajima, *Anal. Sci.*, 5 (1989) 209.
11 P.J.M. Kwakman, H.-P. van Schaik, U.A. Th. Brinkman and G.J. de Jong, *Analyst*, 116 (1991) 1385.
12 J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, 77 (1955) 1067.
13 S. Yamada, Y. Kasai and T. Shioiri, *Tetrahedron Lett.*, (1973) 1595.