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Journal of Chromatography A, 675 (1994) 79–88

JOURNAL OF CHROMATOGRAPHY A

Resolution of enantiomers of alcohols and amines by high-performance liquid chromatography after derivatization with a novel fluorescent chiral reagent

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(First received January 11th, 1994; revised manuscript received March 25th, 1994)

Abstract

4-(2-Chloroformylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*R*(+)-NBD-Pro-COCl and *S*(−)-NBD-Pro-COCl], optically active tagging reagents, have been synthesized for resolution of enantiomers of amines and alcohols by high-performance liquid chromatography. The reagents react with amino and hydroxyl functional groups in the presence of pyridine to produce the corresponding diastereomers. The optimum excitation and emission wavelengths for the diastereomers in water–acetonitrile (1:1) were approximately 485 nm and 530 nm, respectively. The excitation and emission wavelengths were independent of the amine or alcohol portion of the diastereomer. The resulting diastereomers can usually be efficiently resolved by normal-phase chromatography with *n*-hexane–ethyl acetate as the eluent. When *R*(+)-NBD-Pro-COCl was used as the derivatization reagent, the diastereomers corresponding to the *R*-configurations of amines and alcohols were eluted faster than those from the *S*-configuration. The elution order was reversed when the diastereomers were prepared with *S*(−)-NBD-Pro-COCl. The R_s values of the diastereomers derived from amines and alcohols by normal-phase chromatography are in the range of 3.23–4.32 and 2.99–4.10, respectively. After derivatization with NBD-Pro-COCls the alcohol enantiomers were also separated adequately by a reversed-phase column with a water–acetonitrile mixture.

1. Introduction

High-performance liquid chromatography (HPLC) has been widely accepted for the resolution of chiral molecules [1,2]. The resolution of racemates can be done with chiral stationary phase (CSP) columns. The different types of CSPs have been classified as follows: (1) chiral ligand exchange phases [3], (2) affinity phases [4,5], (3) helical polymer phases [6], (4) cavity phases [7,8] and (5) Pirkle-type phases [9]. Another HPLC technique employs formation of diastereomer with a chiral derivatization reagent [10]. Although the compounds having amino functional groups are easily derivatized with various reagents [11], many of the reagents do not have chiral properties [12–15]. The hydroxyl group is one of most difficult to derivatize due to

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0021-9673/94/$07.00
SSDI 0021-9673(94)00319-5

the limited reactivity and the relatively poor stability of the reagent and product [16–18]. Therefore, only a few reagents have been used for the resolution of chiral alcohols. There is a need for chiral reagents for alcohols and amines.

We have developed fluorescent chiral derivatization reagents [*S*(−)- and *R*(+) - 4 - (2 - chloroformylpyrrolidin - 1 - yl) - 7 - (N,N-dimethylaminosulfonyl) - 2,1,3-benzoxadiazole (DBD-Pro-COCl)] for alcohols and amines [19–21]. The enantiomers of some alcohols and amines were well separated by reversed-phase and/or normal-phase HPLC. The reagents are relatively stable and react readily with amines and alcohols. The fluorescence characteristics of the resulting diastereomers with excitation at approximately 450 nm and emission at approximately 560 nm are another advantage because these long wavelengths reduce the likelihood of interference. Although the detection limits (10–50 fmol levels) of the method with DBD-Pro-COCl is not superior to that of other methods, detection is improved with use of an argon-ion laser at 488 nm [20].

The objectives of this work were the synthesis of the chiral derivatization reagents [*S*(−)- and *R*(+)-enantiomers of NBD-Pro-COCl], evaluation of their reactivities toward alcohols and amines and the study of the fluorescence characteristics of the resulting diastereomers. HPLC separations of the diastereomers were also investigated by normal-phase and reversed-phase chromatography.

2. Experimental

2.1. Materials and reagents

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was purchased from Wako Pure Chemicals (Osaka, Japan). 4-(2-Carboxypyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazoles [*R*(+)- and *S*(−)-NBD-Pros] were synthesized as previously described [22]. Prolines [*R*(+)- and *S*(−)-enantiomers] were obtained from Sigma (St. Louis, MO, USA). Enantiomers of 2-hexanol, 2-heptanol, 2-nonanol, 1-phenylethanol, 1-(1-naphtyl)-ethylamine (NEA) and 1-phenylethylamine (PEA) were obtained from Wako. Enantiomers of 1-cyclohexylethylamine (CEA) (Fluka, Buchs, Switzerland), oxalyl chloride (Tokyo Kasei, Tokyo, Japan), methylamine (abs. 30% sol.) and pyridine (Wako) were used as received. Ethyl acetate (AcOEt), *n*-hexane, benzene, acetonitrile and water were of HPLC grade (Wako). All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. Apparatus

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Jemini-300 (Palo Alto, CA, USA) at 300 MHz using tetramethylsilane (0.00 ppm) as the internal standard. For describing NMR characteristics, the following abbreviations are used: s = singlet, d = doublet, m = multiplet and br = broad. Mass spectra (MS) were recorded on JEOL DX-300 [70 eV, electron-impact ionization (EI)] mass spectrometer (Tokyo, Japan). Infrared spectra were measured using potassium bromide (KBr) discs with a Shimadzu Model IR-460 (Kyoto, Japan). For measurement of excitation and emission spectra, a Hitachi 650-60 fluorescence spectrometer with a 1-cm quartz cell was employed without spectral correction. Optical rotations were measured on a DIP-370 Digital Polarimeter (JASCO, Tokyo, Japan) with 50 × 3.5 mm I.D. cylindrical cell. Melting points (mp) were measured by a Yanagimoto micro melting point apparatus (Tokyo, Japan).

The high-performance liquid chromatograph consisted of two LC-10AD pumps (Shimadzu) and an SCL-10A system controller (Shimadzu). Sample solutions were injected with a SIL-10A auto injector (Shimadzu). The analytical columns were an Inertsil ODS-80A (150 × 4.6 mm I.D., 5 μm) for reversed-phase chromatography and an Inertsil SIL (150 × 4.6 mm I.D., 5 μm) (GL Sciences, Tokyo, Japan) for normal-phase chromatography. The columns were maintained at 40°C with a CTO-10AC column oven (Shimadzu). A Shimadzu RF-10A fluorescence monitor equipped with a 12-μl flow cell was

employed for the detection. The excitation and emission wavelengths were fixed at 485 nm and 530 nm, respectively. The peak areas obtained from the fluorescence monitor were calculated with a C-R7A chromatopac (Shimadzu). All mobile phases were de-gassed with an on-line degasser (DGU-3A, Shimadzu). The flow rate of the eluent was 1.0 ml/min.

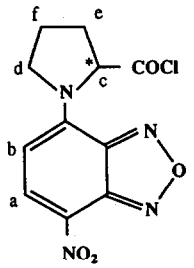
2.3. Synthesis of the chiral derivatization reagents

To *S*(−)-4-(2-carboxypyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*S*(−)-NBD-Pro] (2.6 g, 9.3 mmol) dissolved in 200 ml of anhydrous dichloromethane was added 10 ml of oxalyl chloride and 0.2 ml of dimethylformamide (DMF). The mixture was stirred for 60 min at room temperature. The solvent was evaporated *in vacuo*. The crystalline precipitate obtained was immediately dissolved in 100 ml of anhydrous benzene and the undissolved materials were filtered off. The filtrate solution was then evaporated under reduced pressure. The remaining crystals were dried in a vacuum desiccator over phosphorous pentoxide (P_2O_5).

S(−)-4-(2-Chloroformylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*S*(−)-NBD-Pro-COCl]: red-orange crystals; mp. 103–104°C (decomp.); yield 2.4 g (87%); NMR (ppm) in $CDCl_3$, 8.44 (1H, d, $J = 8.9$ Hz, a), 6.17 (1H, d, $J = 8.9$ Hz, b), 5.67 (1H, br, c), 3.86 (2H, br, d), 2.56–2.70 (2H, m, e), 2.14–2.39 (2H, m, f); EI-MS, m/z 296 (M^+); IR (KBr) 1794, 1615, 1555, 1495, 1447, 1325, 1154, 1111, 999, 959, and 708 cm^{-1}; $[\alpha]_D^{20}$ −204.2°, $c = 0.43$ in $CHCl_3$; Analysis: calculated for $C_{11}H_9N_4O_4Cl$, C 44.53, H 3.06, N 18.88; found, C 44.48, H 2.87, N 18.30.

R(+)-4-(2-Chloroformylpyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*R*(+)-NBD-Pro-COCl] was also obtained from the reaction of *R*(+)-4-(2-carboxypyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*R*(+)-NBD-Pro] and oxalyl chloride in the same manner described above.

R(+)-NBD-Pro-COCl: yield 2.4 g (87%), $[\alpha]_D^{20}$ +208.5°, $c = 0.45$ in $CHCl_3$; Analysis: calculated for $C_{11}H_9N_4O_4Cl$, C 44.53, H 3.06, N 18.88; found, C 44.83, H 2.92, N 18.49. Other instrumental data were the same as those of *S*(−)-NBD-Pro-COCl.



NBD-Pro-COCl

2.4. Reactivity of optically active NBD-Pro-COCl with alcohol and amine enantiomers

Amounts of 50 μl of 10 m*M* NBD-Pro-COCl [*R*(+)- or *S*(−)-enantiomer] in anhydrous benzene, and 50 μl of 2-heptanol or 1-(1-naphtyl)ethylamine (1 m*M* of each enantiomer) in anhydrous benzene containing 2% pyridine were mixed in a 1.5-ml mini-vial (GL Science). The vials were tightly capped and heated for 4 h at 80°C (for derivatization of alcohols) or 50°C (for derivatization of amines). After the fixed time intervals, a vial was removed from the dry heat block, and cooled in ice-water (0–5°C). The reaction was quenched by the addition of 0.9 ml of 1% methylamine in acetonitrile. An aliquot (5 μl) of the diluted solution was automatically injected into an Inertsil ODS-80A, and the fluorescence peak area of the resulting diastereomer was calculated with an integrator. The reagent blanks without alcohols or amines were treated in the same manner.

2.5. HPLC separation of the diastereomers derived from the enantiomers of alcohols or amines

The enantiomers (*ca.* 1 mg each) of alcohols (or amines) were reacted at 80°C (or 50°C) with NBD-Pro-COCl [1 m*M* *R*(+)- or *S*(−)-enantio-

mer] in 1 ml of anhydrous benzene in the presence of 1% pyridine. After 1 h, an aliquot (5 μl) of the solution was injected into Inertsil ODS-80A (reversed-phase column) and Inertsil SIL (normal-phase column). The eluents for reversed-phase and normal-phase chromatography are water–acetonitrile and *n*-hexane–ethyl acetate, respectively. The capacity factor (k'), separation factor (α) and the resolution value (R_s) were calculated from the following equations, respectively.

$$k' = (t_R - t_0)/t_0,\ \alpha = k_2'/k_1',$$
$$R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$$

where t_R, t_{R1} and t_{R2} are retention times of the peaks and t_0 is the dead time of the column ($t = 1.3$ min); w_1 and w_2 are the widths of the bases formed by triangulation of the peaks.

For the fluorescent spectra measurements, 50 μl of the solution was injected onto the column and the peak corresponding to the alcohol or amine derivative was collected from outlet of the detector (*ca.* 2-ml portion).

3. Results and discussion

3.1. Synthesis of NBD-Pro-COCl

DBD-Pro-COCls were synthesized from DBD-Pro enantiomers with PCl_5, as described in a previous paper [19]. The yields of *ca.* 60% were adequate, however, oxalyl chloride provided quantitative yields of the acid chlorides. Therefore, oxalyl chloride was also employed for the synthesis of NBD-Pro-COCls [*R*(+)- and *S*(−)-isomers].

Fig. 1 shows the synthetic pathway of the chiral derivatization reagents and the subsequent reactions with enantiomers of alcohols and amines. The direction of the optical rotation of the chiral reagents are the same as those of the starting materials, NBD-Pros. NBD-Pro-COCls and DBD-Pro-COCls are fairly stable as solids. No degradation was observed after storage of three months at 5°C in a refrigerator. As with other acid chloride type reagents reported previously, these reagents gradually decomposed in solution to produce corresponding acids, NBD-

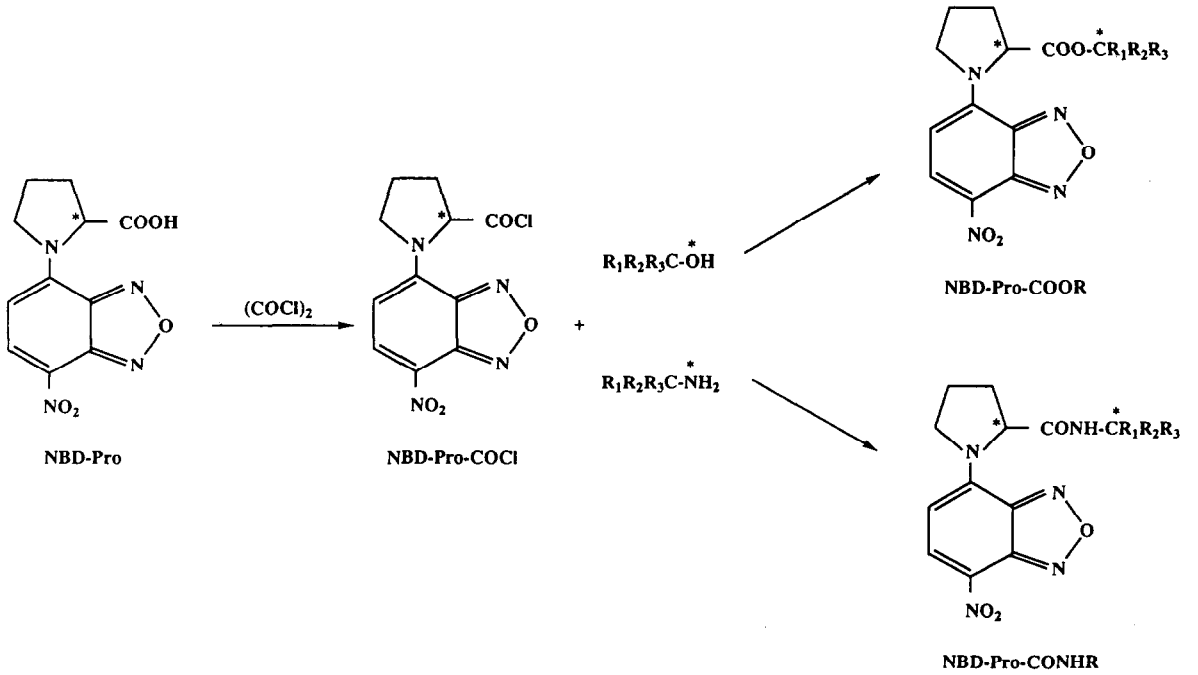


Fig. 1. Synthesis of the chiral derivatization reagents and preparation of diastereomers.

Pro and DBD-Pro. The rates were faster in benzene containing pyridine than without pyridine. Therefore, the reagent solutions should be prepared just prior to use.

Since high optical purity of the tagging reagent is required for the determination of trace amounts of the antipode enantiomer in the presence of a large amount of the enantiomer, the determination of the optical purity of the reagents synthesized was attempted by HPLC with utilizing a few CSP columns such as modified β-cyclodextrin and cellulose as the sorbents. However, the complete separations allowing the determination of the optical purity were not achieved. The limited stability of the reagents in solution is one of difficulties in the analyses. Since no measurable peaks derived from antipode enantiomers are obtained from each pair of the reagents, the optical purities of these reagents seem to be good enough for the determination of racemic alcohols and amines.

3.2. Fluorescence characteristics of the diastereomers

The fluorescence excitation and emission spectra of the diastereomers were measured in acetonitrile–water (1:1). The excitation and emission maxima of the diastereomers derived from amines and alcohols are essentially the same (*ca.* 485 nm and 530 nm, respectively). The results suggest that the fluorescent properties of the diastereomers are dominated by the NBD-Pro structure and independent of the structures of the alcohol or amine analytes. Excitation and emission at long wavelengths provide a distinct advantage in biological samples because there is negligible interference by sample co-extractives, which have no amino and hydroxyl functional groups in the structure.

3.3. Derivatization

As described in our previous work [20], optimal conditions with DBD-Pro-COCls were selected after studies of various parameters affecting the derivatization reaction. The same solvent (benzene), catalyst (pyridine) and temperatures (80 or 50°C) were adopted in the following studies because the reactive site of NBD-Pro-COCl is same of that of DBD-Pro-COCl. The effects of the functional groups (nitro and dimethylaminosufnonyl) at the 7-position of 2,1,3-benzoxadiazole were expected to be negligible. Toluene in the presence of pyridine can also be replaced, instead of the highly toxic benzene. Judging from the results in the previous works [19–21] with DBD-Pro-COCls, it was anticipated that the derivatization of alcohols with NBD-Pro-COCls would be more difficult than the derivatization of amines. In addition, it was necessary to test the reactivity of each reagent enantiomer toward each enantiomer of the chiral molecules because differences in reactivity could give mixtures of diastereomers that would not accurately reflect the isomeric composition. The reactivities of the optically active reagents [*S*(−)- and *R*(+)-NBD-Pro-COCls] toward 2-heptanol and NEA, which were selected as the representative enantiomers of alcohols and amines, were examined separately in benzene solution containing 1% pyridine.

Figs. 2 and 3 show the results of time course studies with 2-heptanol at 80°C and NEA at 50°C. As shown in Fig. 2, the formation of the

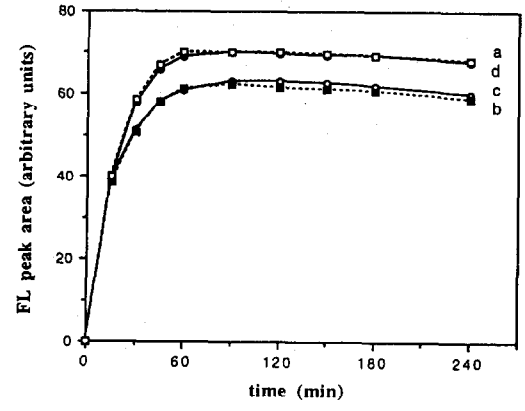


Fig. 2. Time course of diasteromer formations corresponding to 2-heptanol enantiomers with NBD-Pro-COCls at 80°C in benzene containing 1% pyridine. (a) Reaction of *S*(+)-2-heptanol with *R*(+)-NBD-Pro-COCl; (b) *R*(−)-2-heptanol with *R*(+)-NBD-Pro-COCl; (c) *S*(+)-2-heptanol with *S*(−)-NBD-Pro-COCl; (d) *R*(−)-2-heptanol with *S*(−)-NBD-Pro-COCl. HPLC eluent, H_2O–CH_3CN (35:65); other HPLC conditions as in Experimental.

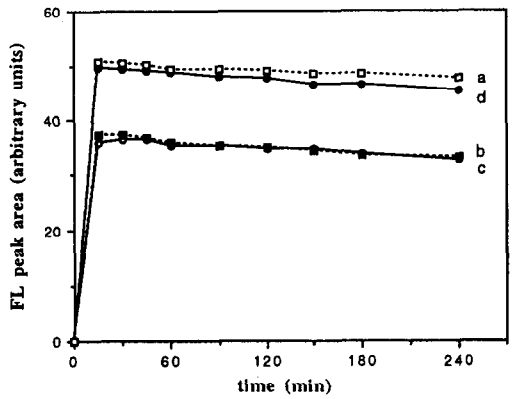


Fig. 3. Time course of diasteromer formations corresponding to NEA enantiomers with NBD-Pro-COCls at 50°C in benzene containing 1% pyridine. (a) Reaction of *S*(−)-NEA with *R*(+)-NBD-Pro-COCl; (b) *R*(+)-NEA with *R*(+)-NBD-Pro-COCl; (c) *S*(−)-NEA with *S*(−)-NBD-Pro-COCl; (d) *R*(+)-NEA with *S*(−)-NBD-Pro-COCl. HPLC eluent, H_2O–CH_3CN (55:45); other HPLC conditions as in Experimental.

diastereomers corresponding to both enantiomers of 2-heptanol increased with heating time and was essentially complete after 90 min. The peak area of the diastereomer, derived from *R*(+)-NBD-Pro-COCl and *S*(+)-2-heptanol, was approximately 10% larger than that of other diastereomer. Similar high intensity was confirmed with the diastereomers derived from *S*(−)-NBD-Pro-COCl and *R*(−)-2-heptanol. Figs. 2 and 3 illustrate that the reaction rates of the NEA isomers with NBD-Pro-COCls were obviously faster than those of 2-heptanol. The reactions were completed after 15 min, even at the temperature of 50°C (Fig. 3). A steady decrease in the peak areas with time was observed for both enantiomers of the reagent. Similar phenomena were observed in the reaction of amine with the reagent. Judging from the curves in Figs. 2 and 3, the derivatives of alcohols and amines seem to be fairly stable. However, exact figures for the stability of the derivatives cannot be given because authentic derivatives are not synthesized yet. The peak areas of the diastereomers, derived from *R*(+)-NBD-Pro-COCl and *S*(−)-NEA or *S*(−)-NBD-Pro-COCl and *R*(+)-NEA, were *ca.* 25% larger than the other diastereomers. The difference of peak areas might be due to the difference of fluorescence quantum yield (ϕ) of the resulting diastereomers. Judging from the reaction curves in Figs. 2 and 3, the reactivities of NBD-Pro-COCls seem to be essentially the same for both enantiomers. Consequently, a 2-h reaction period at 80°C was selected for the derivatization of alcohols and a 30-min reaction period at 50°C in benzene containing 1% pyridine was adopted for the amines.

3.4. HPLC separation of resulting diastereomers

Since intermolecular hydrogen bonding between the derivative and the stationary phase not only contributes to fixation of the conformation, but is also important for the efficient resolution of the diastereomers, the normal-phase column is generally employed together with non-polar organic solvents as a mobile phase. As shown in a previous report [19] some alcohols labelled with DBD-Pro-COCl were well resolved by a silica-gel column with *n*-hexane–ethyl acetate mixture (R_s 3.34–4.48). However, the separations of the diastereomers derived from amines were poor (*e.g.* R_s of PEA, 1.19) [21]. Initially, the separations of each pair of amines labelled with NBD-Pro-COCl were attempted by normal-phase chromatography. The capacity factors (k'), separation factors (α) and resolution values (R_s) for the diastereomers are listed in Table 1. Three amines tested were well resolved by Inertsil SIL column with *n*-hexane–ethyl acetate (55:45) (R_s 3.23–4.32). Both values of R_s and α were independent of the amine. The R_s values obtained with the proposed reagent were higher than those achieved with DBD-Pro-COCl. Similar good resolution was also obtained with the diastereomers of the alcohols (Table 2). The R_s values (2.99–4.10) were slightly smaller than those with DBD-Pro-COCl (R_s 3.23–4.32) [19]. R_s values obtained with the alcohols having greater hydrophobicity, *e.g.* 2-nonanol, were larger than those for alcohols having higher hydrophilicity, *e.g.* 2-hexanol. When *S*(−)-NBD-Pro-COCl was used as the chiral derivatization reagent, the corresponding diastereomers of the *S*-enantiomers of the amines and alcohols

Table 1
HPLC separation of diastereomers derived from *S*(−)-NBD-Pro-COCl by normal-phase chromatography

Amine	*S*-enantiomer		*R*-enantiomer		α	R_s	Eluent
	t_R (min)	k'	t_R (min)	k'			
PEA	10.29	6.91	13.51	9.39	1.36	3.14	A
	13.66	9.51	18.19	12.99	1.37	3.78	B
	19.03	13.64	25.63	18.71	1.37	4.32	C
NEA	8.21	5.32	10.12	6.79	1.28	2.32	A
	10.59	7.15	13.22	9.17	1.28	2.77	B
	14.54	10.19	18.26	13.04	1.28	3.23	C
CEA	9.22	6.09	11.45	7.81	1.28	2.35	A
	11.63	7.94	14.64	10.27	1.29	2.75	B
	15.73	11.10	19.98	14.37	1.29	3.27	C

Column, Inertsil SIL (150 × 4.6 mm I.D., 5 μm) at 40°C; eluent A, *n*-hexane–AcOEt (45:55); eluent B, *n*-hexane–AcOEt (50:50); eluent C, *n*-hexane–AcOEt (55:45); flow rate, 1.0 ml/min, fluorescence detection, ex. 470 nm, em. 540 nm; t_0 = 1.3 min.

elute more rapidly than the *R*-enantiomers. The opposite results were obtained from usage of *R*(+)-DBD-Pro-COCl. No exceptions were observed among all pairs of enantiomers tested. Typical normal-phase chromatograms of the diastereomers formed with *S*(−)-NBD-Pro-COCl are depicted in Fig. 4. The polar compounds, including the hydrolysate of the derivatization reagent, eluted later than the diastereomers. Although the complete resolutions of the enantiomers of amines and alcohols were achieved by normal-phase chromatography, this technique may not be suitable for biological specimens because of sample handling difficulties. There-

Table 2
HPLC separation of diastereomers derived from *R*(+)-NBD-Pro-COCl by normal-phase chromatography

Alcohol	*S*-enantiomer		*R*-enantiomer		α	R_s	Eluent
	t_R (min)	k'	t_R (min)	k'			
2-Hexanol	8.64	5.65	7.48	4.75	1.19	1.73	A
	11.92	8.17	10.17	6.82	1.20	2.33	B
	18.17	12.98	15.33	10.80	1.20	2.99	C
2-Heptanol	8.28	5.37	7.01	4.40	1.22	2.10	A
	11.37	7.75	9.47	6.29	1.23	2.45	B
	17.24	12.26	14.14	9.88	1.24	3.26	C
2-Nonanol	7.74	4.95	6.43	3.95	1.25	2.17	A
	10.60	7.16	8.64	5.65	1.27	2.62	B
	15.93	11.26	12.76	8.82	1.28	3.39	C
1-Phenylethanol	12.26	8.43	10.34	6.95	1.21	2.56	A
	17.91	12.78	14.81	10.39	1.23	3.26	B
	28.55	20.96	23.32	16.94	1.24	4.10	C
	5.80	3.46	5.15	2.96	1.17	1.24	D

Column, Inertsil SIL (150 × 4.6 mm I.D., 5 μm) at 40°C; eluent A, *n*-hexane–AcOEt (70:30); eluent B, *n*-hexane–AcOEt (75:25); eluent C, *n*-hexane–AcOEt (80:20); eluent D, *n*-hexane–AcOEt (55:45); flow rate, 1.0 ml/min; fluorescence detection, ex. 470 nm, em. 540 nm; t_0 = 1.3 min.

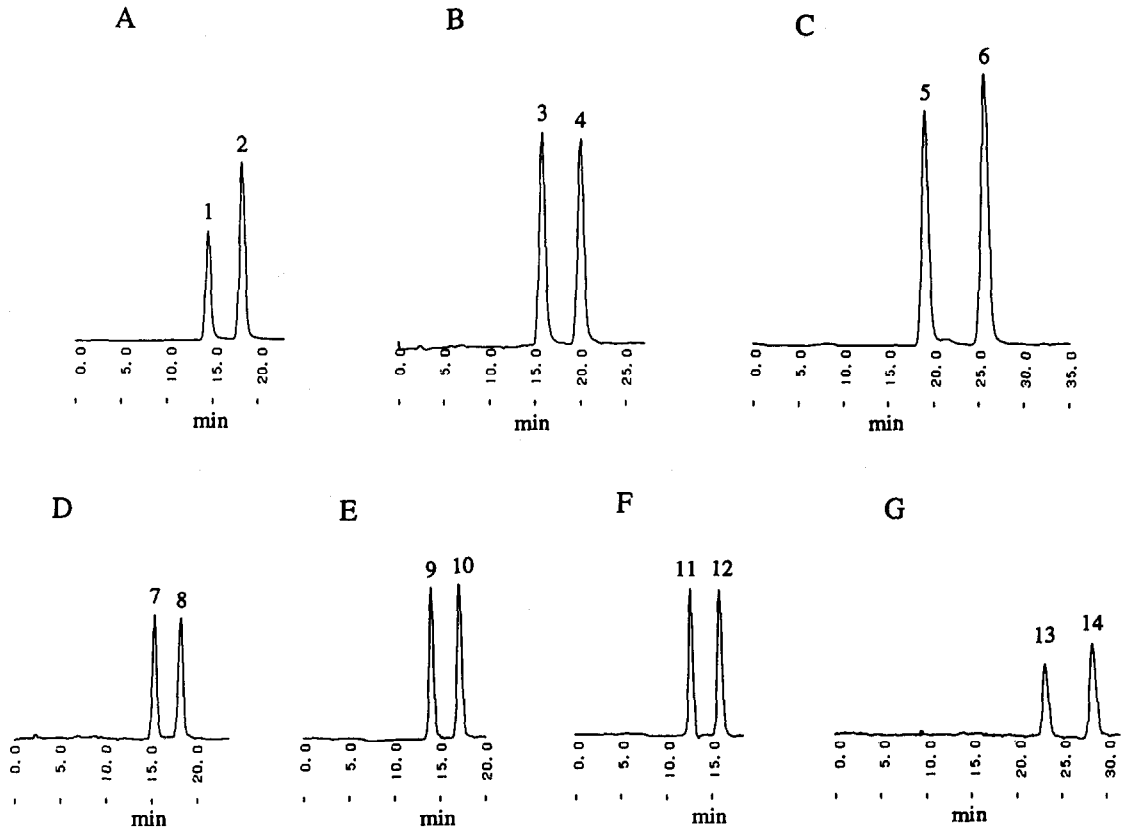


Fig. 4. Chromatograms obtained from the reaction with *S*(−)-NBD-Pro-COCl by normal-phase chromatography. Separation of the resulting diastereomers: (A) NEA; (B) CEA; (C) PEA; (D) 2-hexanol; (E) 2-heptanol; (F) 2-nonanol; (G) 1-phenylethanol. Peaks: 1 = *S*(−)-NEA, 2 = *R*(+)-NEA, 3 = *S*(+)-CEA, 4 = *R*(−)-CEA, 5 = *S*(−)-PEA, 6 = *R*(+)-PEA, 7 = *S*(+)-2-hexanol, 8 = *R*(−)-2-hexanol, 9 = *S*(+)-2-heptanol, 10 = *R*(−)-2-heptanol, 11 = *S*(+)-2-nonanol, 12 = *R*(−)-2-nonanol, 13 = *S*(−)-1-phenylethanol, 14 = *R*(+)-1-phenylethanol. HPLC eluent: *n*-hexane–benzene (55:45) for chromatograms A, B and C; *n*-hexane–benzene (80:20) for chromatograms D, E, F and G. Other HPLC conditions as in Experimental.

fore, the analysis by reversed-phase chromatography with aqueous solvent system was also investigated.

As shown in Table 3, the separation of the diastereomers derived from PEA was incomplete by reversed-phase chromatography; while no separation of the diastereomers obtained from CEA was achieved. Only NEA was resolved, but the R_s value was small (1.65), as compared with normal-phase HPLC. In the case of DBD-Pro-COCl, the R_s values obtained from reversed-phase chromatography were in the following order: CEA > NEA > PEA [21]. On the other hand, the resolution of all pairs of the diastereomers formed from alcohols were perfectly separated (1.55–1.99) (Table 4). However, the R_s values were smaller than those obtained from normal-phase chromatography. The results described above suggest that the formation of hydrogen bonds between the stationary phases and the resulting diastereomers play important roles in the separations. The elution orders were the same as those by normal-phase chromatography, *S*-enantiomers eluted faster than *R*-enantiomers with use of *S*(−)-NBD-Pro-COCl and *R*-enantiomers eluted faster than *S*-enantiomers with use of *R*(+)-NBD-Pro-COCl.

The proposed chiral derivatization reagents [*S*(−)- and *R*(+)-NBD-Pro-COCls] provided excellent resolution of the enantiomers of amines

Table 3
HPLC separation of diastereomers derived from S(−)-NBD-Pro-COCl by reversed-phase chromatography

Amine	S-enantiomer		R-enantiomer		α	R_s	Eluent
	t_R (min)	k'	t_R (min)	k'			
PEA	5.45	3.19	5.24	3.03	1.05	0.42	A
	7.74	4.96	7.35	4.66	1.06	0.77	B
	11.87	8.13	11.87	8.13	1.0	NC [a]	C
NEA	9.58	6.37	9.12	6.02	1.06	0.88	A
	15.58	10.98	14.58	10.21	1.08	1.28	B
	26.68	19.52	24.64	1.96	1.09	1.65	C
CEA	15.46	10.89	15.46	10.89	1.0	NC	B
	26.24	19.19	26.08	19.06	1.01	NC	C

Column, Inertsil ODS-80A (150 × 4.6 mm I.D., 5 μm) at 40°C; eluent A, H_2O–CH_3CN (50:50); eluent B, H_2O–CH_3CN (55:45); eluent C, H_2O–CH_3CN (60:40); flow rate, 1.0 ml/min; fluorescence detection, ex. 470 nm, em. 540 nm; t_0 = 1.3 min
[a] NC = not calculated.

and alcohols by normal-phase and/or reversed-phase HPLC. NBD-Pro-COCl is recommended for the resolution of chiral amines. For the resolution of alcohols, DBD-Pro-COCl might be more suitable judging from the R_s values by normal-phase chromatography (2.99–4.10 *vs.* 3.34–4.48). However, it must be noted that the diastereomers derived from all alcohols and NBD-Pro-COCl can be separated by reversed-phase chromatography. Since the elution order of enantiomers can be changed with the use of the different enantiomer of the chiral reagents

Table 4
HPLC separation of diastereomers derived from R(+)-NBD-Pro-COCl by reversed-phase chromatography

Alcohol	S-enantiomer		R-enantiomer		α	R_s	Eluent
	t_R (min)	k'	t_R (min)	k'			
2-Hexanol	5.90	3.54	5.90	3.54	1.0	NC [a]	E
	7.99	5.15	7.72	4.94	1.04	0.48	F
	11.42	7.79	10.95	7.43	1.05	0.85	A
	17.38	12.37	16.56	11.74	1.05	1.20	B
	28.02	20.55	26.54	19.42	1.06	1.55	C
2-Heptanol	7.75	4.97	7.46	4.74	1.05	0.49	E
	10.98	7.75	10.49	7.07	1.05	0.86	F
	16.39	11.61	15.56	10.97	1.06	1.23	A
	26.17	19.13	24.65	17.96	1.07	1.73	B
	44.39	33.15	41.55	30.96	1.07	1.90	C
2-Nonanol	14.45	10.12	13.67	9.52	1.06	1.25	E
	22.18	16.06	20.85	15.04	1.07	1.61	F
	35.89	26.61	33.51	24.78	1.07	1.99	A
1-Phenylethanol	10.19	6.84	9.81	6.54	1.05	0.66	B
	15.68	11.06	14.92	10.48	1.06	1.17	C
	26.66	19.51	24.99	18.23	1.07	1.75	D

Column, Inertsil ODS-80A (150 × 4.6 mm I.D., 5 μm) at 40°C; eluent A, H_2O–CH_3CN (40:60); eluent B, H_2O–CH_3CN (45:55); eluent C, H_2O–CH_3CN (50:50); eluent D, H_2O–CH_3CN (55:45); eluent E, H_2O–CH_3CN (30:70); eluent F, H_2O–CH_3CN (35:65); flow rate, 1.0 ml/min; fluorescence detection, ex. 470 nm, em. 540 nm; t_0 = 1.3 min
[a] NC = not calculated.

[23,24], the determination of trace amounts of one enantiomer in the presence of much greater amount of the other enantiomer is easily accomplished. The detection limits of the alcohols are in the 10–50 fmol range. Since the excitation wavelengths of the derivatives are close to the argon-ion laser light emission, the determination of the enantiomers of alcohols and amines at attomole level should be possible with laser-induced fluorescence detection [25]. Hence, the proposed method with pre-column derivatization with NBD-Pro-COCl should be suitable for the resolution of chiral amines and alcohols in real samples as is the case with DBD-Pro-COCl. Further studies concerning the resolution of racemic drugs such as β-blockers and herbicides are currently in progress.

Acknowledgement

The authors thank Dr. C.R. Warner, Food and Drug Administration in Washington, DC, for reviewing the manuscript.

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