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N-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-CO-Hz) as a precolumn fluorescent derivatization reagent for carboxylic acids in high-performance liquid chromatography

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Abstract

A new fluorescent reagent for carboxylic acids, *N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-CO-Hz) was synthesized and its applicability as a precolumn derivatization reagent in high-performance liquid chromatography was examined. NBD-CO-Hz reacted with 2-arylpropionic acids (2-APAs), a group of non-steroidal antiinflammatory drugs (NSAIDs) in the presence of a condensing agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and pyridine at room temperature for 2 h to give fluorescent adducts. The reaction solution was subjected to a reversed phase or a chiral stationary phase HPLC and the derivatives were detected fluorometrically at a wavelength of 530 nm with an exitation of 475 nm. The detection limits were in the fmol range on column.

Keywords: *N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-CO-Hz); 2-Arylpropionic acid; HPLC; Fluorescence derivatization; Enantiomeric separation; Chiral stationary phase.

1. Introduction

A number of substances having a carboxylic acid moiety, such as fatty acids, prostaglandins and bile acids, are present in living organisms and in biological fluids. Also the monitoring of the plasma levels is necessary for a number of drugs containing carboxylic acid moiety, such as 2-arylpropionic acids (2-APAs), a group of non-steroidal anti-inflammatory drugs (NSAIDs). High-performance liquid chromatography (HPLC) with fluorometric detection is sensitive and selective, and should be used to quantify these compounds. However, because most of

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these compounds are non-fluorescent, they should be derivatized with a suitable fluorescent derivatization reagent for the sensitive and selective detection. Various reagents have therefore been developed for this purpose [1–3].

In the course of our studies on the development of fluorescent derivatization reagent having a 2,1,3-benzoxadiazole structure, the following were synthesized for carboxylic acids: 4-nitro-7-*N*-piperazino-2,1,3-benzoxadiazole (NBD-PZ); 4-(*N*,*N*-dimethylaminosulphonyl)-7-N-piperazino-2,1,3-benzoxadiazole (DBD-PZ); 4-(*N*,*N*-dimethylaminosulphonyl)-7-cadaverino-2,1,3-benzoxadiazole (DBD-CD) [4]; *N*-(4-dimethylaminosulphonyl-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (DBD-CO-Hz) [5]; 4-*N*,*N*-dimethylaminosulphonyl-7-*N*-(2-aminoethyl)amino-2, 1,3-benzoxadiazole (DBD-ED) [6]; 4-(aminosulphonyl)-7-(1-piperazinyl)-2,1,3-benzoxadiazole (ABD-PZ); 4-(aminosulphonyl)-7-(5-aminopenthylamino)-2,1,3-benzoxadiazole (ABD-AP); 4-(aminosulphonyl)-7-*N*-(2-anlinoethylamino)-2,1,3-benzoxadiazole (ABD-AE) [7] and used for the determination of fatty acids and drugs. The long emission wavelengths of benzoxadiazoyl derivatives are very suitable for the selective detection of carboxylic acids in biological samples containing fluorescent substances of shorter wavelength.

In this paper, we describe the synthesis of a new precolumn fluorescent derivatization reagent for carboxylic acids having a 2,1,3-benzoxadiazole skelton, *N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-CO-Hz) and its application for several 2-APAs such as ibuprofen, ketoprofen, flurbiprofen and pranoprofen.

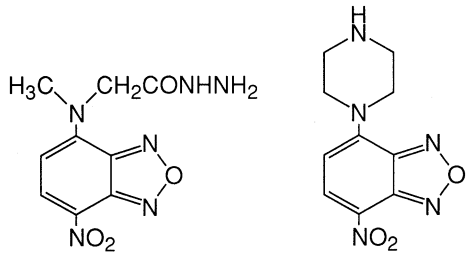
2. Experimental

2.1. Reagents and chemicals

NBD-PZ was purchased from Tokyo Kasei (Tokyo, Japan). (*RS*)-ibuprofen and (*RS*)-ketoprofen were from Wako (Osaka, Japan). (*RS*)-flurbiprofen was from Sigma (St. Louis, MO). (*RS*)- and (*S*)-pranoprofen were from Yoshitomi Pharmaceutical (Fukuoka, Japan). (*S*)-ibuprofen, -ketoprofen, and flurbiprofen were from Nagase Industry (Hyogo, Japan). Acetonitrile and methanol were of HPLC grade (Wako). Hydrazine monohydrate and trifluoroacetic acid (TFA) were from Wako. Water was used after purification by Milli-Q system (Millipore, Waltham, MA). Pyridine, dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 4-nitro-7-chloro-2,1,3-benzoxadiazole (NBD-CI) and *N*-methylglycine were from Tokyo Kasei.

2.2. Apparatus

Melting points were measured on a Yanagimoto micro melting point apparatus (Tokyo, Japan) and uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-EX270 spectrometer (Tokyo, Japan) using tetramethylsilane as an internal standard. Mass spectra were measured on an M-1200 mass spectrometer (atmospheric chemical ionization (APCI) system (Hitachi, Tokyo, Japan). Fluorescence spectra were measured with an Hitachi F-4010 fluorescence spectrometer (Hitachi).



NBD-CO-Hz NBD-PZ

Fig. 1. Chemical structures of NBD-CO-Hz and NBD-PZ.

2.3. Synthesis of NBD-CO-Hz

2.3.1. Synthesis of 4-(N-carboxymethyl-N-methyl)-amino-7-nitro-2,1,3-benzoxadiazole (NBD-COOH)

A 10.4 g (0.052 mol) solution of NBD-Cl in 500 ml of CH_3CN was added dropwise to a solution of 18.5 g (0.21 mol) of *N*-methylglycine in 1.0 l of 0.52 M $NaHCO_3$ solution. The reaction mixture was stirred for 2 h at room temperature and then condensed under reduced pressure. The resultant mixture was added by 140 ml of 6 N HCl solution and stirred for 30 min to give orange crystals (13.2 g, 0.052 mol), ^{1}H-NMR (p.p.m) in d_6-DMSO, 3.45 (broad, 3H), 4.91 (broad, 2H), 6.45 (d, $J = 9$ Hz, 1H), 8.52 (d, $J = 9$ Hz, 1H).

2.3.2. Synthesis of N-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-N-methyl-2-aminoacetyl chloride (NBD-COCI)

A 27 ml (0.32 mol) solution of oxalyl chloride and 0.18 ml of DMF were added to 6.0 g (0.0238 mol) of NBD-COOH in CH_2Cl_2. The reaction mixture was stirred for 1 h at room temperature and evaporated under reduced pressure. The residue was dissolved in 300 ml of toluene and the filtrate was condensed to give orange crystals (5.3 g, yield 82%), ^{1}H-NMR (p.p.m) in C_6D_6, 2.21 (*S*, 2H), 4.30 (*S*, 2H), 5.04 (d, $J = 9$ Hz, 1H), 7.80 (d, $J = 9$ Hz, 1H). Elemental analysis: calculated for $C_9H_7N_4O_4Cl$: C, 39.94; H, 2.61; N, 20.70%; found: C, 40.32; H, 2.30; N 20.28%.

2.3.3. Synthesis of NBD-CO-Hz

A 2.15 g (7.96 mmol) solution of NBD-COCl in 400 ml of acetonitrile was added dropwise to 400 ml of methanol containing 0.8 ml (16.2 mmol) of hydrazine hydrate in the ice bath. After stirring for 20 min, the reaction mixture was added by 100 ml of water and condensed under reduced pressure to give the precipitate. The precipitate was washed with 200 ml of 10% methanol to afford red crystals (1.7 g, yield 80.3%), m.p. 187°C. APCI-MS, m/z 267 $(M+H)^+$; elemental analysis: calculated for $C_9H_{10}N_6O_4$: C, 40.61; H, 3.79; N, 31.57%; found: C, 40.8; H, 3.46; N, 31.04%.

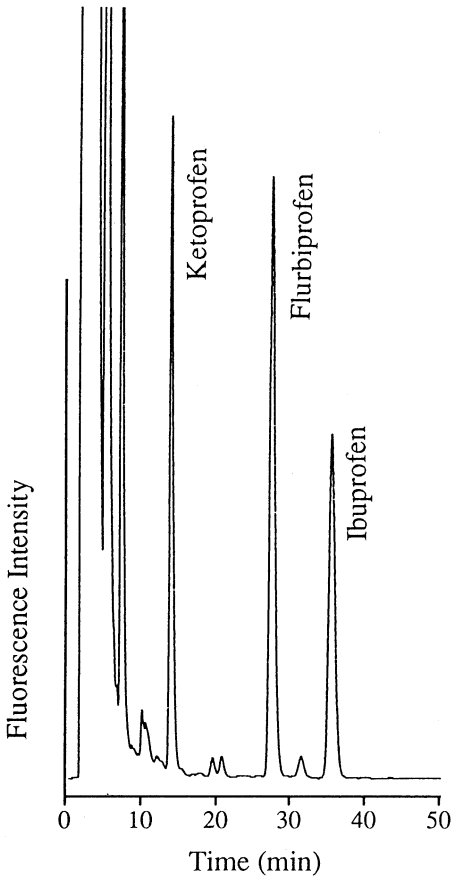


Fig. 2. Chromatogram obtained for a standard mixture of 2-APAs derivatized with NBD-CO-Hz by reversed phase HPLC (5.0 pmol each per 1.0 μl injection volume). HPLC conditions were described in the text.

2.4. Derivatization

To 50 μl of the test solution of 2-APAs in DMF were added 50 μl of 1.0 M EDC in water, 50 μl of 20% pyridine in water and 20 mM NBD-CO-Hz in DMF, successively. The mixture was allowed to stand for 2 h at room temperature and diluted 10 times with mobile phase. A 1.0 μl aliquot of the resulting solution was subjected to HPLC equipped with a reversed phase column.

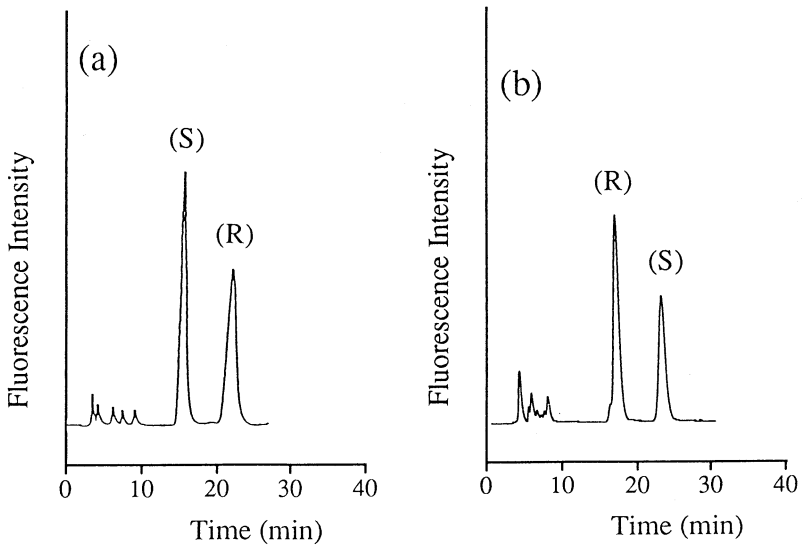


Fig. 3. Chromatograms of ibuprofen derivatized with NBD-CO-Hz (a) and NBD-PZ (b) by chiral stationary phase HPLC. The mobile phase employed are H_2O/CH_3CN (40/60) for NBD-CO-Hz derivatives and H_2O/CH_3OH (0/100) for NBD-PZ derivatives. Other HPLC conditions are described in the text.

For enantiomeric separation study, 50 μl of 0.2 mM solution of NBD-CO-Hz in DMF and 1.0 mM 2-APAs in DMF were used. A 1.0 μl aliquot of the resulting solution was subjected to HPLC equipped with a chiral stationary phase column.

2.5. HPLC

The high-performance liquid chromatograph consisted of an L-6300 intelligent pump (Hitachi), an F-1080 fluorometric detector (Hitachi) and a D-2500 integrator (Hitachi) and a model 7725i sample injector (Rheodyne, Cotati, CA) with a 20 μl sample loop.

The separation of the derivatives was studied on a TSK gel ODS-80 Ts (150 × 4.6 mm, i.d., 5 μm) (Tosoh, Tokyo, Japan). The mobile phase was 0.1% TFA in acetonitrile-water (40:60, v/v) at a flow rate of 1.0 ml min^{-1}.

Enantiomeric separation of the derivatives was studied on a CHIRALCEL OD-RH (a modified cellulose with a 3,5-dimethylphenylcarbamoylation [8–10], 150 × 4.6 mm, i.d., 5 μm) (Daicel, Osaka, Japan). The mobile phase composition employed was acetonitrile–water (60:40, v/v) or methanol–water (100:0, v/v) at a flow rate of 0.5 ml min^{-1}.

The column temperature was ambient. The fluorometric detection was made at 530 nm with excitation at 475 nm.

3. Results and discussion

3.1. Synthesis of NBD-CO-Hz

NBD-CO-Hz was synthesized by the reaction of NBD-COCl and hydrazine hydrate and obtained in a good yield (80.3%). The structure was confirmed as in Fig. 1 by NMR and mass spectroscopy. The NMR spectral data were also consistent with those expected as *N*-(4-nitro-2,1 3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide. The fluorescence emission maximum (λ_{em}) of NBD-CO-Hz in acetonitrile was 530 nm at the excitation wavelength maximum (λ_{ex}) of 475 nm, 530 nm at λ_{ex} of 475 nm in methanol and 532 nm at λ_{ex} of 483 nm in water. The maximum wavelengths for NBD-CO-Hz and its adducts with flurbiprofen were different from each other only by 1–2 nm. In this study $\lambda_{em} = 530$ nm and $\lambda_{ex} = 475$ were selected for all experiments.

Table 1
Capacity factors (k') and separation factors (α) of 2-arylpropionic acid derivatives with NBD-CO-Hz by chiral stationary phase HPLC using H_2O/CH_3CN or H_2O/CH_3OH as a mobile phase.

2-Arylpropionic acid	H_2O/CH_3CN (40/60)			H_2O/CH_3OH (0/100)		
	$k'(R)$	$k'(S)$	α	$k'(R)$	$k'(S)$	α
Flurbiprofen	4.83	3.51	1.38	1.09	0.99	1.10
Ibuprofen	4.69	2.98	1.57	0.78	0.62	1.25
Ketoprofen	3.00	2.05	1.46	1.13	0.97	1.17
Pranoprofen	1.61	1.61	1.00	1.64	1.64	1.00

$k'(S)$ and $k'(R)$ are the capacity factors of the respective enantiomers.

3.2. Derivatization reaction of NBD-CO-Hz with 2-APAs

The carboxylic moiety of the drugs seem to be easily derivatized with NBD-CO-Hz in the presence of EDC and pyridine. Since they have been used previously for the fluorescent derivatization of fatty acids with DBD-CO-Hz [5] and the reactive site of NBD-CO-Hz is the same as that of DBD-CO-Hz the effect of the substituent group at 7-position of 2,1,3-benzoxadiazole was expected to be negligible. The yield of the adducts of NBD-CO-Hz with flurbiprofen at room temperature reached plateau after 2 h. The same reaction pattern was also observed in case of DBD-CO-Hz. The adduct was stable for at least 7 days at 4°C.

3.3. Separation of 2-APAs derivatives on a reversed phase column

The chromatogram for ketoprofen, flurbiprofen and ibuprofen derivatized with NBD-CO-Hz is shown in Fig. 2. The complete separation of the adducts from the reagent was accomplished.

The calibration graph for ketoprofen was linear over the range from 0.1 to 5 pmol per injection ($r = 0.996$ $n = 5$). The relative standard deviations of the peak area for the three drugs for the five replicate measurements at 5 pmol per injection were 1.1–2.0%. The detection limits attained were 2–4 fmol per injection (signal-to-noise ratio = 3). These detection limits were comparable with a method using DBD-CO-Hz [5] and DBD-ED [6]. The detection limits of NBD-CO-Hz derivatives will be improved using the argon laser excitation because the excitation wavelength of NBD-CO-Hz is fitted for emission of the argon laser (488 nm).

3.4. Enantiomeric separation of 2-APAs derivatives

2-APAs are now available as racemates in the treatment of rheumatic and inflammatory diseases although the pharmacological activity of these drugs originate only from the *S*-isomer. Because of the in vivo inversion of non-active *R*-isomer into *S*-isomer [11–13] the enantiomeric determination of 2-APAs is essential in order to keep an appropriate therapy.

In our previous study [14] we have already reported the enantiomeric separation and detection of 2-arylpropionic acids derivatized with DBD-PZ and DBD-CO-Hz on a modified cellulose stationary phase (CHIRALCEL OD-R) and that these derivatives were well separated and the elusion order of enantiomers for each drug was reversed by changing the derivatization reagent. The latter phenomenon was ascribed to the presence and absence of the hydrogen bond interaction derived from the derivatives with the CO-NH-NH-CO- moiety.

In this study we examined the effect of NBD moiety on the enantiomeric separation of the derivatives on the modified cellulose stationary phase CHIRALCEL OD-RH. At first the enantiomeric separation of (*RS*)-ketoprofen derivatives with NBD-CO-Hz was investigated using H_2O/CH_3CN or H_2O/CH_3OH as the mobile

Table 2
Capacity factors (k') and separation factors (α) of 2-arylpropionic acid derivatives with NBD-PZ by chiral stationary phase HPLC using H_2O/CH_3CN or H_2O/CH_3OH as a mobile phase

2-Arylpropionic acid	H_2O/CH_3CN (40/60)			H_2O/CH_3OH (0/100)		
	$k'(R)$	$k'(S)$	α	$k'(R)$	$k'(S)$	α
Flurbiprofen	13.6	13.6	1.00	7.07	9.46	1.34
Ibuprofen	11.3	11.8	1.04	3.57	5.20	1.46
Ketoprofen	7.84	8.01	1.02	6.82	7.97	1.17
Pranoprofen	6.39	6.39	1.00	8.68	9.37	1.08

$k'(S)$ and $k'(R)$ are the capacity factors of the respective enantiomers.

phase. The chromatogram of ibuprofen derivatives with NBD-CO-Hz using H_2O/CH_3CN (40/60) as a mobile phase was shown in Fig. 3(a) and the capacity factors (k') and the separation factors (α) of the derivatives were summarized in Table 1. The derivatives were well separated and greater α values were obtained with H_2O/CH_3CN as the mobile phase although pranoprofen derivatives could not be separated. The (*S*)-enantiomers of the derivatives were eluted first in both mobile phase systems. These results were comparable with those obtained with DBD-CO-Hz [14]. The derivatives with NBD-PZ were well separated with methanol as the mobile phase and larger a values were obtained (Fig. 3(b) and Table 2). The reversal of the elusion order of (*R*)- and (*S*)-derivatives with NBD-PZ were also observed in this study. Therefore the combined use of NBD-CO-Hz and NBD-PZ seems to be useful for the identification of the enantiomers as suggested in the previous paper for DBD-CO-Hz and DBD-PZ [14].

In conclusion NBD-CO-Hz as well as DBD-CO-Hz was a useful precolumn fluorescence derivatization reagent for sensitive detection and enantiomeric separation of the compounds having a carboxylic acid moiety.

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