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Precolumn fluorescence derivatization of carnitine and acylcarnitines with 4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole prior to high-performance liquid chromatography

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Abstract

4-(2-Aminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-ED) was synthesized as a precolumn fluorescence derivatization reagent for the high-performance liquid chromatographic determination of carnitine and acylcarnitines. Carnitine and acylcarnitines were reacted with NBD-ED (2.0 m*M*) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (35 m*M*) in pyridine–dimethylformamide (1:4) at room temperature for 2 h. The NBD-ED derivatives of carnitine and acylcarnitines were separated by gradient elution with water–acetonitrile containing 10 m*M* trifluoroacetic acid on a reversed-phase column and the eluate was monitored with excitation at 470–485 nm and emission at 530–540 nm. Carnitine and sixteen acylcarnitines were determined within 45 min. The detection limits (signal-to-noise ratio = 3) for the carnitine-related compounds were 10–100 fmol. Four other NBD-alkyldiamines were also studied.

1. Introduction

The determination of serum and urinary acylcarnitines in patients with organic acidurias is important for the diagnosis of genetic defects involving mitochondrial dehydrogenases or carboxylases [1,2]. A common feature in these patients is a decrease in free carnitine in serum and an increase in normal or abnormal acylcarnitines in serum and urine. Further, administration of L-carnitine increases considerably the excretion of acylcarnitines into urine, reflecting the washout of coenzyme A esters accumulated in the mitochondrion.

Several post- and precolumn derivatization methods have been developed for the high-performance liquid chromatographic (HPLC) determination of acylcarnitines. HPLC of coenzyme A esters formed by transesterification [3,4] and HPLC of [^{3}H]acylcarnitines produced by added [^{3}H]carnitine in the presence of coenzyme A [5–7] are limited to the catalytic

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reaction of carnitine acetyltransferase to determine short-chain acylcarnitines. HPLC with a carboxylic acid analyser [8,9] or HPLC with mass spectrometry [10,11] requires expensive apparatus. Precolumn derivatization methods such as with bromophenacyl esters with 4′-bromophenacyl triflate [12] or 4-bromophenacyl bromide [13] have been applied to determine carnitine and acylcarnitines in biological samples [14–17].

Various fluorescent precolumn derivatization reagents have been developed for the HPLC determination of carboxylic acids, coumarin-type reagents such as 4-bromomethyl-7-methoxycoumarin [18–20], 4-bromomethyl-7-acetoxycoumarin [21,22] and 3-bromoacetyl-7-methoxycoumarin [23], diazomethane-type reagents such as 9-anthryldiazomethane [24,25] and 1-pyrenyldiazomethane [26], hydrazide-type reagents such as 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide [27] and 4-(5,6-dimethoxy-2-benzimidazoyl)benzohydrazide [28] and aliphatic amine-type reagents such as dansyl semipiperazide [29], monodansylcadaverine [30] and 4-substituted-7-aminoalkylamino-2,1,3-benzoxadiazoles [31,32]. Among these reagents, the aliphatic amine-type reagents seem to be suitable for carboxylic acid determinations owing to their excellent stability and sensitivity.

We have developed an immobilized enzyme reactor of acylcarnitine hydrolase, carnitine dehydrogenase and diaphorase for the sensitive and selective HPLC determination of short-chain acylcarnitines [33]. However, the method needs an immobilized enzyme reactor and cannot determine medium- and long-chain acylcarnitines. In this paper, we report a precolumn derivatization method for the HPLC determination of carnitine and acylcarnitines with 4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-ED).

2. Experimental

2.1. Reagents

Chloride salts of D,L-carnitine and acetyl-, hexanoyl-, heptanoyl-, octanoyl-, nonanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl- and stearoyl-D,L-carnitine were purchased from Sigma (St. Louis, MO, USA). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), ethylenediamine (ED), 1,3-propanediamine (PD), piperazine (PZ), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC), diethyl phosphorocyanidate (DEPC), triphenylphosphine (TPP), N,N′-dimethylformamide (DMF) for fluorescence analysis, pyridine for sequence analysis and methanol and acetonitrile for HPLC analysis were obtained from Wako (Osaka, Japan), putrescine (PS) and cadaverine (CD) from Nacalai Tesque (Kyoto, Japan) and diphenylphosphoroylazide (DPPA), 2,2′-dipyridyl disulphite (DPDS) and 2-chloro-1-methylpyridinium iodide (CMP) from Tokyo Kasei Kogyo (Tokyo, Japan). A TSKgel ODS 80Ts column (150 mm × 4.6 mm I.D., 5 μm particle size) and a Toyopak IC-SP S cartridge (0.15 ml gel bed, 0.06 mequiv.) were purchased from Tosoh (Tokyo, Japan). Other chemicals were of analytical-reagent grade.

2.2. HPLC

The HPLC system from Tosoh was composed of a Model CCPM-8000 pump, a Model AS-48 autosampler equipped with a 100-μl loop injector, a Model FS-8010 fluorescence detector and a Model CP-8000 chromatoprocessor.

2.3. Synthesis of acylcarnitines

Propionyl-, isobutyryl-, butyryl-, isovaleryl-, valeryl- and valproyl-D,L-carnitine hydrochloride were synthesized from the corresponding acid chlorides in trifluoroacetic acid (TFA) according to the method of Bohmer and Bremer [34].

2.4. Synthesis of NBD-alkyldiamine derivatives

NBD-F (10 mg, 55 μmol) in 10 ml of acetonitrile was added dropwise to a stirred solution of each alkyldiamine (550 μmol) in 10 ml of acetonitrile for 30 min at room temperature. After stirring for 2 h at room temperature, acetonitrile was evaporated under reduced pressure. The residue was acidified with 5% HCl and the NBD-alkyldiamine derivatives were purified several

times on a reversed-phase column with a linear gradient of 10 m*M* HCl–acetonitrile.

2.5. Comparison of activation agents for the derivatization reaction

NBD-ED (2.0 m*M*) was reacted with the carnitine mixture (D,L-carnitine and acetyl-, octanoyl- and palmitoyl-D,L-carnitine, 50 μM each) in 100 μl of DMF or acetonitrile in the presence of each activation agent: EDC (35 m*M*)–20% pyridine, DEPC (70 m*M*), DPPA (70 m*M*), DPDS (35 m*M*) and TPP (35 m*M*) or CMP (35 m*M*)–20% triethylamine (TEA). After reaction for 3 h at room temperature, the reaction mixture was diluted with 900 μl of 10 m*M* HCl in DMF–water (4:1, v/v), 100 μl of the solution was injected on to the column and the fluorescent peaks corresponding to the adducts were compared with each other.

2.6. Comparison of derivatization agents

The carnitine mixture (50 μM each) was reacted with EDC (35 m*M*) and the NBD-akyldiamine reagent (NBD-ED, NBD-PD, NBD-CD, NBD-PT or NBD-PZ, 2 m*M* each) at room temperature in 100 μl of pyridine–DMF (1:4, v/v). At fixed time intervals, the reaction was stopped by adding 9 volumes of 10 m*M* HCl in DMF–water (4:1, v/v), 100 μl of the solution were injected on to the column and the fluorescent peak areas corresponding to the adducts were measured at each maximum excitation and emission wavelength.

2.7. Spectral measurement of fluorescent adducts

The carnitine mixture (50 μM each) was reacted with 2 m*M* each of NBD-ED, NBD-PD, NBD-CD, NBD-PT or NBD-PZ in 100 μl of pyridine–DMF (1:4, v/v) in the presence of 35 m*M* EDC at room temperature for 2 h. The fluorescent peaks corresponding to the adducts separated by reversed-phase HPLC were collected. Excitation and emission spectra of the fluorescent adducts were measured with a Model F-3000 spectrofluorimeter (Hitachi, Tokyo, Japan).

2.8. Dependence of fluorescence intensities on organic solvent

The NBD-ED adducts corresponding to carnitine and acetyl-, octanoyl- and palmitoyl-D,L-carnitine separated on the reversed-phase column were collected and the eluates were evaporated to dryness. The residues were dissolved in 10 m*M* TFA with various ratios of acetonitrile and water. The fluorescence intensities were measured with excitation at 485 nm and emission at 540 nm with the spectrofluorimeter.

2.9. Purification of fluorescent adducts with a Toyopak IC-SP S cartridge

The reaction mixture of the NBD-ED adducts with carnitine and acylcarnitines diluted with 9 volumes of 10 m*M* HCl in methanol–water (4:1, v/v) was applied to a Toyopak IC-SP S cartridge, the cartridge was washed with 5 ml of 10 m*M* HCl–methanol (1:1, v/v) and the fluorescent adducts retained on the cartridge were eluted with 3 ml of TEA–acetate (1.0 *M*, pH 7.0) in methanol. The eluent was evaporated to dryness and the residue was dissolved in 1 ml of 0.1 *M* TFA–DMF (1:4, v/v) and an aliquot of the solution was injected on to the reversed-phase column.

2.10. HPLC conditions for NBD-ED adducts with acylcarnitines

The NBD-ED adducts with carnitine and acylcarnitines purified on the Toyopak IC-SP S cartridge and dissolved in 0.1 *M* TFA–DMF (1:4, v/v) were injected on to a TSKgel ODS-80Ts column and separated by the following gradient programme with solvents A = 10 m*M* TFA and B = water–acetonitrile (1:9, v/v) containing 10 m*M* TFA: 0–15% B for 1 min, 15–25% B for 9 min, 25–35% B for 10 min, 35–100% B for 20 min, 100% B for 2 min and 100–0% B for 1 min. The eluate was monitored with excitation at 485 nm and emission at 540 nm.

3. Results and discussion

3.1. Fluorescence properties of the NBD-alkyldiamine adducts with carnitine and acylcarnitines

The excitation and emission wavelengths for maximum fluorescence intensities of the NBD-alkyldiamine adducts with carnitine and acylcarnitines obtained using an eluent composed of acetonitrile–water (1:9 to 9:1, v/v) containing 10 m*M* TFA are given in Table 1. Acetonitrile caused blue shifts of both the excitation and emission maxima of the NBD-alkyldiamine adducts, 9–15 nm shorter in excitation and 4–10 nm shorter in emission of NBD-adducts with carnitine (eluted with 10% acetonitrile) than those of palmitoylcarnitine (in 90% acetonitrile).

Organic solvents markedly affected the fluorescence intensities of the NBD-ED adducts [35]. About a tenfold increase in the fluorescence intensities was obtained in acetonitrile containing 10 m*M* TFA compared with those in 10 m*M* TFA. Although accurate gradient control was necessary for reproducible determination, the dependence of the fluorescence intensities of the NBD-ED adducts on the concentrations of acetonitrile would be favourable for the simultaneous determination of carnitine and acylcarnitines in biological samples that contain higher contents of carnitine and short-chain acylcarnitines than long-chain acylcarnitines.

3.2. Reactivities and fluorescence intensities of NBD-alkyldiamine reagents with carnitine and acylcarnitines

The reactivities of NBD-alkyldiamine reagents with carnitine and acylcarnitines are shown in Fig. 1. The chain length of the acyl groups in acylcarnitines did not affect the derivatization reactivity with NBD-ED and the reaction reached a plateau after 1.5–6 h (Fig. 1a). Comparing the reaction rates of carnitine with five NBD-alkyldiamine reagents, NBD-ED reacted most rapidly and the reaction rate of NBD-PZ seemed to be similar to those of the other NBD-alkyldiamine reagents (Fig. 1b). The relative fluorescence intensities of the NBD-alkyldiamine adducts with carnitine and acylcarnitines were calculated for the peak areas at their maximum excitation and emission wavelengths and are given in Table 2. The fluorescence intensities of the NBD-ED adducts were highest and those of NBD-PZ were lowest among the five NBD-alkyldiamine reagents. These results were different from those for 4-(aminosulphonyl)-2,1,3-benzoxadiazolamine (ABD-amine) adducts with car-

Table 1
Wavelengths for maximum fluorescence intensities of NBD-alkyldiamine adducts with carnitine and acylcarnitines

NBD-diamine	Excitation/ emission	Wavelength (nm)			
		Free	Acetyl	Octanoyl	Palmitoyl
NBD-ED	Ex.	485	483	476	470
	Em.	540	540	536	533
NBD-PD	Ex.	488	487	479	475
	Em.	543	543	535	533
NBD-PS	Ex.	485	490	481	476
	Em.	542	546	536	533
NBD-CD	Ex.	485	484	481	476
	Em.	542	539	535	533
NBD-PZ	Ex.	493	493	487	484
	Em.	542	541	538	538

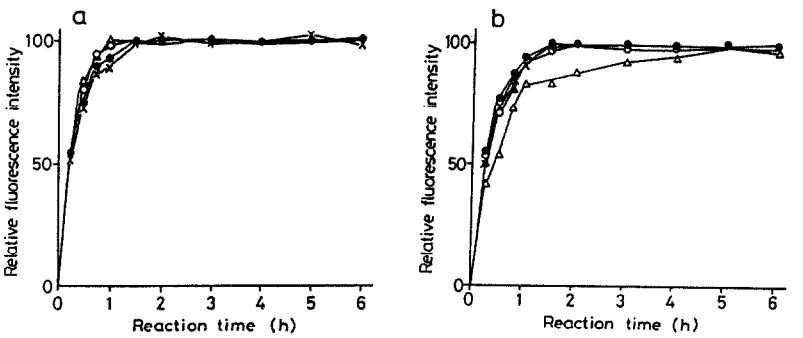

Fig. 1. Reactivities of the NBD-alkyldiamine derivatives with carnitine and acylcarnitines. (a) Reactivities of NBD-ED with carnitine and acylcarnitines in the presence of EDC and pyridine. ● = carnitine; × = acetylcarnitine; △ = octanoylcarnitine; ○ = palmitoylcarnitine. (b) Reactivities of NBD-alkyldiamines with carnitine in the presence of EDC and pyridine. ● = NBD-ED; ○ = NBD-PD; ▲ = NBD-PS; △ = NBD-CD; × = NBD-PZ. The relative fluorescence intensities are expressed with respect to maximum fluorescence intensities = 100.

boxylic acids in the presence of DEPC [32], which indicated that ABD-PZ reacted with carboxylic acids more slowly than ABD-ED and ABD-CD, while the fluorescence intensities of ABD-PZ adducts were highest among the three ABD-amines.

3.3. Derivatization reaction of NBD-ED with carnitine in the presence of activation agents

The five activation agents were compared for the reaction of NBD-ED with carnitine in acetonitrile or DMF and the results are given in Table 3. Among the five activation agents, EDC-pyridine and DPDS-TPP exhibited the highest activation in acetonitrile and DMF, respectively. In the activation of arachidic acid for derivatization with 4-(N,N-dimethylaminosulphonyl)-7-N-piperazino-2,1,3-benzoxazole (DBD-PZ), DEPC and DPDS-TPP exhibited the highest activation in acetonitrile and DMF, respectively [31]. As DPDS-TPP produced side-reaction peaks, EDC-pyridine in DMF was selected as an activation agent.

3.4. Optimized reaction conditions for derivatization of carnitine with NBD-ED

Fluorescent derivatization of carnitine with NBD-ED in the presence of EDC-pyridine was optimized with respect to the concentrations of NBD-ED, EDC, pyridine. The reaction time courses were also compared in acetonitrile and DMF. As shown in Fig. 2, the derivatization reactions reached plateaux with the use of over 2 m*M* of NBD-ED, 20 m*M* of EDC and 10% of

Table 2
Relative fluorescence intensities of NBD-alkyldiamine adducts with carnitine and acylcarnitines

NBD-diamine	Wavelength (nm)		Relative fluorescence intensity			
	Ex.	Em.	Free	Acetyl	Octanoyl	Palmitoyl
NBD-ED	485	540	100	100	100	100
NBD-PD	488	543	92	82	63	82
NBD-PS	485	542	74	59	27	55
NBD-CD	485	542	76	57	25	49
NBD-PZ	493	542	39	50	28	24

Table 3
Reactivities of activation agents in NBD-ED adducts with carnitine

Activation agent	Relative fluorescence intensity	
	Acetonitrile	Dimethylformamide
EDC	100	100
DPDS–TPP	89	101
CMP–TEA	24	48
DEPC	23	67
DPPA	56	61

pyridine in DMF at room temperature for 3 h. The reaction time courses reached plateaux after 1.5 and 2 h in acetonitrile and DMF, respectively. The derivatization conditions selected were 2 m*M* NBD-ED and 35 m*M* EDC in 20% pyridine–DMF at room temperature for 2 h.

3.5. HPLC of the NBD-ED adducts with carnitine and acylcarnitines

The main impurity peaks due to the side-reactions were eluted with the peaks of acetylcarnitine and myristoylcarnitine. Minor impurity peaks due to carboxylic acids hydrolysed from acylcarnitines were also detected after the peaks of the corresponding acylcarnitines. Pretreatment with a Toyopak IC-SP S cartridge was effective in eliminating these impurity peaks. The impurity peaks were easily washed out from the Toyopak IC-SP S cartridge with 5 ml of 10 m*M* HCl–methanol (1:1, v/v), whereas the NBD-ED adducts of carnitine and acylcarnitines retained on the cartridge were eluted with 3 ml of TEA–acetate (1.0 *M*, pH 7.0) in methanol.

As shown in Fig. 3, the NBD-ED adducts with carnitine and sixteen acylcarnitines were clearly separated within 45 min. The isomeric acylcarnitines such as isobutyryl- and butyryl-D,L-carnitine, isovaleryl- and valeryl-D,L-carnitine and valproyl- and octanoyl-D,L-carnitine were also clearly separated with faster retention times for the former acylcarnitines than for the latter. The detection limits for the NBD-ED adducts with carnitine and acylcarnitines were 100 fmol for carnitine and 10 fmol for stearoylcarnitine. The reproducibilities obtained in eight assays were 1.8–3.6, 1.6–4.4 and 1.6–3.2% (relative standard

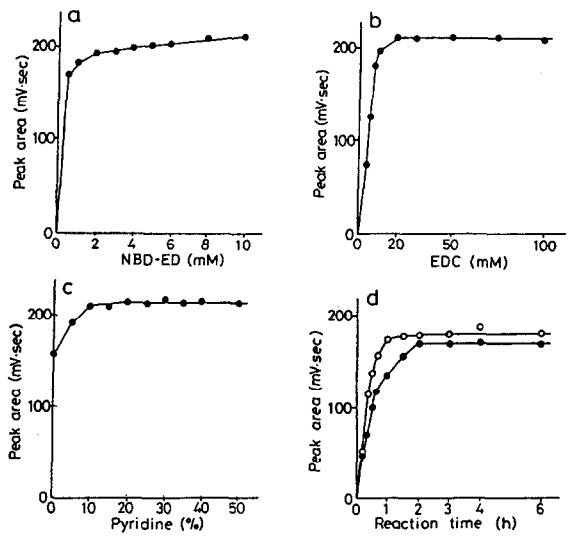

Fig. 2. Optimized reaction conditions for derivatization of carnitine with NBD-ED in the presence of EDC and pyridine. (a) Concentration of NBD-ED; (b) concentration of EDC; (c) concentration of pyridine; (d) reaction time course in acetonitrile and DMF. ● = DMF; ○ = acetonitrile.

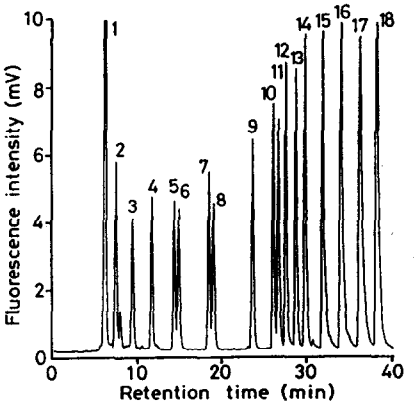

Fig. 3. Chromatogram of carnitine and acylcarnitines derivatized with NBD-ED. Carnitine and acylcarnitines (50 pmol each) were injected. HPLC conditions as described in the text. Peaks: 1 = NBD-ED; 2 = carnitine; 3 = acetylcarnitine; 4 = propionylcarnitine; 5 = isobutyrylcarnitine; 6 = butyrylcarnitine; 7 = isovalerylcarnitine; 8 = valerylcarnitine; 9 = hexanoylcarnitine; 10 = heptanoylcarnitine; 11 = valproylcarnitine; 12 = octanoylcarnitine; 13 = nonanoylcarnitine; 14 = decanoylcarnitine; 15 = lauroylcarnitine; 16 = myristoylcarnitine; 17 = palmitoylcarnitine; 18 = stearoylcarnitine.

deviations) for 25, 50 and 250 pmol of NBD-adducts with carnitine and sixteen acylcarnitines, respectively. Carnitine dicarboxylic acid esters such as glutarylcarnitine could not be eluted using the present HPLC conditions.

This method should be applicable to the simultaneous determination of carnitine and acylcarnitines in human urine and serum samples in combination with suitable pretreatment procedures [14–17].

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