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High-performance liquid chromatographic determination of short-chain aliphatic aldehydes using 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole as a fluorescence reagent

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Abstract

High-performance liquid chromatographic determination of four short-chain aliphatic aldehydes using fluorescence detection was carried out with 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H). DBD-H derivatives with three aliphatic aldehydes – formaldehyde, acetaldehyde and propionaldehyde – were synthesized and their fluorescence properties were examined. Relative fluorescence intensities of these compounds in acetonitrile were ca. ten-fold larger than those in aqueous acetonitrile. DBD-hydrazones could be separated by reversed-phase chromatography using aqueous acetonitrile as eluent and detection at 560 nm with excitation at 445 nm. Submicromolar levels of formaldehyde, acetaldehyde, propionaldehyde and butylaldehyde could be determined. The HPLC procedure using propionaldehyde as internal standard was applied to the measurement of acetaldehyde levels in normal human plasma before and 30 min after ingestion of ethanol.

1. Introduction

Aliphatic aldehydes occur during autoxidation of lipids and contribute to a rancid smell [1,2]. Among the aliphatic aldehydes, acetaldehyde being produced during ethanol metabolism is known to be extremely toxic and to cause alcoholic liver disease [3]. Therefore a sensitive and simple method for the determination of aliphatic aldehydes is very important not only in food chemistry but also in biological or biomedical chemistry.

For the determination of acetaldehyde, several methods have been developed, e.g. high-performance liquid chromatography (HPLC) with fluorescence detection using 1,3-cyclohexanedione and ammonium ion [4], HPLC with ultraviolet detection using 2,4-dinitrophenylhydrazine [5,6], and gas chromatography [3,7]. Recently, we have developed a new fluorescent derivatization reagent for carbonyl compounds, i.e. 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) [8]. DBD-

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H easily reacts with acetone or propionaldehyde to form fluorescent hydrazones at room temperature.

In this paper, we describe the preparation of fluorescent derivatives of short-chain aliphatic aldehydes with DBD-H and their HPLC separation on a reversed-phase column. Furthermore, a study on the determination of free acetaldehyde in human plasma is also described.

2. Experimental

2.1. Apparatus

Fluorescence spectra were measured with a Hitachi 650-10S fluorescence spectrophotometer (Tokyo, Japan) with a 10-mm quartz cell. The HPLC system consisted of a CCPD HPLC pump (Tosoh, Tokyo, Japan), a 7125 injector (Rheodyne, Cotati, CA, USA) with 20-μl sample loop, a SP-120-ODS analytical column (5 μm, 250 × 4.6 mm I.D.; Daiso, Osaka, Japan), a RF550 fluorescence detector (Shimadzu, Kyoto, Japan), and a R-61 recorder (Rikadenki, Tokyo, Japan). The eluent used was acetonitrile–water (46:54, v/v). The flow-rate was set at 1 ml/min. Analytes were detected at an emission wavelength of 560 nm and an excitation wavelength of 445 nm.

2.2. Reagents

DBD-H was prepared as described in a previous paper [8]. Trifluoroacetic acid (TFA), formaldehyde, acetaldehyde, propionaldehyde, butylaldehyde, furfural, cinnamaldehyde, *p*-hydroxybenzaldehyde, *p*-dimethylaminobenzaldehyde, tetraethoxypropane (as malondialdehyde), glutaraldehyde, and terephthalaldehyde were purchased from Wako Pure Chemicals (Osaka, Japan). Acetonitrile was HPLC grade (Wako). Water was deionized and once distilled.

2.3. Preparation of DBD-hydrazones

DBD-hydrazones were prepared according to our previously published method [8].

DBD-hydrazone of formaldehyde: to 50 mg DBD-H (0.195 mmol) in 20 ml of acetonitrile 100 μl of 37% formaldehyde (ca. 1.23 mmol) were added and the mixture was stirred for 4 h at room temperature in the dark. After evaporation, the residue was recrystallized from chloroform to give orange prisms: yield 13 mg (25%), mp 167–167.5°C. Elemental analysis: calculated for $C_9H_{11}O_3N_5S$: C, 40.15; H, 4.12; N, 26.02; S, 11.91%; found: C, 39.70; H, 4.09; N, 25.51; S, 12.18%.

DBD-hydrazone of acetaldehyde: To 20 mg DBD-H (0.078 mmol) in 10 ml of acetonitrile 34.5 mg of acetaldehyde (0.78 mmol) was added and refluxed for 30 min. After cooling to room temperature, the mixture was evaporated. The residue obtained was recrystallized from benzene–*n*-hexane to give orange crystals; yield 16 mg (72%), mp 184–185°C. Elemental analysis: calculated for $C_{10}H_{13}N_5O_3S$: C, 42.40; H, 4.63; N, 24.73; S, 11.32%; found: C, 42.69; H, 4.53; N, 24.66; S, 11.37%.

DBD-hydrazone of propionaldehyde was prepared as described previously [8]: mp 167–168°C.

2.4. Fluorescence spectra of DBD-hydrazones

Each hydrazone was dissolved in acetonitrile or aqueous acetonitrile to give a 10^{-3} *M* stock solution. For the measurement of fluorescence spectra, each stock solution was diluted with the same solvent to give a concentration of $1 \cdot 10^{-7}$ *M* for the DBD-hydrazones of acetaldehyde and propionaldehyde, and $1 \cdot 10^{-8}$ *M* for the DBD-hydrazone of formaldehyde.

2.5. Labeling procedure for DBD-hydrazones

To 20 μl of sample solution in acetonitrile were subsequently added 20 μl of 1.0% TFA in acetonitrile and 40 μl of 10 m*M* DBD-H and mixed well. After standing for 30 min at room temperature, a 20-μl aliquot of the mixture was injected onto the HPLC system.

2.6. Subjects and blood specimens

Samples were obtained from seven healthy volunteers (mean age 23.4, range 21–34 years). Venous blood was collected in EDTA Vacutainer

tubes and centrifuged at 1500 *g* for 20 min at 4°C. The supernatant was immediately treated or frozen and stored at −20°C.

2.7. Determination of acetaldehyde in human blood plasma

To 50 μl of plasma in a 1.5-ml plastic tube were added 10 μl of aqueous propionaldehyde solution as internal standard (I.S.) (or aqueous acetaldehyde solution for a recovery test), 40 μl of 10 m*M* DBD-H in acetonitrile, and 20 μl of 1% TFA in acetonitrile. After vortex-mixing for 30 s, the mixture was left for 30 min at room temperature. After centrifugation at 400 *g* for 10 min (4°C), the upper layer was passed through a membrane filter (0.45 μm), and a 20-μl aliquot of the filtrate was injected onto the HPLC system.

Aqueous acetaldehyde solutions as well as the aqueous I.S. solution were prepared by diluting the stock solution in acetonitrile [$(1.1–22.0)\cdot 10^{-2}$ *M* for acetaldehyde and $5.62\cdot 10^{-2}$ *M* for I.S.] 500-fold with water.

3. Results and discussion

3.1. Fluorescence spectra of DBD-H derivatives of aldehydes

Fluorescence spectra of three synthetic standard DBD-hydrazones were measured in acetonitrile or aqueous acetonitrile to select the wavelengths for the fluorescence detector of the HPLC system. As shown in Table 1, these hydrazones have an excitation maximum and an emission maximum at 425–435 nm and 535–545 nm in acetonitrile, respectively. In acetonitrile–water (46:54, v/v), used as eluent in this study, both wavelengths showed red shifts of 15–35 nm for excitation and 5–25 nm for emission. Consequently, we selected 445 and 560 nm as excitation and emission wavelengths, respectively. The relative fluorescence intensity (RFI) of DBD-hydrazone of acetaldehyde in acetonitrile was the largest; the RFI in aqueous acetonitrile was lower than that in acetonitrile. This suggested that a larger water content in the mobile phase would decrease the peak intensities of the chromatogram of the hydrazones.

3.2. Derivatization conditions

Derivatization of DBD-H with aldehydes was examined according to the previously published method [8] using acetonitrile solutions of formaldehyde, acetaldehyde, propionaldehyde and butylaldehyde. Fig. 1 shows the effect of DBD-H concentration on RFI measured as peak height of the chromatogram; 10 m*M* DBD-H was used in the subsequent experiments. The effect of TFA on the derivatization yield was examined over the range 0–5%; the largest and almost constant RFI was obtained for a TFA concentration of >1%.

The reaction of DBD-H with aldehydes was very rapid and hydrazones are immediately formed at room temperature except for formaldehyde (Fig. 2); the derivatization reaction was

Table 1
Fluorescence spectra and relative intensities of synthetic standard DBD-hydrazones in acetonitrile and aqueous acetonitrile

Aldehyde for DBD-hydrazone	Fluorescence spectrum (λ_{max}, nm)					
	Acetonitrile			Acetonitrile–water (46:54, v/v)		
	λ_{ex}	λ_{em}	RFI[a]	λ_{ex}	λ_{em}	RFI[a]
Formaldehyde	425	535	96.3	460	540	6.9
Acetaldehyde	435	545	100	450	565	10.8
Propionaldehyde	430	545	88.9	445	570	5.0

[a] RFI = relative fluorescence intensity; RFI of DBD-H hydrazone with CH_3CHO was arbitrarily taken as 100.
Sample: DBD-hydrazone with HCHO (0.05 μ*M*), CH_3CHO (0.1 μ*M*), C_2H_5CHO (0.1 μ*M*) in acetonitrile.

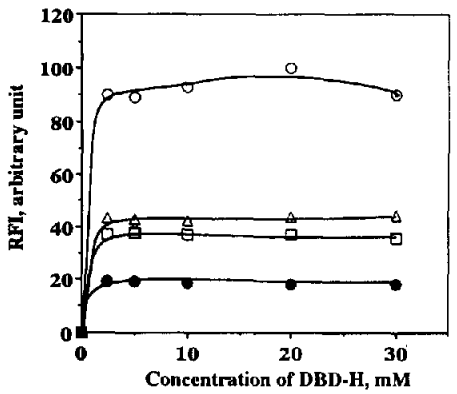

Fig. 1. Effect of DBD-H concentration on RFI. Sample (concentration): (○) HCHO (20.4 μM); (△) CH_3CHO (28.6 μM); (□) C_2H_5CHO (40.8 μM); (●) $CH_3CH_2CH_2CHO$ (41.2 μM). TFA: 1.0%.

performed at room temperature for 30 min. At room temperature the DBD-hydrazones were stable for at least 10 h after derivatization under protection from light; the stability of the DBD-hydrazone of formaldehyde was slightly lower than those of the other hydrazones.

3.3. HPLC conditions

HPLC separation of DBD-hydrazones of several kinds of aldehydes was examined on a reversed-phase analytical column (SP-120-ODS, Daiso). As eluent aqueous acetonitrile was used; a preliminary experiment showed that acetonitrile–water (46:54, v/v) gave good separation of the DBD-hydrazones of short chain aliphatic aldehydes (Fig. 3). As shown in Fig. 3, a small peak corresponding to that of the DBD-hydrazone of formaldehyde or acetaldehyde was obtained with the reagent blank; these peaks probably originated from environmental aldehydes and could not be eliminated. By using the above eluent, DBD-hydrazones of several other kinds of aldehydes could be separated. Table 2 shows the retention times and RFIs for each hydrazone. Among these, hydrazones of aromatic aldehydes, i.e. cinnamaldehyde, *p*-hydroxybenzaldehyde and *p*-dimethylaminobenzaldehyde and *o*-phthalaldehyde, showed substantially lower RFIs than those of the aliphatic aldehydes. The DBD-hydrazone of glutaraldehyde gave two peaks at retention times of 43 and 50 min; the later peak showed a large RFI in spite of its longer retention time. This result

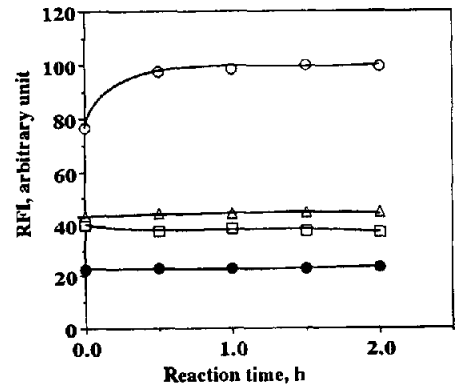

Fig. 2. Effect of reaction time at room temperature on RFI. Sample (concentration): (○) HCHO (20.4 μM); (△) CH_3CHO (28.6 μM); (□) C_2H_5CHO (40.8 μM); (●) $CH_3CH_2CH_2CHO$ (41.2 μM). DBD-H: 10 mM, TFA: 1.0%.

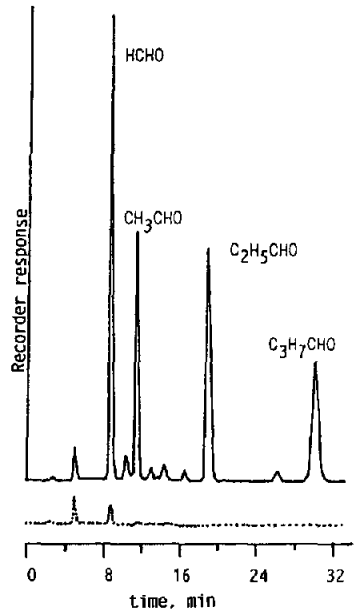

Fig. 3. Chromatogram of DBD-hydrazones with four short-chain aliphatic aldehydes. Sample concentration: 102 μM (HCHO); 143 μM (CH_3CHO), 204 μM (C_2H_5CHO); 206 μM ($CH_3CH_2CH_2CHO$). Eluent: acetonitrile–water (46:54, v/v). Chromatogram for the reagent blank is represented by the dotted line.

Table 2
Retention times and RFIs of DBD-hydrazones with several kinds of aldehyde

Aldehyde[a]	t_R (min)	RFI[b]
Monoaldehydes		
Formaldehyde	9	376
Acetaldehyde	11	153
Propionaldehyde	18	100
Butylaldehyde	30	43.1
Furfural	18	5.4
Cinnamaldehyde	64	4.2
p-Hydroxybenzaldehyde	13	2.7
p-Dimethylaminobenzaldehyde	63	0.012
Dialdehydes		
Malondialdehyde	25, 34	26.1(t_R = 25), 3.8(t_R = 34)
Glutaraldehyde	43, 50	5.5(t_R = 43), 64.5(t_R = 50)
Terephthalaldehyde	30, 120	1.2(t_R = 30), 2.5(t_R = 120)

[a] Sample concentration: 20 μM (formaldehyde, acetaldehyde, propionaldehyde), 40 μM (butylaldehyde, glutaraldehyde), 100 μM (malondialdehyde), 200 μM (furfural, cinnamaldehyde), 400 μM (*p*-hydroxybenzaldehyde), 500 μM (terephthalaldehyde), 2000 μM (*p*-dimethylaminobenzaldehyde).
[b] RFI (as relative peak height) of DBD-hydrazone with propionaldehyde was arbitrarily taken as 100.

suggests that DBD-H should be the preferable labeling reagent for a high-sensitive determination of glutaraldehyde.

3.4. Calibration curve, detection limit and precision

Calibration curves for four aldehydes were prepared; linear relationships were obtained over the range of 2.0–102 μM for formaldehyde ($y = 0.778x + 1.68$, $r = 0.998$), 2.8–280 μM for acetaldehyde ($y = 0.297x - 0.24$, $r = 1.000$), 4.1–408 μM for propionaldehyde ($y = 0.197x + 0.01$, $r = 1.000$) and 4.1–412 μM for butylaldehyde ($y = 0.103x + 0.04$, $r = 1.000$); where y is the relative peak height (cm) at a detector sensitivity of high × 1, and x is the sample concentration (μM) in acetonitrile. Detection limits at a signal-to-noise ratio of 3 were 0.08, 0.2, 0.3 and 0.6 μM for formaldehyde, acetaldehyde, propionaldehyde and butylaldehyde, respectively. For synthetic standard hydrazones, the detection limits were 0.04 (formaldehyde), 0.048 (acetaldehyde) and 0.092 (propionaldehyde) μM. The reproducibility was examined with five samples containing known concentration of the aldehydes in acetonitrile; the relative standard deviations (R.S.D.) were 2.6% (20.4 μM, formaldehyde), 1.9% (28.6 μM, acetaldehyde), 2.0% (40.8 μM, propionaldehyde) and 3.5% (41.2 μM butylaldehyde).

3.5. Determination of acetaldehyde in blood

As a simple method is preferable for the determination of acetaldehyde in blood: direct derivatization of free plasma acetaldehyde with DBD-H was examined without any pretreatment. Working curves were prepared with 50 μl of spiked plasma by an internal standard method; propionaldehyde was used as an I.S. When aldehyde solutions in acetonitrile were added to plasma, the recoveries seemed to be lower than those obtained with aqueous aldehyde solutions. Thus, we used aqueous acetaldehyde or I.S. solution for the determination of acetaldehyde in plasma. A linear relationship was obtained up to at least 87.6 μM of acetaldehyde in plasma ($y = 0.071x + 0.032$, $r = 1.000$), where y is the peak-height ratio of sample to I.S., and x is the sample concentration (μM) in plasma. The lower detection limit was 0.3 μM (signal-to-noise ratio of 2). The sensitivity is comparable to that of a gas chromatographic method (1 μM) [9] or HPLC-

UV (0.45 μM) [6], but lower than that of HPLC with fluorometric detection (0.2 μM [10], 0.1 μM [4]). The reproducibility of the method was examined with six spiked plasma samples; the R.S.D. was 6.4% for plasma spiked with 24.6 μM of acetaldehyde. The percentage recovery of acetaldehyde was calculated from the slopes of calibration curves obtained from standard solutions and spiked plasma according to our previous method [11]; the recovery was 102%.

As an application of the developed method, acetaldehyde levels were determined in healthy volunteers 30 min after ingestion of ethanol (180 ml per person as sake, 15–16% ethanol). Representative chromatograms are shown in Fig. 4; the peak corresponding to the DBD-hydrazone of acetaldehyde significantly increased after ingestion of ethanol. The aldehyde levels for the seven healthy volunteers were 0.89 ± 0.41 μM and 29.5 ± 13.6 μM before and 30 min after ingestion of ethanol, respectively. The control acetaldehyde levels were two-times larger than those reported by Lucas et al. [6] (0.41 ± 0.13 μM), and lower than those by Pezzoli et al. [5] (12.2 ± 1.7 μM). The aldehyde levels measured 30 min after ingestion were comparable with those reported by Palmer and Jenkins [3] (ca. 20 μM), but were 10 times larger than those reported by Lucas et al. [6]; this may be partly caused by differences in amount of ethanol ingested, but the true reason is still unclear.

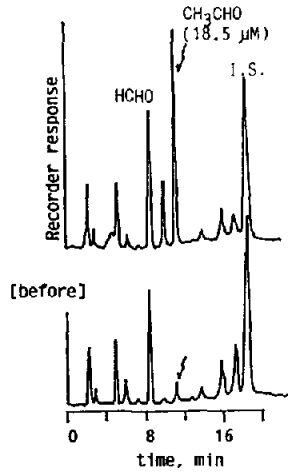

Fig. 4. Chromatogram of plasma before and 30 min after ingestion of ethanol. I.S.: 22.5 μM in plasma. Eluent: acetonitrile–water (46:54, v/v).

4. Conclusions

DBD-H was shown to be a useful fluorescent derivatization reagent for aldehydes in HPLC analysis. As the proposed method is very simple and needs no pretreatment for the determination of free plasma acetaldehyde, it might be useful for biomedical or pharmaceutical studies. An HPLC method with peroxyoxalate chemiluminescence detection for aldehydes using DBD-H is under investigation to increase the sensitivity. The detailed will be reported elsewhere.

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