ELSEVIER

Journal of Chromatography A, 695 (1995) 11–18

JOURNAL OF CHROMATOGRAPHY A

Determination of aldehydes by high-performance liquid chromatography with fluorescence detection after labelling with 4-(2-carbazoylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole

Toshimasa Toyo'oka[a,*], Yi-Ming Liu[b]

[a]*Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422, Japan*

[b]*Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan*

First received 19 August 1994; revised manuscript received 15 November 1994; accepted 15 November 1994

Abstract

The utility of the fluorescent labelling reagent 4-(2-carbazoylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-ProCZ) for the determination of aldehydes was evaluated. The labelling conditions were optimized with model aldehydes (benzaldehyde and *n*-butanal). Under the relatively mild reaction conditions of 65°C for 10 min in acidic medium, all the aldehydes tested were quantitatively derivatized with the reagent to yield stable and highly fluorescent hydrazone derivatives. The maximum excitation (ca. 450 nm) and emission (ca. 540 nm) wavelengths were essentially the same for all the aldehydes. The fluorescence intensity was substantially affected by the solvents in the medium, being higher in organic than protic solvents. However, pH in the range 2.7–11.5 had little effect on the fluorescence properties. The derivatives obtained from six aliphatic aldehydes with DBD-ProCZ were completely separated by reversed-phase liquid chromatography with aqueous acetonitrile. The on-column detection limit (signal-to-noise ratio = 3) with fluorescence detection is at the sub-picomole level.

1. Introduction

Fluorescence detection is the method of choice in numerous determinations of target materials at their real-life levels. The recent upsurge of interest in this area has resulted in many new fluorescence labelling reagents, as documented by the review literature [1,2]. A number of such reagents, e.g., 6,7-dimethoxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-yl-propionohydrazide (DMEQ-hydrazide) [3], fluoren-9-yl-methoxycarbonylhydrazine (Fmoc-hydrazine) [4], anthracenecarboxylic acid hydrazides [5], O-(anthrylmethyl)hydroxylamines [5], 4-hydrazino-7-substituted-benzoxadiazoles [6,7] and 5-hydrazino-N,N-dimethylnaphthalene-1-sulfonamide (Dns-hydrazine) [8–11], have been developed for the determination by high-performance liquid chromatography (HPLC) of carbonyl compounds such as aldehydes and ketones. Many of the reagents for labelling of aldehydes and ketones possess a hydrazino group ($-NHNH_2$) as

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)01130-3

the reactive site [3–13]. These reagents have been applied to the determination of carbonyl compounds at trace levels. However, reliable derivatization procedures based on novel labelling reagents that offer high sensitivity, high selectivity and good fluorescence properties in addition to good stabilities of the reagents and the derivatives are still needed.

Recently, we have developed the benzoxadiazole-based fluorescence labelling reagents 4 - (2 - carbazoylpyrrolidin - 1 - yl) - 7 - (N,N - dimethylaminosulfonyl)-2,1,3-benzoxadiazole [(R)-(+)- and (S)-(−)-DBD-ProCZ] and 4-(2-carbazoylpyrrolidin - 1 - yl) - 7 - nitro - 2,1,3 - benzoxadiazole [(R)-(+)- and (S)-(−)-NBD-ProCZ] for the precolumn derivatization of carbonyl compounds for HPLC analysis [14]. These reagents have been successfully applied to the stereoselective resolution of several racemic ketones by reversed-phase and/or normal-phase chromatography. This paper deals with the precolumn derivatization of achiral aldehydes with DBD-ProCZ and the separation and fluorescence detection of the derivatives by reversed-phase HPLC.

2. Experimental

2.1. Materials and reagents

4-(2-Carbazoylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-ProCZ) was synthesized as described previously [14]. Benzaldehyde, *n*-butanal, *n*-pentanal, *n*-hexanal, *n*-heptanal, *n*-octanal and *n*-nonanal were purchased from Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), acetonitrile, methanol, ethanol and water were of HPLC grade (Wako, Osaka, Japan). All other chemicals were of analytical-reagent grade and were used as received.

2.2. Apparatus

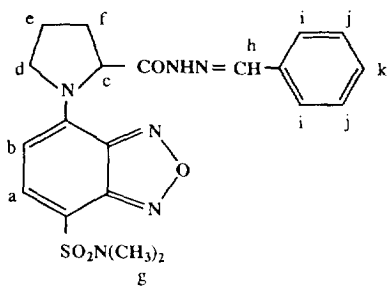
Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian (Palo Alto, CA, USA) Jemini-300 instrument at 300 MHz using tetramethylsilane (0.00 ppm) as the internal standard. For describing NMR characteristics, the following abbreviations are used: s = singlet, bs = broad singlet, d = doublet and m = multiplet. Mass spectrometry (MS) was carried out on a JEOL (Tokyo, Japan) DX-300 instrument [70 eV, electron-impact (EI) ionization]. For measurement of excitation and emission spectra, a Shimadzu (Kyoto, Japan) RF-5000 spectrofluorimeter with a 1-cm quartz cell was employed without spectral correction. Melting points (m.p.) were measured with a Yanagimoto (Tokyo, Japan) micro melting point apparatus.

2.3. HPLC

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 autoinjector (Shimadzu). The analytical column was an Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm) (GL Sciences, Tokyo, Japan). The column was maintained at 40°C with a CTO-10AC column oven (Shimadzu). A Shimadzu RF-550 fluorescence monitor equipped with a 12-μl flow cell was used for the detection of the derivatives. The excitation and emission wavelengths were fixed at 450 and 540 nm, respectively. The peak areas obtained from the fluorescence monitor were computed with a C-R7A Chromatopac (Shimadzu). All mobile phases were degassed with a DGU-3A on-line degasser (Shimadzu). The flow-rate of the eluent was 1.0 ml/min.

2.4. Synthesis of benzaldehyde derivative with DBD-ProCZ

A 2% solution of TCA in acetonitrile (0.5 ml) was added to a mixture of benzaldehyde (21 mg, 0.2 mmol) and DBD-ProCZ (14 mg, 0.04 mmol) dissolved in 9 ml of acetonitrile. After stirring at room temperature for 30 min, the solution was heated at 50°C for 15 min. The solvent in the reaction mixture was evaporated under reduced pressure. Acetonitrile (1 ml) and water (30 ml) were added to the remaining residues, then the



Benzaldehyde derivative

excess amount of benzaldehyde was extracted with light petroleum (b.p. 30–60°C) (20 ml). Benzaldehyde labelled with DBD-ProCZ in the aqueous layer was extracted three times with ethyl acetate (25 ml). The combined ethyl acetate extract was evaporated in vacuo and the residue was recrystallized from acetonitrile as orange crystals, m.p. ca. 130°C, yield 16 mg (90%). NMR (ppm) in $CDCl_3$, 9.52 (1H, bs, h), 7.88 (2H, m, i). 7.74 (1H, d, a + k), 7.46 (2H, m, j), 6.07 (1H, m, b + c), 3.7–4.1 (2H, bs, d), 2.44 (2H, m, f), 2.23 (2H, m, e), 2.81 (6H, s, g); EI-MS, m/z 442 (M^+).

2.5. Labelling procedure for aldehydes and HPLC separation

A 50-μl volume of 0.5% TCA in acetonitrile was added to a mixture of 50 μl of DBD-ProCZ (1 mM) in acetonitrile and 50 μl of aldehydes (5–50 μM) in water–acetonitrile (7:3). After heating at 65°C for 10 min, the reaction solution was cooled in ice–water to stop the reaction. An aliquot (5 μl) of the derivatization solution was subjected to HPLC. The reagent blanks without aldehydes were also treated in the same manner.

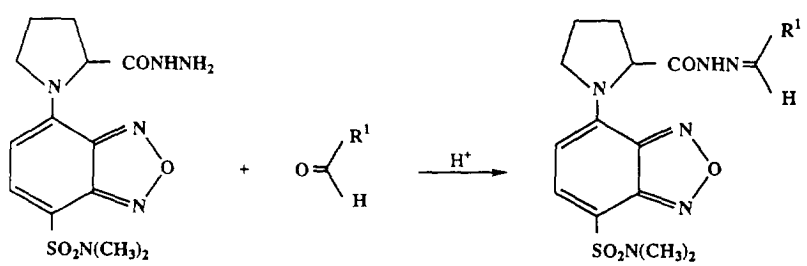
2.6. Fluorescence properties

Stock standard solutions (1 mM each) of the reagent (DBD-ProCZ) and its benzaldehyde derivative were prepared in acetonitrile. For studies of the effects of organic solvents or pH on the fluorescence properties, 50-μl portions of the stock standard solutions were added to 2 ml of the organic solvents or aqueous solutions at various pHs (2.7–11.5). The fluorescence spectra and fluorescence intensities were measured with a spectrofluorimeter within 5 min after preparation of the solutions.

3. Results and discussion

3.1. Fluorescence properties of the reagent and the derivatives

Fig. 1 shows the labelling reaction of aldehydes with DBD-ProCZ. The fluorescence properties of the reagent and its benzaldehyde derivative in various solvents are shown in Table 1. The excitation maxima of these compounds were essentially the same in protic, aprotic and organic solvents, whereas the emission maxima were shifted towards the short-wavelength region



DBD-ProCZ Hydrazone Derivative

Fig. 1. Reaction of DBD-ProCZ with aldehydes.

Table 1
Effect of solvent on the fluorescence properties of the reagent and benzaldehyde derivative

Solvent	λ_{max} (nm)		FI[a]	
	Excitation	Emission	DBD-ProCZ	Derivative
Water	450	550	5.5	4.7
Dimethyl sulfoxide	451	541	49	145
Methanol	451	541	92	90
Acetonitrile	450	535	195	220
Acetone	451	531	390	290
Benzene	450	522	260	310
Ethyl acetate	450	525	250	350

[a] FI = Fluorescence intensity.

with increasing hydrophobicity of the medium. The fluorescence intensities (FI) of the reagent and the derivative were higher in organic solvents such as benzene and ethyl acetate than protic solvents. Although the FI of the reagent in acetone was higher than that of the derivative (390 versus 290), the reason is not obvious. The excitation and emission wavelengths were stable over a wide pH range (2.7–11.5) (λ_{max}: excitation 450 nm and emission 550 nm). Table 2 shows the FI at various pHs. The FI was relatively stronger in neutral and slightly alkaline solutions. The lower intensity at pH 11.5 does not appear to be due to decomposition, but to changes in ionization, because the intensity recovered on changing the pH from 11.5 to 4.0. This suggests that the derivative exhibits good stability over a wide pH range. As essentially the same fluorescence properties were observed for the reagent and its derivatives, it is likely that the fluorescence is due to the fluorophore of the benzofurazan moiety.

Table 2
Effect of pH on the fluorescence properties of the reagent and benzaldehyde derivative

pH	FI[a]	
	DBD-ProCZ	Derivative[b]
2.7	280	280
3.5	290	290
4.7	280	310
5.5	290	330
6.7	290	350
7.7	310	400
8.7	305	405
9.5	330	400
10.4	330	360
11.5	290 (290)[c]	200 (290)[c]

[a] FI = Fluorescence intensity.
[b] Maximum wavelengths of the derivative at all pHs tested are ca. 450 nm (excitation) and ca. 550 nm (emission).
[c] FI data in parentheses are the values after addition of dilute HCl to change the pH from 11.5 to 4.0.

3.2. Optimization of the derivatization

The labelling reaction is affected by various parameters, such as the concentration of the reagent, the pH of the reaction solution and the reaction temperature. Therefore, the derivatization conditions were optimized using benzaldehyde and *n*-butanal, which were selected as the representative aromatic and aliphatic aldehydes, respectively. The labelling reactions of carbonyl compounds with hydrazines such as DBD-ProCZ are usually accelerated by an acid in the medium to yield the corresponding hydrazone derivatives via addition to the carbonyl group followed by dehydration.

Initially, the relative yields of hydrazone derivatives with various acid catalysts were studied by reversed-phase HPLC. Essentially the same yields of the derivatives were obtained with the four acids tested (acetic acid, hydrochloric acid, TFA and TCA). No reaction was observed in the

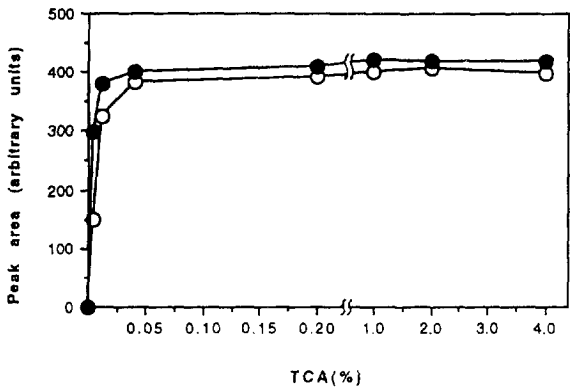


Fig. 2. Effect of TCA concentration on the tagging reaction. ○ = *n*-Butanal; ● = benzaldehyde.

absence of the catalyst. TCA was selected as the derivatization catalyst for subsequent experiments. With respect to the concentration, a small amount of TCA provided quantitative yields of the derivatives, as shown in Fig. 2. The yields were almost the same in the concentration range 0.05–4%. Some unknown peaks appeared with TCA concentrations higher than 5%.

The solvent in the reaction mixture also affected the labelling reaction. As illustrated in Fig. 3, the yields of the derivatives were highest in acetonitrile and lowest in DMF. The labelling reactions in benzene and tetrahydrofuran were not investigated because the reagent has poor solubility in these solvents.

The concentration of DBD-ProCZ is critical for the labelling reaction; higher concentrations gave higher reaction yields. Quantitative labelling was achieved with the use of a 20 molar excess of the reagent.

Reaction temperature is also a critical parameter for the derivatization. The effect was tested in acetonitrile–water (7:3) containing 0.05% TCA. As depicted in Fig. 4A, the labelling reaction proceeds under the mild condition of room temperature (ca. 25°C), and the reactions of both aldehydes were complete after 20 min. Judging from the curves in Fig. 4A, the reaction of the aliphatic aldehyde (*n*-butanal) appears to be faster than that of the aromatic aldehyde (benzaldehyde). The derivatization reaction proceeds faster at elevated temperature (65°C). As shown in Fig. 4B, the reaction of benzaldehyde was completed after 5 min with little additional change over 15 min. The same effect was noted with *n*-butanal. The labelling of the

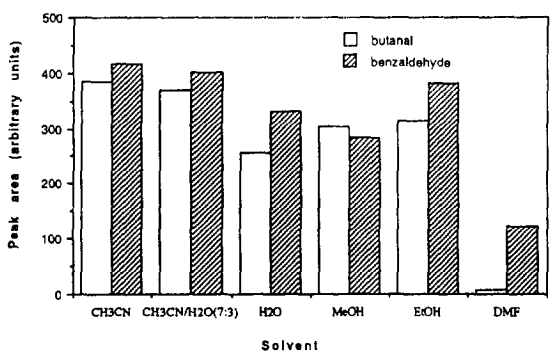


Fig. 3. Effect of solvent on the derivatization.

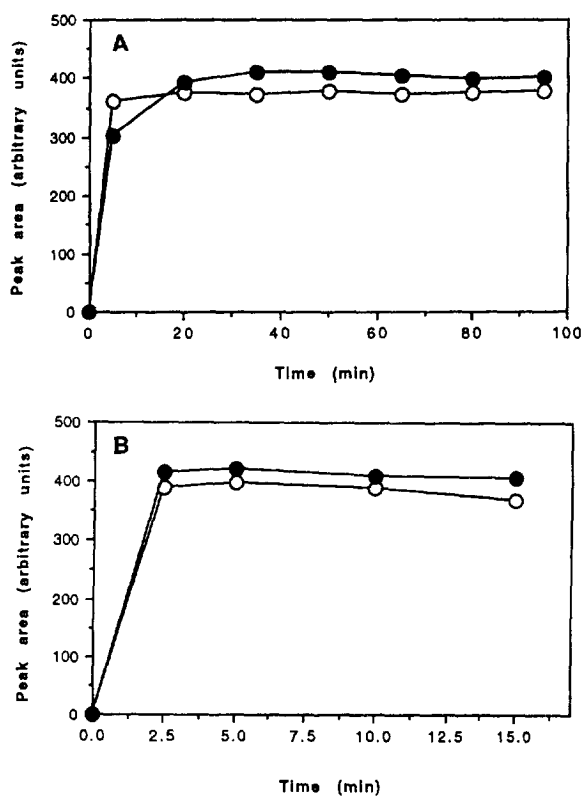


Fig. 4. Derivatization reaction as a function of time: (A) at room temperature; (B) at 65°C. ○ = *n*-Butanal; ● = benzaldehyde.

aldehyde proceeds to greater than 95% as determined by comparison of the peak areas obtained from the authentic derivative and the reaction mixture. Further, no degradation of the derivatives was observed after storage for 90 min at room temperature. The reagent and its derivative in acetonitrile are also stable for at least 3 weeks in a refrigerator at 5°C. Based on these observations, a reaction period of 10 min with a 100 molar excess of DBD-ProCZ at 65°C in water–acetonitrile (7:3) containing 0.05% TCA was selected for the reaction. Under these conditions, a linear relationship was obtained between the peak area of the derivative and the concentration of the aldehyde (1–12 nmol/ml of benzaldehyde or *n*-butanal) in the reaction medium. The slope, intercept and correlation coefficient (γ) were 52.09, 4.35 and 0.999 (benzaldehyde) and 48.38, −4.68 and 0.999 (*n*-butanal), respectively. The relative standard deviations (R.S.D.) for benzaldehyde and *n*-butanal (10 nmol/ml each) with the proposed procedure were 1.0% and 3.1% ($n = 5$), respectively.

3.3. HPLC separation of labelled aliphatic aldehydes

Fig. 5 shows the chromatographic separation of six aliphatic aldehydes (C_4–C_9) after derivatization with DBD-ProCZ. The resulting hydrazone derivatives were completely separated by a reversed-phase ODS column with simple linear gradient elution with aqueous acetonitrile (50–70% for 20 min) with a mobile phase containing 10 m*M* sodium phosphate buffer (pH 7.1). The large peak at ca. 2.5 min is unreacted DBD-ProCZ. The earlier peak is a system peak. The unnumbered peaks appear to be due to impurities in the analytes. The detection limit (signal-to-noise ratio = 3) for both the aliphatic and aromatic aldehydes is in the range 100–150 fmol per 5-μl injection volume (corresponding to 1 pmol of aldehyde). The detection limits were calculated from the peak heights of the derivatives, obtained from ten-fold dilution of the derivatized solution (2 nmol/ml of each aldehyde). The sensitivity is comparable to those of methods using labelling reagents reported by other research groups [3,4,6].

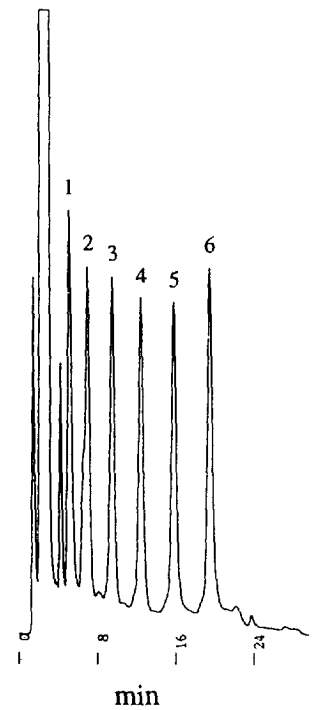


Fig. 5. Chromatographic separation of aliphatic aldehydes labelled with DBD-ProCZ. Peaks: 1 = *n*-butanal; 2 = *n*-pentanal; 3 = *n*-hexanal; 4 = *n*-heptanal; 5 = *n*-octanal; 6 = *n*-nonanal. Each peak except the reagent corresponds to 15 pmol. Eluent A, 10 m*M* phosphate buffer (pH 7.1); eluent B, acetonitrile; linear gradient elution from A–B (50:50) to A–B (30:70) in 20 min. For other HPLC conditions, see Experimental.

3.4. Preliminary application

As an application of the proposed method, the determination of carbonyl compounds in French lotion was tried. The chromatograms shown in Fig. 6 were obtained from derivatized samples of (A) the lotion and, (B) the reagent blank without lotion, and from the underivatized lotion samples with (C) FL detection at 540 nm (excitation at 450 nm) and (D) UV detection at 200 nm. Four main carbonyl components (aldehydes and/or ketones) seem to be present in the lotion (Fig. 6A). No attempt was made to characterize the four carbonyl-containing compounds because

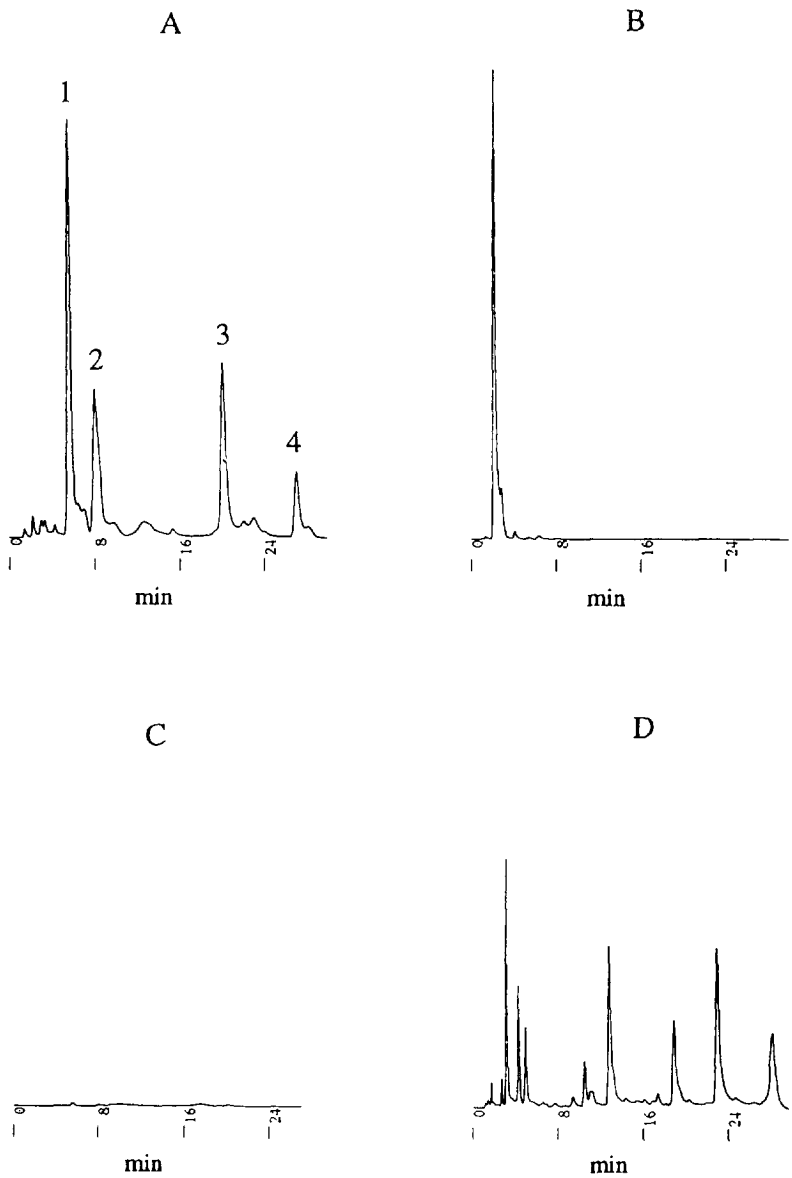


Fig. 6. Chromatogram obtained from a French lotion sample before and after derivatization with DBD-ProCZ. Peaks 1–4 = derivatives of carbonyl compounds. (A) Derivatized solution of the lotion sample; (B) derivatized solution without lotion; (C) underivatized solution with FL detection (excitation at 450 nm and emission at 540 nm); (D) underivatized solution with UV detection at 220 nm. HPLC conditions as in Fig. 5.

this was outside the scope of the present research. Highly sensitive fluorescence detection seems not be necessary in this instance, as the contents in the sample are very high. However, it should be noted that the fluorescence method is much superior to those with UV detection in terms of selectivity, as can be seen by comparing chromatograms C and D in Fig. 6. many interfering peaks arise from the UV-absorbing compounds present in the sample when UV detection is used. However, these compounds usually do not fluoresce at relatively long excitation and

emission wavelengths such as those used is the proposed method. Hence fluorescence detection at long wavelengths is preferable even if highly sensitive detection is not critical for the analysis.

4. Conclusions

The proposed HPLC–fluorescence detection procedure for the trace determination of aliphatic and/or aromatic aldehydes offers advantages in terms of the ease of reaction, the sensitivity of the fluorescence properties of the resulting hydrazone and the stabilities of the reagent and the derivatives. The detection limits are similar to the levels achieved with the methods reported previously [3,4,6]. However, the selectivity seems to be much superior to those of the other methods, because the fluorescence maxima of the derivatives resulting from DBD-ProCZ are longer than those for the derivatives obtained from Fmoc-hydrazine ($\lambda_{ex.}$ 270 nm and $\lambda_{em.}$ 320 nm) [4], DMEQ-hydrazide ($\lambda_{ex.}$ 362 nm and $\lambda_{em.}$ 442 nm) [3] and Dns-hydrazine ($\lambda_{ex.}$ 350 nm and $\lambda_{em.}$ 500 nm) [9]. In addition, the sensitivity may be improved with peroxyoxalate chemiluminescence (CL) detection, as the determination of trace amounts of carboxylic acids after labelling with similar tagging reagents having a DBD moiety was successfully achieved with the CL method [15]. Studies of the application of the method to environmental pollutants and the quality control of drugs are in progress.

Acknowledgement

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

References

[1] H. Lingeman, W.J.M. Underberg, A. Takadate and A. Hulshoff, *J. Liq. Chromatogr.*, 8 (1985) 789.
[2] K. Imai and T. Toyo'oka, in R.W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in High-performance Liquid Chromatography (Journal of Chromatography Library*, Vol. 39A) Elsevier, Amsterdam, 1988, p. 209.
[3] T. Iwata, T. Hirose, M. Nakamura and M. Yamaguchi, *Analyst*, 118 (1993) 517.
[4] R.-E. Zhang, Y. Cao and W.M. Hearn, *Anal. Biochem.*, 195 (1991) 160.
[5] J. Goto, Y. Saisho and T. Nambara, *Anal. Sci.*, 5 (1989) 399.
[6] S. Uzu, S. Kanda, K. Imai, K. Nakashima and S. Akiyama, *Analyst*, 115 (1990) 1477.
[7] G. Gubitz, R. Wintersteiger and R.W. Frei, *J. Liq. Chromatogr.*, 7 (1984) 389.
[8] R. Chayen, R. Duir, S. Gould and A. Harrel, *Anal. Biochem.*, 42 (1971) 283.
[9] M. Takeda, M. Maeda and A. Tsuji, *J. Chromatogr.*, 244 (1982) 347.
[10] K. Mopper and L. Johnson, *J. Chromatogr.*, 256 (1983) 27.
[11] F.M. Eggert and M. Jones, *J. Chromatogr.*, 333 (1985) 123.
[12] K. Muramoto, R. Goto and H. Kamiya, *Anal. Biochem.*, 162 (1987) 435.
[13] J-K. Lin and S.-S. Wu, *Anal. Chem.*, 59 (1987) 1320.
[14] T. Toyo'oka and Y.-M. Liu, *Anal. Proc.*, 31 (1994) 265.
[15] T. Toyo'oka, M. Ishibashi and T. Terao, *J. Chromatogr.*, 627 (1992) 75.