Journal of Chromatography A, 661 (1994) 105–112
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2936

Sensitive determination of N-terminal prolyl peptides by high-performance liquid chromatography with laser-induced fluorescence detection

Toshimasa Toyo'oka*, Mumio Ishibashi and Tadao Terao

Division of Drugs, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158 (Japan)

ABSTRACT

Short-chain peptides with an N-terminal proline (Pro–Gly, Pro–Ile, Pro–Gly–Gly, Pro–Leu–Gly–NH_2, and Pro–Thr–Pro–Ser–NH_2, etc.) were determined by HPLC with laser-induced fluorescence (LIF) detection. The peptides were quantitatively labelled with 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) at 50°C after 1 h in a 0.1 *M* borax (pH 9.3)–acetonitrile mixture. The rate of reaction decreases inversely with the molecular weight of the peptides. The mean value of fluorescent emission of the resulting DBD–peptides and DBD–peptide amides was 573 nm (excitation, 453 nm). The proline peptides, including bioactive peptides such as Pro–Leu–Gly–NH_2 (release inhibitor of melanocyte-stimulating hormone), Pro–Thr–Pro–Ser–NH_2 (IgA_1 proteinase inhibitor) and Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2 [FMRF amide-like (Phe–Met–Arg–Phe–NH_2) neuropeptide], were well separated by reversed-phase HPLC with water–acetonitrile containing 0.1% trifluoroacetic acid (TFA). The acetonitrile concentration in the mobile phase had a profound effect upon the retention times, and the capacity factors (k') were dependent on the hydrophobicity of the peptides. The structure of DBD–Pro–Leu–Gly–NH_2 was identified by LC–atmospheric pressure chemical ionization MS. The chromatographic detection limits ($S/N = 2$) of the peptides with a 15-mW argon-ion laser at 488 nm were in the 6–28 fmol range. The detection limits were improved to 2–5 fmol with a microbore column. The detectability was two orders of magnitude higher than with a conventional fluorescence detector using xenon arc lamp.

INTRODUCTION

A number of fluorogenic labelling reagents, *e.g.* 5-N,N-dimethylaminonaphthalenesulphonyl chloride (Dns-Cl), 4-nitro-7-fluoro-2,1,3-benzoxadiazole (NBD-F), fluorescamine, *o*-phthalaldehyde (OPA) and 2,3-naphthalenedicarboxyaldehyde (NDA), have been developed for the amino functional group [1,2]. Many of these reagents are currently used for the determination of various amines, such as biogenic amines, and amino acids. The following characteristics of the labelling reagent are required: (1) the reagent and its hydrolysate exhibit no or negligible fluorescence, (2) the reagent reacts with target compound selectively and rapidly, (3) the resulting derivative is sufficiently stable and fluoresces strongly and (4) the derivative preferably has fluorescence characteristics at long wavelengths (excitation, more than 400 nm; emission, more than 500 nm).

The fluorescence properties are important for the analysis of real samples, because other interfering substances in the samples that fluoresce at 300–400 nm will prevent accurate and reproducible determinations.

In previous papers, we described the synthesis of fluorescence labelling reagent, 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2, 1, 3-benzoxadiazole (DBD-F) [3], and evaluated the reactivity of the reagent toward thiols and amines [4]. Secondary amines such as proline react more rapidly with the reagent than a primary amine such as alanine, whereas the reaction with thiol compound proceeds quantitatively under the

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E0941-M

selected conditions. A reaction product with alcohols was not found. Therefore, the reactivity of DBD-F with functional groups seems to be in this order: $-SH > -NH > -NH_2 \gg -OH$. Reagents (fluorescamine, OPA and NDA, etc.) [5–7] reported have been used as the labelling reagents for primary amines. Primary and secondary amino groups have been derivatized with fluorescein isothiocyanate, Dns-Cl and NBD-F, etc. [8–10]. Only a few reagents, such as 4-nitro-7-chloro-2,1,3-benzoxadiazole (NBD-Cl), react predominantly with secondary amines [11]. Since the derivatization conditions with NBD-Cl are quite rigorous, the derivatives decompose in the course of the reaction. In contrast, the DBD derivatives obtained from secondary amine are stable in the reaction medium [12,13]. The sensitivity of detection of the derivatives with conventional fluorescence detection using a xenon arc lamp is limited to sub-pmol level [4]. To achieve better sensitivity, excitation by a laser source has recently been developed. Laser-induced fluorescence (LIF) detection offers some definite potential advantages over conventional light sources [14,15], *i.e.* production of a very high photon flux (high excitation energy), improvement of the signal-to-noise ratios and the possibility of an accurate positioning and focusing of the beam. The purpose of present paper is highly sensitive determination of N-terminal prolyl peptides including bioactive amides by HPLC with LIF detection.

EXPERIMENTAL

Materials and reagents

DBD-F was obtained from Tokyo Kasei (Tokyo, Japan). L-Proline (Pro), hydroxy-L-proline (OH-Pro), L-prolyl-glycine (Pro–Gly), L-prolyl-glycyl-glycine (Pro–Gly–Gly), L-prolyl-L-leucine (Pro–Leu), L-prolyl-L-isoleucine (Pro–Ile), L-prolyl-L-leucyl-glycine (Pro–Leu–Gly), L-prolyl-L-leucyl-glycinamide (Pro–Leu–Gly–NH_2) (release inhibitor of melanocyte-stimulating hormone) [16], L-prolyl-L-threonyl-L-prolyl-L-serinamide (Pro–Thr–Pro–Ser–NH_2) (IgA_1 proteinase inhibitor) [17] and L-prolyl-L-asparaginic acidyl-L-valyl-L-asparaginic acidyl-L-histidyl-L-valyl-L-phenylalanyl-L-leucyl-L-arginyl-L-phenylalaninamide (Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2) [FMRF amide-like (Phe–Met–Arg–Phe–NH_2) neuropeptide] [18] were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) was also used as received (Dojindo, Kumamoto, Japan). Trifluoroacetic acid (TFA), acetonitrile and water were of HPLC grade (Wako, Tokyo, Japan). All other chemicals were of analytical-reagent grade and were used without further purification.

HPLC

The high-performance liquid chromatograph consisted of two LC-9A pumps (Shimadzu) and an SCL-6B system controller (Shimadzu). Sample solutions were injected by an SIL-6B autoinjector (Shimadzu). The analytical column was an Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm) (GL Sciences, Tokyo, Japan) and a TSK-gel PTH-Pak (250 × 2.0 mm I.D., 5 μm) (Tosoh, Tokyo, Japan). The column was maintained at 40°C with a 655A-52 column oven (Hitachi, Tokyo, Japan). A Shimadzu RF-550 fluorescence monitor equipped with a 12-μl flow cell was employed for the detection. The wavelengths of excitation and emission were fixed at 450 and 560 nm, respectively. A Tosoh LF-8010 monitor, equipped with a 5-μl flow cell and an interference filter at 540 ± 20 nm, was employed for the LIF detection. The peak areas obtained from the fluorescence and LIF monitors were determined with a C-R4A Chromatopac (Shimadzu). All mobile phases were degassed with an on-line degasser (DGU-3A, Shimadzu). The flow-rate of the eluent for the conventional column and microbore column was 1.0 ml/min and 0.2 ml/min, respectively.

LC–MS

The apparatus used was a Hitachi L-6200 HPLC instrument equipped with an Inertsil ODS-2 (150 × 4.6 mm I.D., 5 μm) column and connected to a Hitachi M-1000 mass spectrometer [atmospheric pressure chemical ionization (APCI) system] [19,20]. The vaporizer temperature was 280°C, and the drift voltage was 160 V. The separation of DBD–Pro–Leu–Gly–NH_2, DBD-OH and DBD-F was carried out with a

mobile phase of water–acetonitrile (7:3) containing 0.1% TFA at a flow-rate of 1.0 ml/min.

Time course of the reaction of peptide or peptide-amide with DBD-F

A 0.1-ml aliquot of DBD-F (10 m*M*) in acetonitrile [or dimethyl formamide (DMF)] and 0.2 ml of peptide or peptide-amide (2.5 μM) in 0.1 *M* borax (pH 9.3) containing (or without) 1 m*M* Na_2EDTA were mixed in a 1.5-ml mini-vial (GL Science). The vials were tightly capped and heated at 50°C for 4 h. At fixed time intervals, one vial was taken out from dry heat block, and cooled in ice-water (0–5°C). Then a 627 μl of water were added to 40 μl of the reaction mixture. An aliquot (10 μl, corresponding to 1 pmol) of the diluted solution was automatically injected into the Inertsil ODS-2 column, and the LIF peak area of the resulting diastereomer was determined with an integrator. The reagent blanks without peptide or peptide-amide were treated in the same manner.

For the fluorescent spectra measurements, 50 μl of the reaction solution before dilution were injected onto the column, monitored with a conventional fluorescence detector, and the peak corresponding to the derivative was collected from outlet of the detector (*ca.* 2-ml portion).

Separation of peptide and peptide-amide derivatives

To 0.5 ml (low nmol to pmol) of a test solution in 0.1 *M* borax containing 1 m*M* Na_2EDTA placed into a 1.5-ml mini-vial were added 0.25 ml of 10 m*M* DBD-F in acetonitrile. The vials were tightly capped and heated at 50°C for 1 h in the dark. After cooling in ice-water (0–5°C), a suitable volume of water was added to the reaction mixture, an aliquot of the diluted solution was chromatographed and the fluorescence peak area of the derivative obtained from the LIF detector was determined with an integrator. The reagent blanks without peptide or peptide-amide were treated in the same manner.

RESULTS AND DISCUSSION

Fluorescence characteristics of the derivatives

Fig. 1 shows the labelling reaction of proline with DBD-F. DBD-F is not itself fluorescent; however, the derivatives with amines fluoresce at relatively long wavelengths. Initially, the fluorescence excitation and emission maxima of DBD derivatives were determined in acetonitrile–water containing 0.1% TFA, which has been widely used as an eluent for peptide separations by reversed-phase HPLC. As shown in Table I, the maximal wavelengths of excitation and emission were *ca.* 453 nm and *ca.* 573 nm, respectively. The results suggest that the fluorescence is independent of the peptides or amides. Although the excitation maximum (453 nm) is not well suited to the light emission of argon ion at 488 nm, it is possible to achieve sensitive detection of the peptides and their amides with the laser source.

Derivatization

The reactivity to N-terminal prolyl peptides was compared with N-terminal prolyl peptide-amides in aqueous acetonitrile (pH 9.3) containing 1 m*M* Na_2EDTA at 50°C. Figs. 2 and 3 show the time course of the reaction of the peptides and the peptide-amides, respectively. Judging from the curves in Fig. 2, the reaction rate seems to be dependent on the molecular

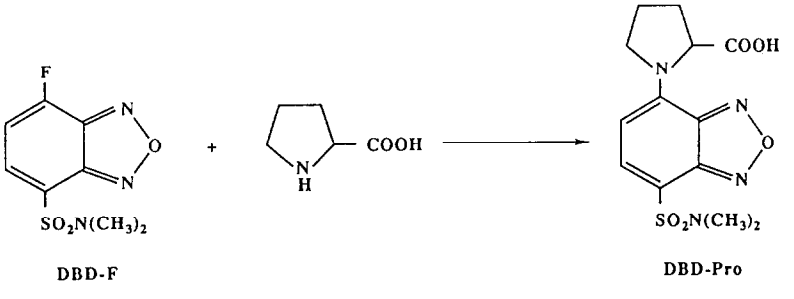

Fig. 1. The reaction of DBD-F and proline.

TABLE I

FLUORESCENCE PROPERTIES OF DBD–PEPTIDES IN WATER–ACETONITRILE (7:3) CONTAINING 0.1% TFA

Derivative	λ_{max} (nm)	
	Excitation	Emission
Pro	453	574
Pro–Gly	452	574
Pro–Gly–Gly	453	576
Pro–Leu–Gly	452	572
Pro–Leu	454	572
Pro–Ile	454	573
Pro–Leu–Gly–NH_2	453	573
Pro–Thr–Pro–Ser–NH_2	453	573
Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2	452	568

mass, Pro > Pro–Gly > Pro–Gly–Gly. A similar phenomenon was also observed in the comparison of peptide-amides (Pro–Thr–Pro–Ser–NH_2 *versus* Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2) (Fig. 3). However, the rates to Pro–Leu–Gly–NH_2 and Pro–Thr–Pro–Ser–NH_2 are essentially the same. Therefore, the rate seems to be a function not only of molecular weight, but also of hydrophilicity. Differences in the reactivities of peptides and peptide-amides were not observed. Even though a 2000-fold excess of the reagent relative to the peptides and peptide-amides was added to the reaction solution, a peak due to DBD-OH was not apparent on the chromatograms obtained with the LIF detector. The negligible fluorescence of DBD-OH is a predominant consideration for the determination of peptides and peptide-amides because a large amount of the labeling reagent must be added to the sample solution to drive the reaction to completion. As described in a previous paper [21], when the derivatization reaction of amino acids with NBD-F, which has a similar structure to DBD-F, is carried out without EDTA in the medium, the yield of some derivatives is reduced compared with the reaction in the presence of EDTA. EDTA probably inhibits intramolecular and/or intermolecular chirate formation between amino acids, such as Asp and His, and metals such as Cu^{2+} and Ni^{2+}. Therefore, a small amount of EDTA was added to the reaction medium to scavenge the metal ions. Fig. 4 shows that the reaction rates without EDTA are slightly slower than the rates in the presence of EDTA. The results suggest that the contribution of metal ions to the reaction of peptides and/or peptide-amides is not significant. To improve the solubility of peptides and/or peptide-amides in biological specimens, the reaction in DMF–water was substituted for the acetonitrile–water mixture. As depicted in Fig. 5, the required heating time is slightly reduced;

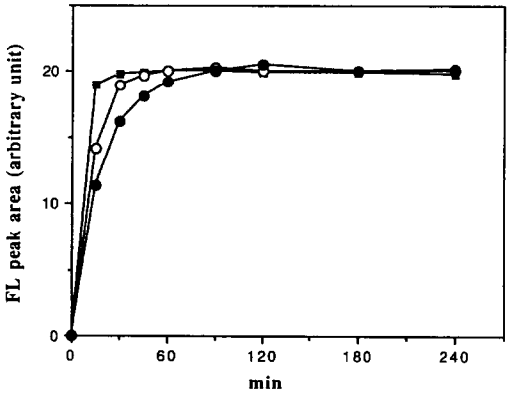

Fig. 2. Time course of the derivatization reaction of N-terminal prolyl peptides with DBD-F. ■ = Pro; ○ = Pro–Gly; ● = Pro–Gly–Gly. Eluent, water–acetonitrile (7:3) containing 0.1% TFA; fluorescence (FL) detection, 560 nm (excitation at 450 nm). Other HPLC conditions are given in the Experimental section.

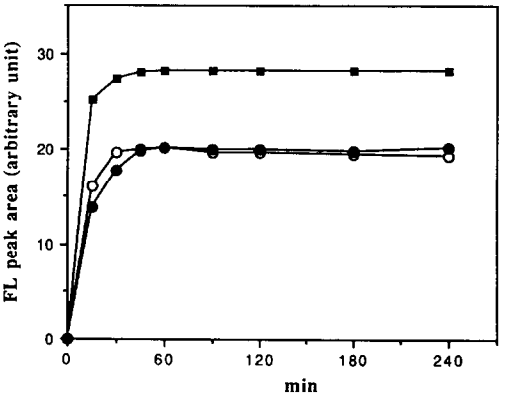

Fig. 3. Time course of the derivatization reaction of N-terminal prolyl peptide-amides with DBD-F. ○ = Pro–Thr–Pro–Ser–NH_2; ■ = Pro–Leu–Gly–NH_2; ● = Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2. HPLC conditions as in Fig. 2.

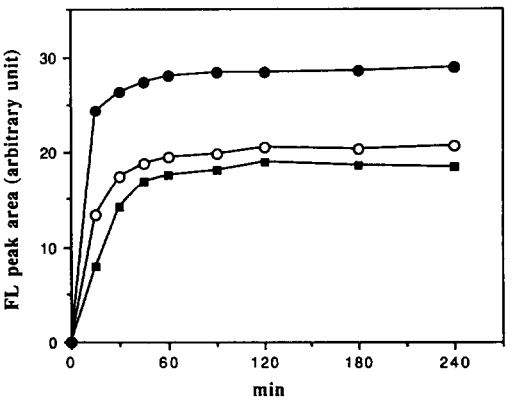

Fig. 4. Time course for the derivatization reaction of N-terminal prolyl peptides and the amides with DBD-F in acetonitrile–water without Na_2EDTA. ○ = Pro–Gly; ■ = Pro–Leu–Gly; ● = Pro–Leu–Gly–NH_2. HPLC conditions as in Fig. 2.

however, the curves obtained from the reaction in aqueous DMF are almost superimposable on the curves obtained from the reaction in aqueous acetonitrile. Therefore, both solvents can be used as reaction medium by combination with alkaline solution. Thus, 50°C for 1 h in aqueous acetonitrile (pH 9.3) containing 1 m*M* Na_2EDTA is recommended for the derivatization of peptides and the corresponding amides.

Structural elucidation of the derivatives with LC–MS

The structure of DBD–Pro–Leu–Gly–NH_2 was identified by LC–APCI-MS. Figs. 6 and 7 show the mass chromatogram and mass spectra of the derivative, the hydrolysate (DBD-OH) and the reagent (DBD-F). From the analysis with a UV detection at 220 nm, the derivative, DBD-OH and DBD-F elute at 8.5, 6.0 and 15.5 min, respectively. In the APCI-MS system, quasimolecular ions $[M + H]^+$ of the compounds were observed as base peaks: $m/z = 510$ for DBD–Pro–Leu–Gly–NH_2, $m/z = 244$ for DBD-OH and $m/z = 246$ for DBD-F.

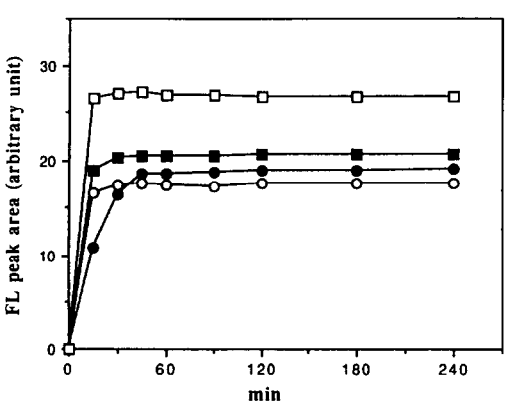

Fig. 5. Time course for the derivatization reaction of N-terminal prolyl peptides and the amides with DBD-F in DMF–water. ■ = Pro; ○ = Pro–Gly; ● = Pro–Leu–Gly; □ = Pro–Leu–Gly–NH_2. HPLC conditions as in Fig. 2.

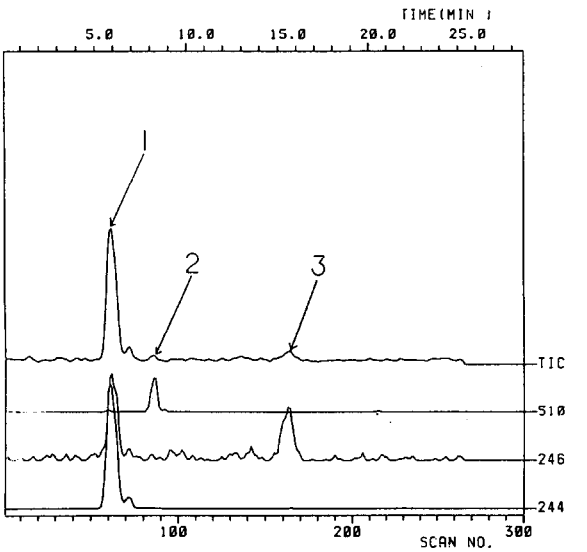

Fig. 6. Mass chromatograms of DBD derivatives. 1 = DBD-OH; 2 = DBD–Pro–Leu–Gly-NH_2; 3 = DBD-F. TIC = Total ion current.

HPLC separation of the derivatives

The separation of the DBD derivatives obtained from peptides and peptide-amides with an N-terminal proline was studied with reversed-phase chromatography with aqueous acetonitrile containing 0.1% TFA. Figs. 8 and 9 show the correlation between acetonitrile concentration in the mobile phase and capacity factor (k'). The acetonitrile concentration in the eluent influences the retention times of the derivatives and larger k' values are obtained with a lower concentration of acetonitrile. In the case of the peptides, C-terminal glycyl peptides elute faster than leucine or isoleucine. Therefore, the elution order is dependent on the kind of C-terminal amino acid, but independent of molecular mass.

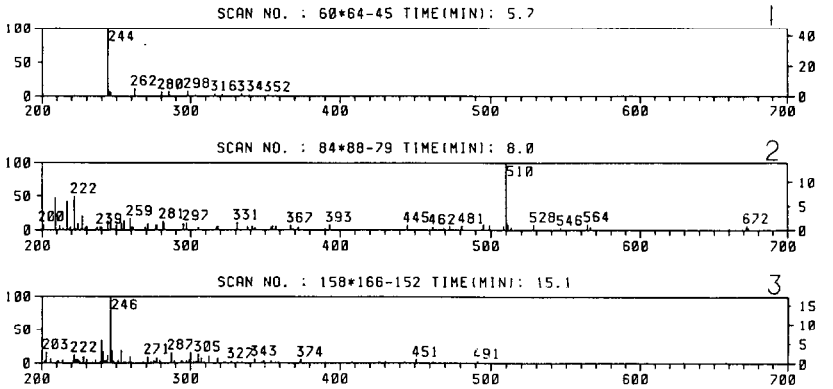

Fig. 7. Mass spectra scanned at the peak tops of the mass chromatograms (see Fig. 6).

In other words, the order might be defined by the hydrophobicity of the peptides. Similar results were obtained with the separation of peptide-amides (Fig. 9). A high degree of dependency on acetonitrile concentration was observed with decapeptide-amide (Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2). The peptide-amide with high hydrophobicity such as Pro–Leu–Gly–NH_2 is strongly influenced by the concentration of acetonitrile in the mobile phase; however, a hydrophilic peptide-amide, such Pro–Thr–Pro–Ser–NH_2, is not significantly changed over a relatively wide concentration range. Consequently, the acetonitrile concentration should be carefully controlled to obtain precise and accurate results.

Figs. 10 and 11 show typical chromatograms of DBD derivatives obtained from peptides and peptide-amides. Six DBD–peptides and three DBD–peptide amides were completely separated by isocratic elution with a water–acetonitrile mixture in the presence of 0.1% TFA. The detection limits (signal-to-noise ratio of 2) of DBD–peptides, calculated from the chromatogram, were from 7 fmol (Pro–Gly–Gly) to 28 fmol (Pro–Leu), while those of DBD–peptide-amides were between 6 fmol (Pro–Thr–Pro–Ser–NH_2) and 83 fmol (Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2). The detection limits were about two orders of magnitude lower than those with conventional fluorescence detection. The detectability improved with the use of

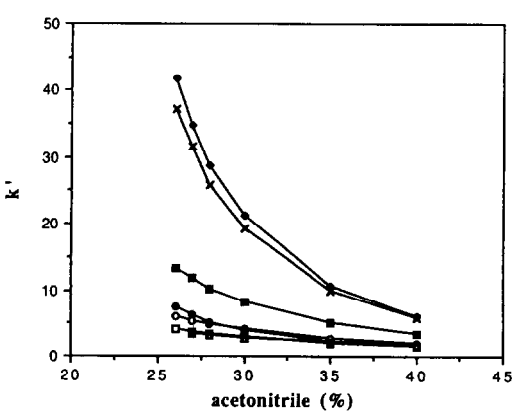

Fig. 8. The effect of acetonitrile concentration in the mobile phase on the retention time of DBD–peptides. ■ = Pro; ○ = Pro–Gly; □ = Pro–Gly–Gly; ◆ = Pro–Leu; × = Pro–Ile; ● = Pro–Leu–Gly; △ = OH-Pro. HPLC conditions as in Fig. 2, except for the eluent composition.

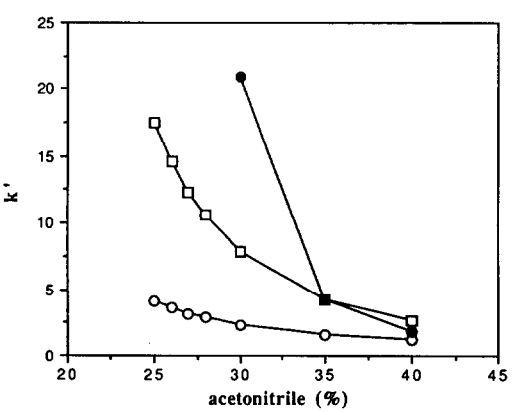

Fig. 9. The effect of acetonitrile concentration in the mobile phase on the retention time of DBD–peptide amides. □ = Pro–Leu–Gly–NH_2; ○ = Pro–Thr–Pro–Ser–NH_2; ● = Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2. HPLC conditions as in Fig. 2, except for the eluent composition.

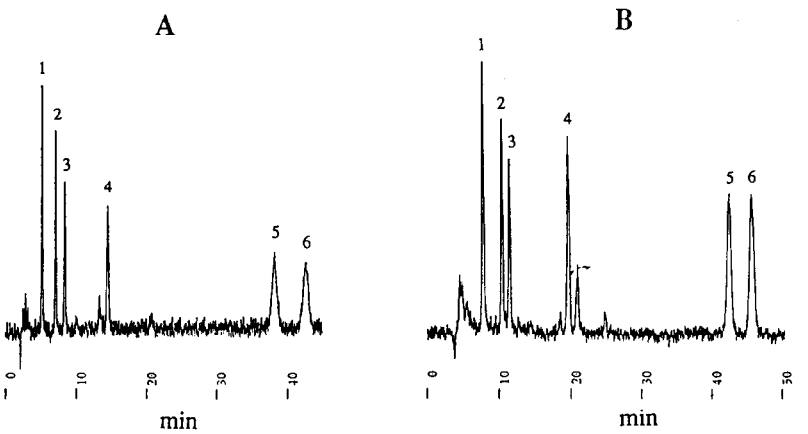

Fig. 10. Chromatograms of DBD–peptides using a reversed-phase column with LIF detection. (A) Inertsil ODS-2, each peak corresponding to 100 fmol. (B) TSK-gel PTH-Pak, each peak corresponding to 50 fmol. Peaks: 1 = Pro–Gly–Gly; 2 = Pro–Gly; 3 = Pro–Leu–Gly; 4 = Pro, 5 = Pro–Ile; 6 = Pro–Leu. Eluents: A = water–acetonitrile (74:26) containing 0.1% TFA; B = water–acetonitrile (70:30) containing 0.1% TFA. Flow-rate: A = 1.0 ml/min; B = 0.2 ml/min. Detection: A = 15 mW; B = 10 mW. Other HPLC conditions are given in the Experimental section.

a microbore column of 2.0 mm diameter relative to a conventional column with a 4.6 mm diameter. The chromatograms obtained with the microbore column are shown in Figs. 10B and 11B. The detection limits of DBD–peptides were 2.7 fmol (Pro–Gly–Gly), 3.4 fmol (Pro–Gly), 4.3 fmol (Pro–Leu–Gly), 3.6 fmol (Pro), 5.1 fmol (Pro–Ile) and 5.1 fmol (Pro–Leu), whereas those of DBD–peptide amides were 2.6 fmol (Pro–Thr–Pro–Ser–NH_2), 2.3 fmol (Pro–Leu–Gly–NH_2) and 14 fmol (Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2). The low sensitivity for the decapeptideamide might be due to the quantum yield of the derivative.

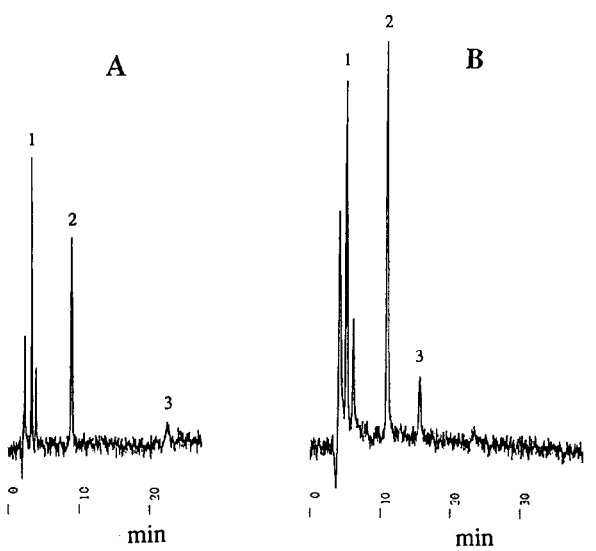

Fig. 11. Chromatograms of DBD–peptide amides using a reversed-phase column with LIF detection. (A) Inertsil ODS-2, each peak corresponding to 100 fmol. (B) TSK-gel PTH-Pak, each peak corresponding to 50 fmol. Peaks: 1 = Pro–Thr–Gly–Ser–NH_2; 2 = Pro–Leu–Gly–NH_2; 3 = Pro–Asp–Val–Asp–His–Val–Phe–Leu–Arg–Phe–NH_2. Eluents: A = water–acetonitrile (70:30) containing 0.1% TFA; B = water–acetonitrile (65:35) containing 0.1% TFA. Flow-rate: A = 1.0 ml/min; B = 0.2 ml/min. Detection: 15 mW. Other HPLC conditions are given in the Experimental section.

In conclusion, ultra trace analysis at the low fmol level is possible with DBD-F using the recommended procedure, even though the excitation and emission wavelengths do not match the light emission of the laser source and the interference filter wavelengths. The sensitivity may be improved with a suitable interference filter (570 nm) and an increase in the output laser power. Hence, the proposed procedure using DBD-F may be applicable to sensitive determination of peptides and/or peptide amides in biological specimens. Further study is under way.

ACKNOWLEDGEMENTS

The authors thank Mr. T. Takahashi, Hitachi Instruments Engineering Co., for LC–MS measurement. Thanks are also due to Dr. C.R.

Warner, Food and Drug Administration, Washington, DC, USA for reviewing the manuscipt.

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. Toyo'oka, T. Suzuki, Y. Saito, S. Uzu and K. Imai, *Analyst*, 114 (1989) 413.
4 T. Toyo'oka, T. Suzuki, Y. Saito, S. Uzu and K. Imai, *Analyst*, 114 (1989) 1233.
5 P. Bohlen, S. Stein, J. Stone and S. Udenfriend, *Anal. Biochem.*, 67 (1975) 438.
6 P.E. Hare, *Methods Enzymol.*, 47 (1977) 3.
7 B.K. Matuszewsky, R.S. Givens, K. Srinivasachar, R.G. Carlson and T. Higuchi, *Anal. Chem.*, 59 (1987) 1102.
8 K. Muramoto, H. Kamiya and H. Kawauchi, *Anal. Biochem.*, 141 (1984) 446.
9 C. De Jong, G.J. Hughes, E. Van Wieringen and K.J. Wilson, *J. Chromatogr.*, 241 (1982) 345.
10 Y. Watanabe and K. Imai, *J. Chromatogr.*, 239 (1982) 723.
11 L. Johnson, S. Lagerkvist, P. Lindroth, M. Ahnoff and K. Martinsson, *Anal. Chem.*, 54 (1982) 939.
12 T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu and K. Imai, *J. Chromatogr.*, 588 (1991) 61.
13 T. Toyo'oka, M. Ishibashi and T. Terao, *Analyst*, 117 (1992) 727.
14 E.S. Yeung and M.J. Sepaniak, *Anal. Chem.*, 52 (1980) 1465A.
15 R.B. Green, *Anal. Chem.*, 55 (1983) 20A.
16 R. Walter, R.F. Ritzmann, H.N. Bhargava and L.B. Flexner, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 518.
17 S.G. Wood, M. Lynch, A.G. Plaut and J. Burton, *J. Med. Chem.*, 32 (1989) 2407.
18 S. Robb, L.C. Packman and P.D. Evans, *Biochem. Biophys. Res. Commun.*, 160 (1989) 850.
19 M. Sakairi and H. Kambara, *Anal. Chem.*, 60 (1988) 774.
20 H. Kodama, H. Nakamura, K. Sugahara and Y. Numajiri, *J. Chromatogr.*, 527 (1990) 279.
21 H. Miyano, T. Toyo'oka, K. Imai and T. Nakajima, *Anal. Biochem.*, 150 (1985) 125.