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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TYROSINE-CONTAINING PEPTIDES BY PRE-COLUMN DERIVATIZATION INVOLVING FORMYLATION FOLLOWED BY FLUORESCENCE REACTION WITH 1,2-DIAMINO-4,5-DIMETHOXYBENZENE

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## SUMMARY

A pre-column fluorescence derivatization method is described for the high-performance liquid chromatographic determination of tyrosine-containing peptides. A tyrosyl residue in the peptide is first formylated in an alkaline medium in the presence of chloroform, and the resulting aldehyde is then converted into a fluorescent derivative by reaction with 1,2-diamino-4,5-dimethoxybenzene. The derivative is separated on a reversed-phase column (LiChrosorb RP-18) by isocratic elution with an aqueous acetonitrile-containing potassium chloride–hydrochloric acid buffer (pH 2.2) and sodium 1-hexanesulphonate. The method is selective and fairly sensitive; the lower limits of detection for the tyrosine-containing peptides tested are in the range 3.4–26.2 pmol in a 100-$\mu$l injection volume.

## INTRODUCTION

Several physiologically active peptides containing a tyrosyl residue in their molecules, such as enkephalins[1] and angiotensins[2], occur in body fluids. Many methods, involving radioimmunoassay[3,4], bioassay[5,6] or high-performance liquid chromatography (HPLC)[7,8], have been proposed for the determination of the peptides. Radioimmunoassay generally offers a high sensitivity and selectivity for the peptides, but it is difficult to obtain specific antibodies especially for small molecular peptides[3]. Bioassay for peptides such as angiotensins cannot provide good precision and specificity in quantification[5].

On the other hand, HPLC methods can determine various peptides simultaneously, if the method gives complete separation. Hitherto, detection in HPLC has usually been done by either ultraviolet (UV) absorption at wavelengths between 200 and 230 nm[9,10] or post-column on-line fluorescence derivatization with a fluorogenic reagent, such as fluorescamine[11] or *o*-phthalaldehyde[12]. Since these detection methods are not selective for tyrosine-containing peptides, various other peptides in crude samples interfere with the determination of the peptides. Although electrochemical detection has recently introduced for selective determination of tyrosine-

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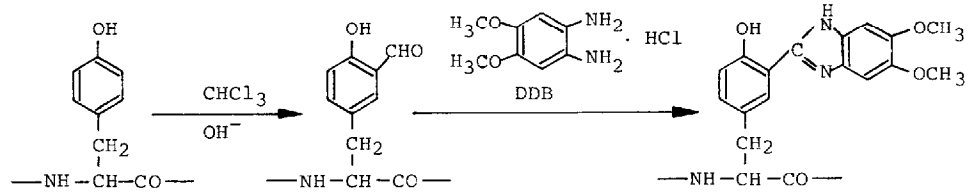
containing peptides by HPLC[13,14], oxidizing and/or reducing components in biological samples should be removed before subjecting the samples to HPLC.

We reported previously a reversed-phase HPLC method with pre-column fluorescence derivatization for selective and sensitive determination of *p*-hydroxybestatin in human serum, which has a tyrosyl moiety in its molecule[15]. This derivatization is based on formylation of the tyrosyl moiety in an alkaline medium by the Reimer–Tiemann reaction[16] and then conversion of the resulting aldehyde into a highly fluorescent derivative with 1,2-diamino-4,5-dimethoxybenzene (DDB), a fluorogenic reagent for aromatic aldehydes, in a weakly acidic solution[17,18] (Scheme 1). This study aims to apply this fluorescence derivatization technique to the selective HPLC determination of tyrosine-containing peptides.




Scheme 1.

## EXPERIMENTAL

### *Chemicals and solutions*

All chemicals were of analytical reagent grade, unless indicated otherwise. Deionized and distilled water was used. The peptides used, Tyr-Gly, Gly-Tyr, Tyr-Arg, Tyr-Phe, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, methionine enkephalin (MEK; Tyr-Gly-Gly-Phe-Met), leucine enkephalin (LEK; Tyr-Gly-Gly-Phe-Leu), angiotensin I (ANG I; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), angiotensin II (ANG II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and angiotensin III (ANG III; Arg-Val-Tyr-Ile-His-Pro-Phe), were purchased from Sigma (St. Louis, MO, U.S.A.). DDB monohydrochloride was obtained from Dojindo (Kumamoto, Japan).

DDB solution (1.3 m*M*) was prepared by dissolving 5.3 mg of DDB monohydrochloride in 20 ml of water. This solution should be used within 2 h. Potassium chloride–hydrochloric acid buffer (0.2 *M*, pH 2.2) was prepared by dissolving 14.9 g of potassium chloride in 950 ml of water, adjusting the pH to 2.2 with concentrated hydrochloric acid and diluting the solution with water to 1.0 l.

### *Apparatus and HPLC conditions*

Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical pathlength, 10 × 10 mm).

The HPLC system consisted of a Toyo Soda 803D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Shimadzu RF 530 fluorescence spectrometer fitted with a

12-$\mu$l flow-cell operating at an emission wavelength of 425 nm and an excitation wavelength of 350 nm. The column was LiChrosorb RP-18 (150 × 4.0 mm I.D.; particle size, 5 $\mu$m; Japan Merck, Tokyo, Japan). The mobile phase was composed of acetonitrile, 0.2 *M* potassium chloride–hydrochloric acid buffer (pH 2.2) and 50 m*M* sodium 1-hexanesulphonate. The flow-rate was 0.8 ml/min throughout. The column temperature was ambient (20–27°C).

*Fluorescence derivatization*

To a 200-$\mu$l portion of peptide solution placed in a test tube (10.5 × 1.2 cm I.D.) were added 100 $\mu$l of chloroform and 50 $\mu$l of 3.0 *M* potassium hydroxide. The mixture was warmed at 60°C for 10 min to formylate the tyrosyl residue. The mixture was cooled in ice-water for *ca.* 1 min. To the mixture, 50 $\mu$l of 14.0 *M* acetic acid and 300 $\mu$l of DDB solution were successively added with cooling in ice-water. The mixture was warmed at 60°C for 18 min to derivatize the formylated residue, and then cooled. A 100-$\mu$l portion of the final reaction mixture was injected into the chromatograph.

## RESULTS AND DISCUSSION

*HPLC separation*

Reversed-phase HPLC is the most powerful method for the separation of intact peptides and it is simple and reproducible[9]. We thus studied reversed-phase HPLC for the separation of the DDB derivatives of tyrosine-containing peptides. The DDB derivatives of di- and tripeptides (Tyr-Gly, Gly-Tyr and Tyr-Gly-Gly) were successfully resolved on LiChrosorb RP-18 column by isocratic elution (Fig. 1). Peak 4 in

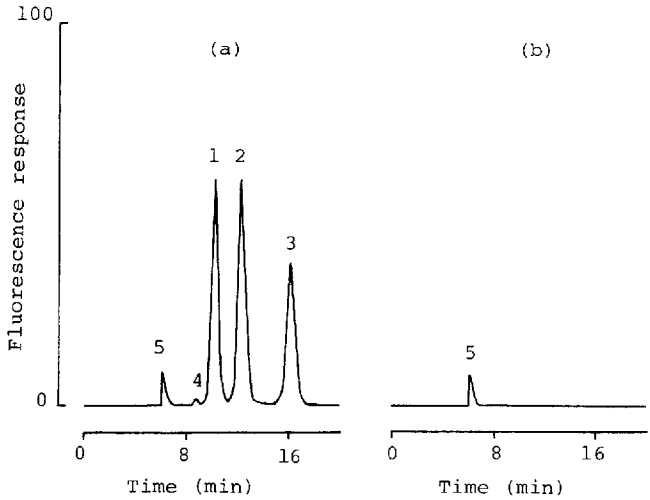



Fig. 1. Chromatograms of (a) a mixture of Tyr-Gly, Gly-Tyr and Tyr-Gly-Gly and (b) reagent blank. A 200-$\mu$l portion of a peptide mixture (10 nmol/ml each) or water for the blank was treated according to the procedure in the Experimental section. HPLC conditions: mobile phase, acetonitrile–0.2 *M* potassium chloride–hydrochloric acid buffer (pH 2.2) 50 m*M* sodium 1-hexanesulphonate (9:31:10). Peaks: 1 = Tyr-Gly-Gly; 2 = Tyr-Gly; 3 = Gly-Tyr; 4 = by-product (tyrosine) from all the peptides tested; 5 = reagent blank.

Fig. 1a was due to a by-product, tyrosine produced from these peptides under the formylation conditions. As shown in Fig. 2, the derivatives from relatively large molecular peptides, MEK, LEK, ANGs I, II and III, were also well resolved on the same column. Although some other by-products were also yielded from these peptides during the formylation (Fig. 2a, peaks 6, 7 and 8), the by-product peaks were separated completely from those for the peptides, and did not disturb the determination of the peptides.

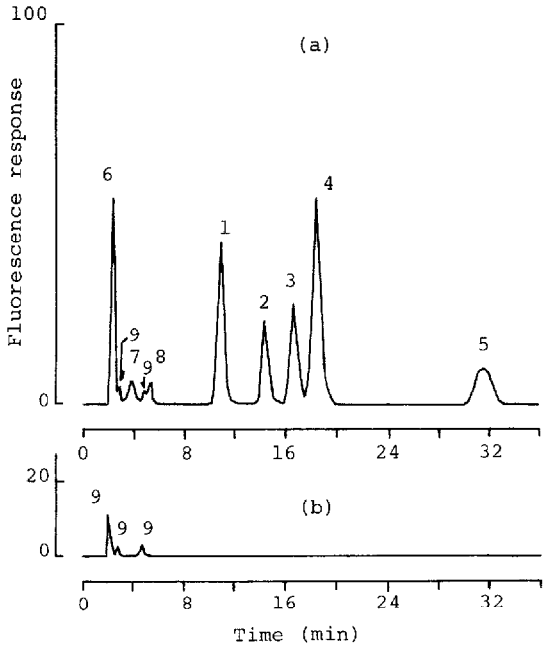



Fig. 2. Chromatograms of (a) a mixture of MEK, LEK, ANGs I, II and III and (b) the reagent blank. A 200-$\mu$l portion of a peptide mixture (20 nmol/ml each) or water for the blank was treated according to the procedure in the Experimental section. HPLC conditions: mobile phase, acetonitrile–0.2 *M* potassium chloride–hydrochloric acid buffer (pH 2.2)–50 m*M* sodium 1-hexanesulphonate (13:32:5). Peaks: 1 = MEK; 2 = ANG II; 3 = ANG III; 4 = LEK; 5 = ANG I; 6 = reagent blank and by-products from all the peptides tested; 7 = by-products from all the peptides tested; 8 = by-product from MEK; 9 = reagent blank.

The elution conditions described are similar to those for conventional HPLC of intact peptides[7,14]. A greater concentration of acetonitrile in the mobile phase was required for the separation of the larger molecular peptides (Figs. 1a and 2a). When methanol was used instead of acetonitrile in the mobile phase, the peaks for the peptides were badly broadened.

Sodium 1-hexanesulphonate in the mobile phase was required as an ion-interaction reagernt for better separation of the DDB derivatives of the peptides. In its absence, the derivatives were eluted faster and could not be separated completely, even when the concentration of acetonitrile in mobile phase was decreased. Sodium 1-pentanesulphonate or sodium 1-heptanesulphonate, both at 50 m*M*, had a similar

effect on the separation for the derivatives. The effect becomes more remarkable as the carbon number of ion-interaction reagent increases.

The retention times of the DDB derivatives increased with decreasing pH of the potassium chloride–hydrochloric acid buffer in the mobile phase in the range 1.0–6.0. The DDB derivatives of the peptides fluoresced most intensely at pH 1.0–3.0. The potassium chloride in the buffer did not have any significant effect on the separation of the peaks at a concentrations in the range 0.1–0.4 *M*; 0.2 *M* potassium chloride–hydrochloric acid buffer (pH 2.2) was employed as a component of the mobile phase. Phosphate buffer (50 m*M*, pH 2.2) or acetate–hydrochloric acid buffer (50 m*M*, pH 2.2) may also be used in place of the potassium chloride–hydrochloric acid buffer.

*Formylation and derivatization*

The formylation of the tyrosine-containing peptides tested (Tyr-Gly, MEK and LEK) proceeded rapidly at temperatures higher than 50°C. The peak heights from the peptides reached maximum and constant values after warming at 60 or 70°C for 9 min or longer. The peak heights of the by-products from the peptides, however, increased as the reaction temperature inversed and also with longer reaction time; 10-min warming at 60°C was employed.

Potassium hydroxide at a concentration higher than 0.6 *M* in the formylation mixture decreased the peak heights from all the peptides, probably because of alkaline hydrolysis of the peptides; 0.6 *M* potassium hydroxide was used to obtain maximum peak heights (Fig. 3).

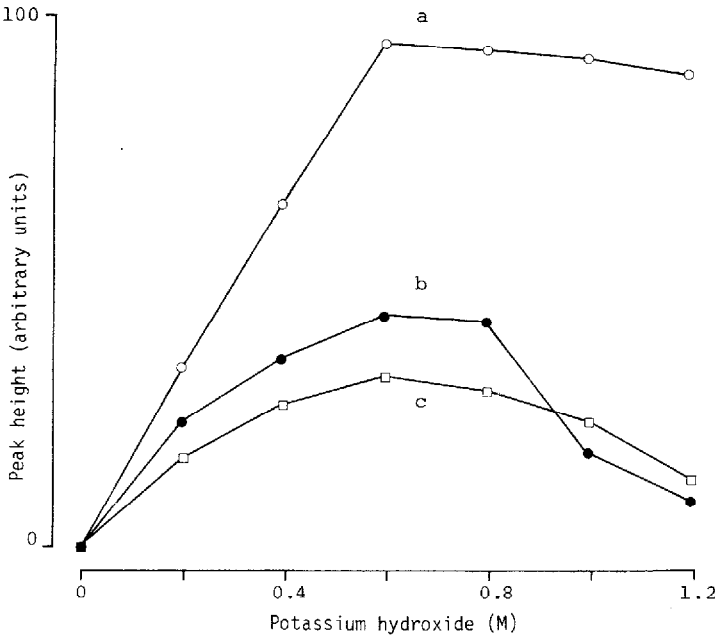



Fig. 3. Effect of potassium hydroxide concentration on the formylation. Portions (200 μl) of peptide solution (10 nmol/ml) were treated by the recommended procedure at various concentrations of potassium hydroxide, and the pH values of the after formylation reaction mixtures were adjusted to 3.3 with 5–17 *M* acetic acid. Curves: (a) Tyr-Gly; (b) LEK; (c) MEK.

Chloroform in a volume range 75–200 μl in the formylation mixture gave maximum and almost constant peak heights for the peptides; a volume of 100 μl was selected for the recommended procedure. The peak heights of the by-products from the peptides were not varied by the amount of chloroform (10–200 μl). Shaking the reaction mixture during the formylation did not affect the peak heights. Excess chloroform was evaporated during the formylation. When a capped test-tube (45 × 12 mm I.D.) was used for the formylation, the remaining chloroform was removed before the fluorescence derivatization with DDB, otherwise the derivatization reaction with DDB was not initiated for 20 min or more.

The fluorescence derivatization with DDB occurred most effectively at pH 3.3 in aqueous acetic acid. At a pH higher than 5.0 or lower than 1.8, the reaction did not proceed. The peak heights of the fluorescent derivatives reached maximum and constant values after warming at 60°C for 15 min or longer; 18-min warming at 60°C was employed as optimum.

Other peptides having no tyrosyl residue in their molecules, such as tuftsin (Thr-Lys-Pro-Arg), kallidin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and substance-P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$), did not provide any fluorescent peaks under the conditions described.

*HPLC determination*

Table I shows the detection limits and the retention times obtained by the present reversed-phase HPLC method for the DDB derivatives from the tyrosine-containing peptides tested, tyrosine and tyramine. Peptides that contain a tyrosyl

TABLE I

DETECTION LIMITS AND RETENTION TIMES OF THE DDB DERIVATIVES OF TYROSINE-CONTAINING PEPTIDES, TYROSINE AND TYRAMINE

| *Compound* | *HPLC conditions A** | | *HPLC conditions B** | |
|---|---|---|---|---|
| | *Detection limit (pmol)* | *Retention time (min)* | *Detection limit (pmol)* | *Retention time (min)* |
| Tyrosine | 3.4 | 8.8 | N.D.** | 2.8 |
| Tyramine | 3.8 | 14.2 | N.D. | 2.8 |
| Tyr-Arg | 4.0 | 13.8 | N.D. | 2.8 |
| Gly-Tyr | 5.1 | 16.3 | N.D. | 2.8 |
| Tyr-Phe | 25.9 | 79.2 | N.D. | 4.3 |
| Tyr-Gly | 3.4 | 12.4 | N.D. | 2.8 |
| Tyr-Gly-Gly | 3.4 | 10.4 | N.D. | 2.8 |
| Tyr-Gly-Gly-Phe | 24.0 | 78.8 | N.D. | 4.2 |
| Methionine enkephalin | – | N.E.*** | 7.6 | 10.9 |
| Leucine enkephalin | – | N.E. | 6.8 | 18.4 |
| Angiotensin I | – | N.E. | 26.2 | 31.5 |
| Angiotensin II | – | N.E. | 11.3 | 14.5 |
| Angiotensin III | – | N.E. | 10.3 | 16.8 |

* HPLC conditions A and B, see Fig. 1 and Fig. 2, respectively.

** N.D. = not determined, because their peaks overlapped with those of the by-products from the peptides and of the reagent blank.

*** N.E. = not eluted.

residue at any position in their molecules can be converted into DDB derivatives, and single fluorescent peaks for these peptides were observed in the chromatograms.

The amounts of the peptides applicable to the derivatization procedure ranged from *ca.* 0.02 to 60 nmol. The calibration graphs were all linear for the relationship between the peak heights and the amounts of the peptides used for the derivatization. The lower limits of detection of the peptides tested are further range 3.4–26.2 pmol in a 100-$\mu$l injection volume at a signal-to-noise ratio of two (Table I). The coefficients of variation were between 1.8 and 2.2%, and 2.0 and 3.0% for the peptides in Figs. 1a and 2a, respectively (300 pmol, $n = 10$ in each case).

The proposed HPLC method is selective and fairly sensitive for tyrosine-containing peptides, and may be applied to the assay of the enzyme activity of enkephalinase[19], renin[20] or angiotensin-converting enzyme[21]; further study is in progress in our laboratory.

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