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FLUORIMETRIC DETERMINATION OF AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A HOLLOW-FIBRE MEMBRANE REACTOR

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of primary amino acids. The method involves separation of primary amino acids on a C_{18} column using sodium heptanesulphonate as an ion-pairing agent, post-column derivatization with *o*-phthalaldehyde and 2-mercaptoethanol introduced into the main flow stream using a sulphonated hollow-fibre membrane reactor immersed in their solutions and fluorimetric detection of derivatives (λ_{ex} = 340 nm, λ_{em} = 450 nm). A higher or similar sensitivity was obtained compared with the conventional post-column derivatization method. The detection limits were 0.2–2.3 pmol at a signal-to-noise ratio of 3. For amounts of 48–110 pmol, the precision was of the order of 1.1–4.0% (relative standard deviation, n = 20).

INTRODUCTION

Since the introduction of *o*-phthalaldehyde (OPA) by Roth[1], various high-performance liquid chromatographic (HPLC) methods have been developed for the assay of amino acids using the reaction with OPA in the presence of a reducing agent such as 2-mercaptoethanol (2-ME) as pre-column[2–23] or post-column[23–40] derivatization. The post-column derivatization method is now being used for the detection system of an amino acid analyzer together with the ninhydrin reaction. The drawback of the method is that it needs one or more pumps for delivering the reagent and mixing units and reactors for the post-column reaction, which lead to an increase in detector noise, band broadening and dilution (which reduce the sensitivity gained through derivatization and deteriorate the ultimately obtainable limit of detection[41,42]).

A passive hollow-fibre membrane reactor was first used in ion chromatography for decreasing the background conductivity, *i.e.*, as a suppressor[43,44]. Recently, passive and active membrane reactors were employed for post-column derivatization in HPLC[42,46–48] and flow injection analysis[41]. They introduce the desired reagents into the main flow stream by use of a concentration gradient and pressure, respectively. These membrane reactors can eliminate or control the problems mentioned above.

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This paper deals with a fluorimetric HPLC method for the determination of primary amino acids using two passive membrane reactors (sulphonated hollow-fibre membranes) for adjustment of the eluent pH and introduction of OPA and 2-ME.

EXPERIMENTAL

Reagents and materials

4-Amino-*n*-butyric acid (4-AB), L-ornithine monohydrochloride (Orn) and sodium heptanesulphonate were obtained from Nakarai Chemicals (Kyoto, Japan), DL-methionine sulphone (Met-S) and taurine (Tau) from Sigma (St. Louis, MO, U.S.A.) and Wako (Osaka, Japan), respectively. Other amino acids (L-form) were obtained from Protein Research Foundation (Minoh, Osaka, Japan). OPA and 2-ME were supplied from Nakarai. A sulphonated hollow-fibre membrane (AFS-2) was obtained from Dionex Co. (Sunnyvale, CA, U.S.A.).

De-ionized, glass-distilled water and distilled methanol were used for the preparations of sample solutions and HPLC eluents.

The OPA reagent was prepared as follows: 600 mg of OPA were dissolved in 5 ml ethanol, and after addition of 1.0 ml of 2-ME the solution was diluted to 100 ml in 0.4 *M* borate buffer solution (pH 9.2).

Chromatography

The experimental set-up is illustrated in Fig. 1: P, a Bip-I pump (Japan Spectroscopic, Tokyo, Japan) for delivering the eluent; I, a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a 100-μl loop for loading of the samples; C, a 150 mm × 4.6 mm I.D. column packed with Develosil ODS-5 (particle size 5 μm, Nomura Chemicals, Seto, Aichi, Japan) for separations; M_1 and M_2, sulphonated hollow-fibre membrane reactors of lengths 30 cm and 1 m, respectively, for post-column reaction; D, an RF-540 spectrofluorophotometer (Shimadzu, Kyoto, Japan) equipped with a 12-μl flow-through cell for detection; R, a C-R3A recorder-integrator (Shimadzu) for recording and integrating chromatographic peaks. The eluents used were as follows: A, 15 m*M* sodium heptanesulphonate–27 m*M* phos-

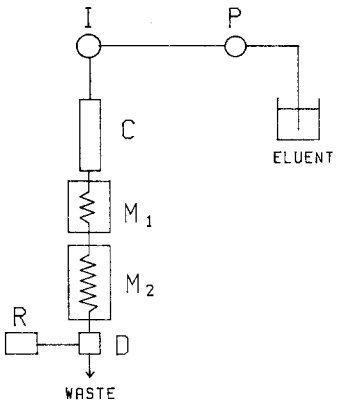

Fig. 1. Experimental set-up used: P = pump; I = injector; C = column; M_1 and M_2 = sulphonated hollow-fibre membrane reactors; D = fluorimetric detector; R = recorder-integrator.

phoric acid–3 m*M* sodium dihydrogenphosphate–methanol (40:40:40:3); B, 15 m*M* sodium heptanesulphonate–27 m*M* phosphoric acid–3 m*M* sodium dihydrogenphosphate–methanol (1:1:1:1.5). The flow-rate was maintained at 0.8 ml/min. The hollow-fibre membrane reactors inserted between the column and the detector were immersed in 100-ml beakers containing 0.2 *M* sodium tetraborate and 0.6 *M* sodium hydroxide solution (M_1), and 6 mg/ml OPA and 1.0% (v/v) 2-ME solution (M_2). The vessels were kept in a water-bath thermostatted at 60°C. Detection was performed with excitation at 340 nm and emission at 450 nm.

Comparison of detection methods

The peak broadening due to the post-column reactor was estimated by the following methods: A, fluorimetric detection (λ_{ex} = 340 nm, λ_{em} = 450 nm) with an open-tubular post-column reactor; B, fluorimetric detection with hollow-fibre membrane reactors. For method A, the additional reaction devices were as follows: a double plunger pump (NP-DX-2; Nihon Seimitu Kagaku, Tokyo, Japan) for delivering the post-column reagent (80 mg OPA and 0.2 ml 2-ME in 100 ml of borate buffer solution) at a flow-rate of 0.8 ml/min; a mixing tee and the reaction coil, 1 m × 0.5 mm I.D. PTFE tube, maintained at 60°C in a thermostatted water-bath for a post-column reaction. In method B the post-column reaction conditions were the same as described above. The eluent pH after the column was 9.3 in both methods. The peak broadening, σ_t, due to the column and the reactor was calculated. The peak broadening due to the column was estimated by direct UV detection of amino acids.

RESULTS AND DISCUSSION

Reaction conditions for the hollow-fibre post-column reactor

Sulphonated hollow-fibre membrane reactors (which have negatively charged membrane matrices) permit inward transport of the uncharged and cationic forms of a molecule due to the existing concentration gradient[41,42]. Preliminary studies revealed that the reaction of amino acids with OPA and 2-ME occurred in a flow system using a sulphonated hollow-fibre membrane as a post-column reactor. Therefore, we tried to develop an HPLC method for the fluorimetric determination of amino acids using a hollow-fibre membrane reactor.

The post-column reaction conditions for amino acids were examined with respect to the various factors affecting the post-column reaction; reaction temperature, pH at reaction, concentrations of OPA and 2-ME and length of the hollow-fibre membrane reactor. Eluents A (for Ala and Asp) and B (for Arg) were delivered at a flow-rate of 0.8 ml/min. A 20-μl portion of a typical amino acid solution (Ala, Asp and Arg) (0.5 μg/ml) was loaded onto the column and the peak height was measured. Two hollow-fibre membrane reactors (M_1 and M_2) were connected in series for adjustment of the eluent to alkaline pH and introduction of OPA and 2-ME, respectively. M_1 (whose length was fixed at 30 cm) and M_2 were immersed in sodium tetraborate and sodium hydroxide solution, and in borate buffer solution (pH 9.2) containing OPA and 2-ME, respectively. The fluorescence intensity increased with increasing reaction temperature from room temperature to 65°C. Thus, the reaction temperature was set at 60°C. To adjust the eluent pH, the concentration of sodium hydroxide was varied from 0.5 to 0.9 *M* at a sodium tetraborate concentration of 0.2

M. As a result, the eluent pH after the column changed from 8.9 to 10.9. Although the fluorescence intensity of other amino acids was scarcely affected by the reaction pH, the optimum reaction pH for Orn was ≈9.3. Thus, the concentration of sodium hydroxide was set to 0.6 *M* (pH 9.3 after the column). Fig. 2 shows the effect of the OPA concentration on the fluorescence intensity, when the concentration of 2-ME was 1.0% and the length of the hollow-fibre reactor (M_2) was 1 m. At ⩾ 6 mg/ml OPA a maximum and constant fluorescence intensity was obtained. The concentration of 2-ME was changed from 0.2 to 1.5% at an OPA concentration of 6 mg/ml (Fig. 3). The effect of 2-ME was not as large as that of OPA. The optimum 2-ME concentration was 1.0%. Fig. 4 shows the effect of the length of the hollow-fibre reactor on the fluorescence intensity, when the concentrations of OPA and 2-ME were 6 mg/ml and 1.0%, respectively. The maximum peak height was obtained at a length of ⩾ 1 m. Thus, the post-column reaction conditions described in Experimental were selected for the assay of amino acids.

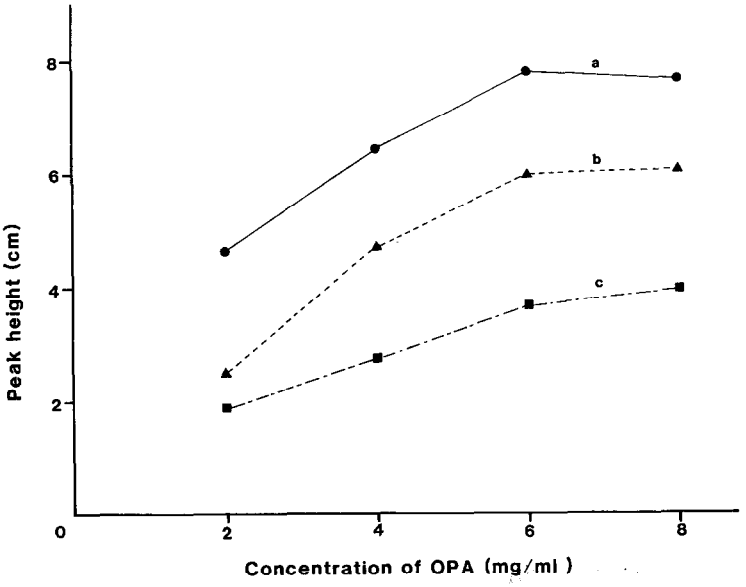

Fig. 2. Effect of the OPA concentration on the fluorescence intensity of amino acids. M_1 (30 cm) was immersed in 0.2 *M* sodium tetraborate and 0.6 *M* sodium hydroxide solution, and M_2 (1 m) in OPA and 1.0% 2-ME solution. The OPA concentration was changed from 2 to 8 mg/ml. A 20-μl portion of a typical amino acid solution (Ala, Asp and Arg) was loaded onto the column, and the peak height was measured. Detection: λ_{ex}, 340 nm; λ_{em}, 450 nm. a, Ala; b, Asp; c, Arg. Amounts injected: Ala, 110 pmol; Asp, 75 pmol; Arg, 48 pmol. Other conditions as in the text.

After 24 h, the decrease in the eluent pH after the column was only about 0.1 unit, while the OPA and 2-ME solution could be used again without decreasing the reactivity (although a decrease in the volume of the solution was observed; this shows that the flux of water occurs together with that of OPA and/or 2-ME).

The concentrations of OPA and 2-ME employed for the conventional post-column reaction of amino acids were 0.8 mg/ml and 0.2%, respectively[24,25]. The

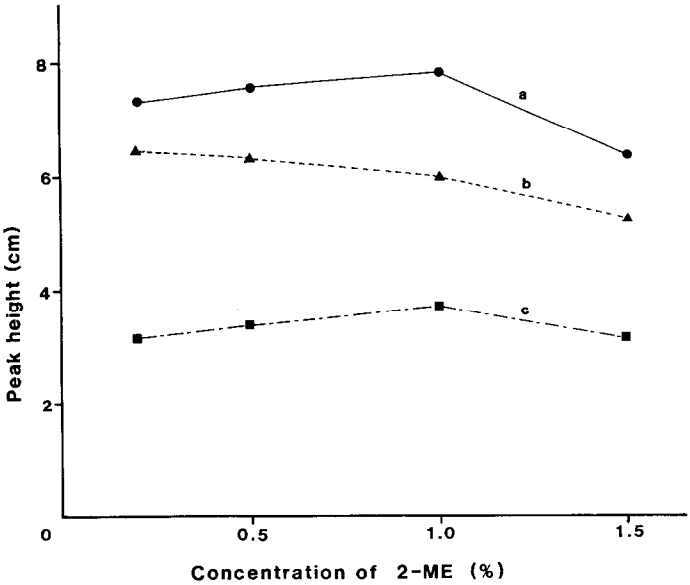

Fig. 3. Effect of the 2-ME concentration on the fluorescence intensity of amino acids. Conditions as in Fig. 2 except that the OPA concentration was 6 mg/ml. The concentration of 2-ME was changed from 0.2 to 1.5%.

proposed method needs 7.5 times the OPA and 5 times the 2-ME concentrations compared with the conventional method. However, the depleting of these reagents was much the same between the proposed and conventionial methods in 1-day.

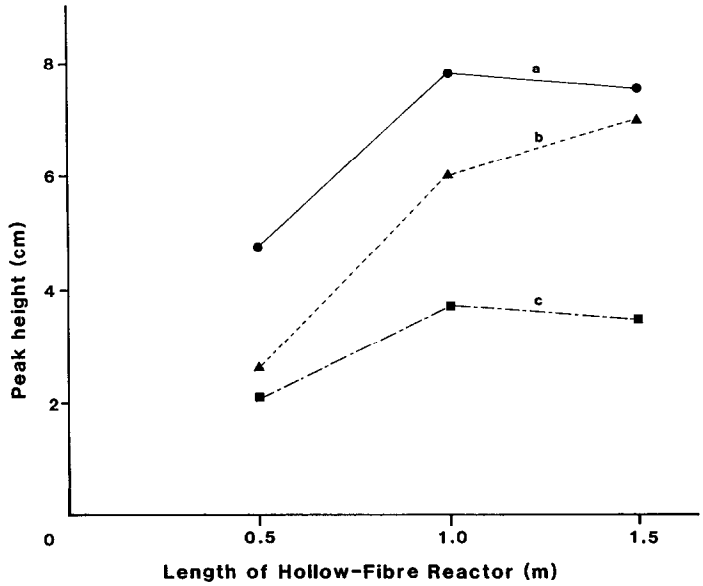

Fig. 4. Effect of the length of the hollow-fibre reactor on the fluorescence intensity of amino acids. Conditions as in Fig. 2 except that the OPA concentration was 6 mg/ml. The length of reactor M_2 was changed from 0.5 to 1.5 m.

Comparison of reactor designs

The separation of amino acids was performed on a C_{18} column using sodium heptanesulphonate as an ion-pairing agent. Fig. 5 shows chromatograms of Tau, Asn, Asp, Met-S, Thr and Ala obtained with the conventional (A) and hollow-fibre membrane reactor (B). Fig. 6 shows corresponding chromatograms of 4-AB, Met, Orn, Arg, Leu and Phe. Table I shows the peak broadening, σ_t, due to the column and the reactor. The hollow-fibre membrane reactor gave the same or higher response, except for Arg, and better resolution, R_s, between Asn and Asp compared with the conventional reactor (with the former, $R_s = 1.24$ and with the latter, $R_s = 0.94$). This shows that the reaction of amino acids with OPA and 2-ME proceeds to much the same extent in both post-column reactors, and that the hollow-fibre reactor has less band broadening than the conventional one. The large peak broadening and low peak height of Arg (which is a basic amino acid) with the hollow-fibre membrane reactor might be due to the interaction of Arg with the sulphonated membrane and/or the outward transport.

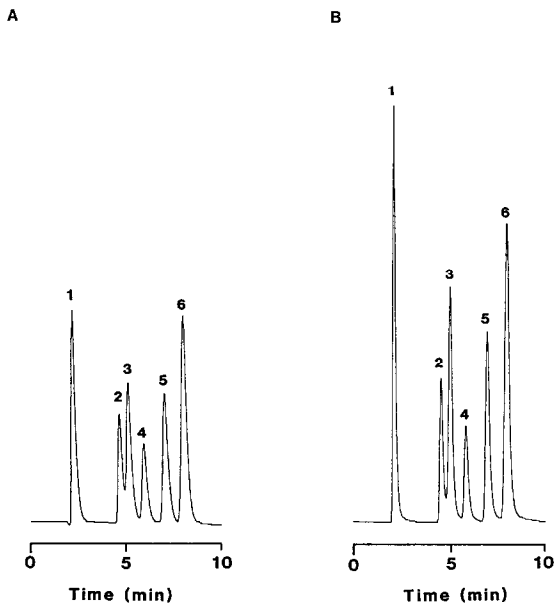

Fig. 5. Chromatograms of Tau (1), Asn (2), Asp (3), Met-S (4), Thr (5) and Ala (6) with the conventional (A) and hollow-fibre membrane reactors (B). Eluent A. Amounts injected: Tau, 80 pmol; Asn, 65 pmol; Asp, 75 pmol; Met-S, 55 pmol; Thr, 85 pmol; Ala, 110 pmol. Other conditions as in the text.

Reproducibility, linearity and detection limits

Table II lists the relative standard deviation (R.S.D.) ($n = 20$) for the measured peak heights of standard amino acids. The results reveal good reproducibility for all amino acids studied. Peak heights were found to be randomly scattered around a mean value; that is, no trends (constant decrease in peak height with time) were observed. This shows that the optimum pH and concentrations of OPA and 2-ME are maintained in spite of continuous depletion of Na^+, OPA and 2-ME. The cali-

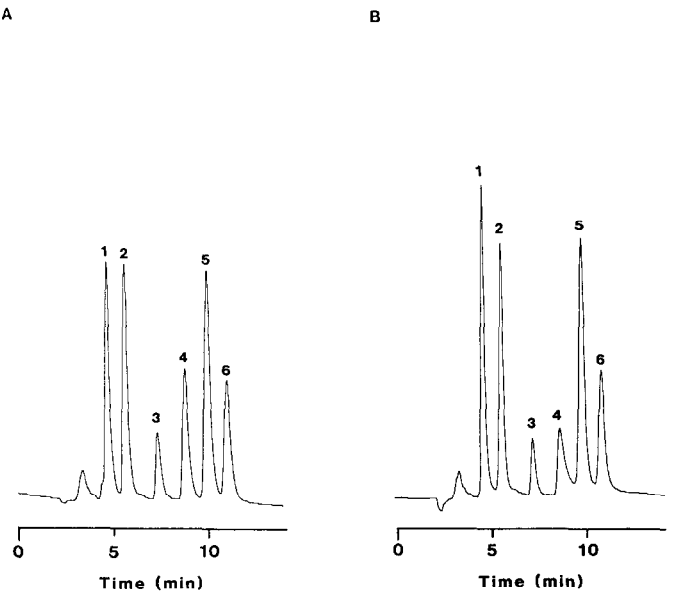

Fig. 6. Chromatograms of 4-AB (1), Met (2), Orn (3), Arg (4), Leu (5) and Phe (6) with the conventional (A) and hollow-fibre membrane reactors (B). Eluent B. Amounts injected: 4-AB, 49 pmol; Met, 65 pmol; Orn, 60 pmol; Arg, 48 pmol; Leu, 75 pmol; Phe, 60 pmol. Other conditions as in the text.

bration graphs of peak height *versus* absolute amount for each amino acid were linear in the ranges between a few and about one hundred pmol and between about one hundred and a few thousand pmol injected with a correlation coefficient of $\geqslant 0.999$, and passed through the origin. The wide linear dynamic range was obtained for all amino acids studied. The detection limits of the proposed method were 0.2–2.3 pmol at a signal-to-noise ratio of 3 (Table II). Although the peak height of Asp was higher than those of Asn, Met-S, Thr, Orn, Arg and Phe, the appearance of the system peak at around the retention time of Arg at low concentrations resulted in the highest detection limit.

TABLE I

PEAK BROADENING, σ_t(s), IN THE POST-COLUMN REACTOR

Open-tubular and hollow-fibre post-column reactors were used in methods A and B, respectively.

Amino acid	*Column*	*Reactor*		*Total*	
		Method A	*Method B*	*Method A*	*Method B*
4-AB	4.6	5.0	3.9	6.8	6.0
Met	5.0	5.1	4.0	7.2	6.4
Orn	5.9	5.6	3.1	8.2	6.7
Arg	6.1	6.9	8.5	9.2	10.5
Leu	7.9	3.9	2.6	8.8	8.3
Phe	9.2	5.9	3.7	10.9	9.9

TABLE II

REPRODUCIBILITY AND DETECTION LIMITS FOR AMINO ACIDS

The detection limit was based on a signal-to-noise ratio of 3.

Amino acid	*Injected amount (pmol)*	*Average peak height (cm) (n = 20)*	*R.S.D. (%)*	*Detection limit (pmol)*
Tau	80	11.0	1.4	0.2
Asn	65	3.8	4.0	0.7
Asp	75	6.0	3.4	2.3
Met-S	55	2.5	1.6	1.2
Thr	85	5.0	1.1	0.5
Ala	110	7.8	2.4	0.5
4-AB	49	8.5	2.0	0.6
Met	65	6.9	2.6	0.7
Orn	60	1.5	4.0	1.5
Arg	48	1.9	3.3	1.4
Leu	75	6.8	2.9	0.5
Phe	60	3.2	2.9	0.9

CONCLUSION

It has been demonstrated that the hollow-fibre membrane reactor is useful for the fluorimetric determination of amino acids using the post-column reaction with OPA and 2-ME. The method is as sensitive as previously reported methods, with good reproducibility.

The advantage of the proposed method is that it does not require an additional pump for delivering the post-column reagent and a mixing tee and reactor for post-column reaction. This leads to sensitive and reproducible assays of amino acids. We are now investigating applications of the proposed method to the simultaneous determination of primary and secondary amino acids, and to real samples.

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