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Separation of free amino acids by reversed-phase ion-pair chromatography with column switching and isocratic elution

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SUMMARY

Free amino acids (FAA) were separated by reversed-phase ion-pair liquid chromatography using a column-switching technique with isocratic elution. The eluted FAA were monitored by fluorescence detection using *o*-phthalaldehyde reagent. A 5-μm phenyl-bonded silica gel column (5 cm × 4.6 mm I.D.) was used for the hydrophobic and basic amino acids, which were separated within 20 min. A 5-μm octadecyl-bonded silica gel column (25 cm × 4.6 mm I.D.) was used for polar acidic and small amino acids, which were separated within 11 min. The system was applied to the FAA analysis of hydrolysate of bovine serum albumin.

INTRODUCTION

The chromatographic determination of amino acids is frequently used in many fields, and shortening of the analysis time is required. Reversed-phase ion-pair chromatography (RP-IPC) has been demonstrated to give more rapid and efficient separations of ionizable compounds than ion-exchange liquid chromatography (IEC)[1]. Free amino acids (FAA) were separated by RP-IPC with copper(II) ion. A column-switching technique with a combination of different alkyl-bonded silica gel columns under isocratic elution made it easy to achieve a rapid separation of a mixture of FAA[2]. However, the sensitivity was not satisfactory for application of the system to the determination of amino acids in biological samples.

o-Phthalaldehyde (OPA) reagent reacts with primary amines and amino acids in alkaline medium in the presence of a reducing agent such as 2-mercaptoethanol[3]. The post-column method was applied to IEC[4]. The OPA reagent in combination with sodium hypochlorite (NaOCl) oxidation made it possible to detect imino acids such as proline[5,6], and N-acetyl-L-cysteine was more suitable than mercaptoethanol be-

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cause of the stable thio-substituted isoindole derivative[7,8]. The OPA post-column detection method has been widely employed in reversed-phase liquid chromatography[9–21] for highly sensitive detection.

In this work, a column-switching technique and a post-column reaction detection system using OPA were combined for the rapid determination of FAA by RP-IPC.

EXPERIMENTAL

The liquid chromatograph consisted of an ERC-3310 degasser from ERMA (Tokyo, Japan) two Model 576 HPLC pumps and a Model FA-1040 flow-injection analysis pump from Gasukuro Kogyo (Tokyo, Japan), a Model F-1000 fluorescence detector from Hitachi (Tokyo, Japan) a Model 7125 injector, a Model 7000 six-way valve and a Model 7040 four-way valve from Rheodyne (Cotati, CA, U.S.A.) and a Sysmac PO time sequencer from Omron (Osaka, Japan). The six- and four-way valves were operated by Model 5701 pneumatic actuators (Rheodyne).

An octadecyl-bonded (ODS), an octyl-bonded (C_8) and a phenyl-bonded (Ph) silica gel (Inertsil ODS-2, C_8 and Ph respectively) and a diethyl-bonded silica gel (C_4) were obtained from Gasukuro Kogyo. These columns were 250 and 50 mm × 4.6 mm I.D. and were thermostated at 40 or 45°C. The eluent was prepared by dissolving appropriate amounts of a given sodium hexane- (C_6), heptane- (C_7) and octanesulphonate (C_8) and dodecyl sulphate (SDS) together with sodium chloride and an organic solvent (methanol, acetonitrile or ethanol) in phosphate buffer. OPA–N-acetyl-L-cysteine (N-AcCys) reagent was prepared by dissolving of 1.6 g of OPA and 2.0 g of N-AcCys in 10 ml of methanol and diluting with 1 l of 0.2 *M* sodium borate buffer, prepared by dissolving 24.6 g of boric acid and 15.2 g of sodium hydroxide in 2 l of doubly distilled water. Sodium hypochlorite solution was prepared by diluting 0.4 ml of commercial Antiformin (5% aqueous NaOCl solution) in 0.2 *M* sodium borate buffer[8].

All reagents were purchased from Wako (Osaka, Japan) and amino acids from Ajinomoto (Tokyo, Japan).

Protein hydrolysis was effected by the following method[8]. The protein sample (*ca.* 100 μg) [bovine serum albumin, crystallized four times, from ICN Pharmaceuticals (Cleveland, OH, U.S.A.)] was dissolved in 50 μl of 6 *M* hydrochloric acid in a Reacti-Vial, and the vial was placed in an oven at 150°C for 2 h. After hydrolysis, the vial was cooled. The hydrolysate were evaporated to dryness in a desiccator over potassium hydroxide pellets, then the residue was dissolved in 200 μl of the eluent.

RESULTS AND DISCUSSION

The effects of several factors on the retention behaviour of amino acids in RP-IPC was examined, *viz.*, alkyl chain length of the ion-pair (IP) reagent, concentration of IP reagent and organic modifier, pH, column temperature, phosphate and sodium ion concentrations in the eluent and selectivity of bonded phases of the silica gel packings.

First, the retention behaviour of polar acidic and small amino acids (Asp, Ser, Glu, Gly, Thr, Ala, Pro and Cys) was examined, because of their very weak retention in RP-IPC. These polar amino acids were more strongly retained as a result of forming

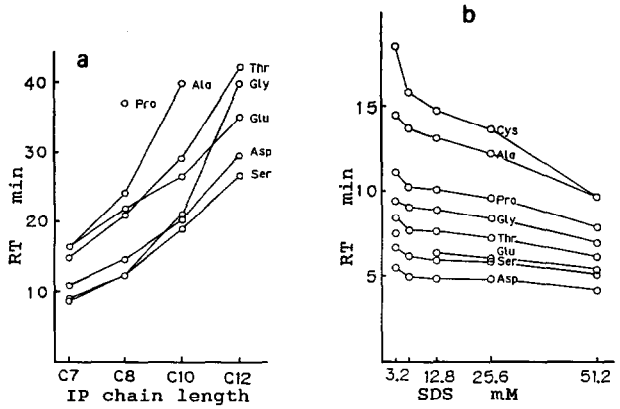

Fig. 1. Effect of (a) ion-pair reagent and (b) concentration. C7, C8, C10 = heptane-, octane- and decane-sulphonate respectively; C12 = dodecylsulphate. Column, Inertsil ODS-2 (250 mm × 4.6 mm I.D.); detector, UV (200 nm); flow-rate, 1.0 ml/min; column temperature, (a) 20°C and (b) 45°C; eluents, see text. RT = Retention time.

more hydrophobic ion pairs with a longer alkyl chain IP reagent, as shown in Fig. 1a where the eluent was 0.05 *M* sodium phosphate buffer (pH 2.0) with 6.4 m*M* IP reagent added. However, no simple behaviour was observed for the amino acids, and the alkyl chain length of IP reagent was potentially effective on the retention of glycine. Their retention became constant with more than 6.4 m*M* of SDS, as shown in Fig. 1b where the eluent was a combination of 0.05 *M* sodium phosphate buffer containing SDS (pH 2.7) and 16% methanol containing SDS. The column was easily re-equilibrated with highly concentrated IP reagent solution. Therefore, 25.6 m*M* SDS solution was selected as giving a good separation and shorter equilibration time.

Increasing the organic modifier concentration shortened the retention time, and this effect was readily observed on the retention of relatively hydrophobic amino acids such as proline. However, no specificity was observed between methanol and acetonitrile (ACN) as shown in Fig. 2.

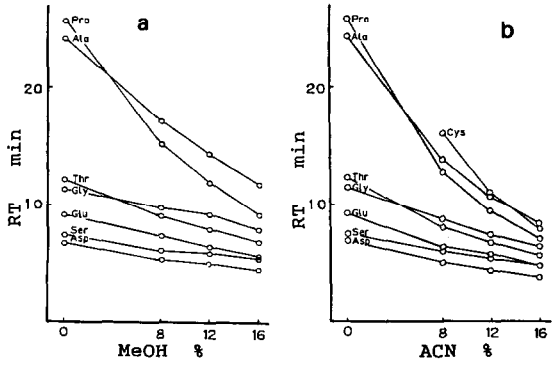

Fig. 2. Effect of organic modifier, methanol (MeOH) and acetonitrile (ACN) on the retention parameters of various amino acids. Eluent, 0.05 *M* sodium phosphate buffer containing 25.6 m*M* SDS (pH 2.7) plus methanol or ACN containing 25.6 m*M* SDS; column temperature, 45°C; other conditions as in Fig. 1.

The pH was the most sensitive factor in the separation of amino acids. After examining its effect with eluents of pH 2.0–5.0, pH 2.7 was selected as giving the best separation of polar amino acids. The concentration of sodium phosphate buffer was an important factor in shortening the retention time, but was not critical for separating less retained amino acids (Fig. 3a). The effect of sodium chloride concentration was examined on an ODS column. After injection of amino acids on to a column equilibrated with a conditioning eluent [0.05 *M* sodium phosphate buffer containing 25.6 m*M* SDS (pH 2.7) plus 12% methanol containing 25.6 m*M* SDS], these amino acids were eluted with a separation eluent [0.05 *M* sodium phosphate buffer (pH 2.7) containing 5% ethanol and ACN and some sodium chloride]. As seen in Fig. 3b, an increase in sodium chloride concentration had a greater effect on both the retention time and elution order, especially for basic amino acids. An increase in column temperature shortened the retention times. The retention times on bonded-phases from C_4 to ODS groups increased with increase in the alkyl chain length. However, the lifetime of the C_4 phase was too short to make separation system. The separation of acidic amino acids required an ODS column, and that of valine and methionine was achieved on a Ph column.

A column-switching system was established from the above results. As shown in Fig. 4, two switching valves and one fluorescence detector were used instead of one switching valve and two ultraviolet absorption detectors as in a previous study[2].

The time sequence is given in Table I. After injection on to a Ph column, the sample was separated into polar acidic and small (less retained) and hydrophobic and basic (retained) amino acids. The less retained amino acids were transferred to an ODS column by eluent 2 from pump 2. Eluent 2 was 0.05 *M* sodium phosphate buffer containing 25.6 m*M* SDS (pH 2.7) plus 12% methanol and 0.094 *M* sodium chloride. The flow-rate was 1.0 ml/min (process I). The ODS column was removed from the line at the first column switch 3.5 min after injection. The retained amino acids were first separated on the Ph column by eluent 1 from pump 1. Eluent 1 was 0.05 *M*

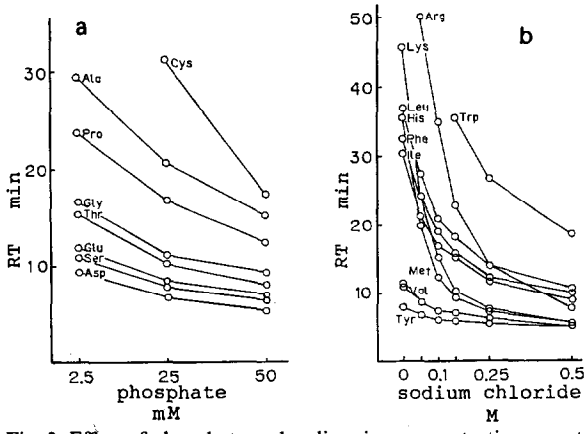

Fig. 3. Effect of phosphate and sodium ion concentration on retention of amino acids. (a) Eluent, sodium phosphate buffer (pH 2.7) plus 12% methanol and 25.6 m*M* SDS; other conditions as in Fig. 1. (b) Column, Inertsil ODS-2 (50 mm × 4.6 mm I.D.); flow-rate, 1.0 ml/min; column temperature, 45°C; eluent, see text.

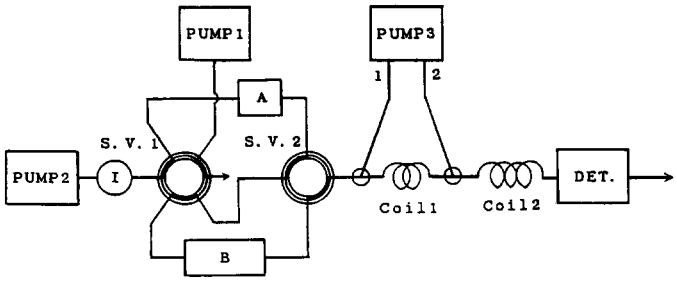

Fig. 4. Flow diagram of column-switching system. Column A, Inertsil Ph (50 mm × 4.6 mm I.D.); column B, Inertsil ODS-2 (250 mm × 4.6 mm I.D.); DET, fluorescence detector (excitation at 340 nm, emission at 450 nm); PUMP 1 and 2, HPLC pumps; PUMP 3-1, NaOCl reagent pump, flow-rate 0.9 ml/min; PUMP 3-2, OPA reagent pump, flow-rate 0.9 ml/min; coil 1, 60 cm × 0.5 mm I.D.; coil 2, 600 cm × 0.5 mm I.D., column and reaction coil temperature, 40°C; I, injector; S.V.1 and 2, six- and four-way switching valves. Eluent and reaction reagents, see text.

sodium phosphate buffer (pH 2.7) containing 6.5% ethanol, 6.5% ACN and 0.11 *M* sodium chloride. The flow-rate was 1.0 ml/min (process II).

After 23.5 min, less retained amino acids were separated on the ODS column by eulent 2, and the separation was completed within 34.5 min (process III). Finally, the Ph column was reconditioned with eluent 2 and the ODS column was recombined at 37.5 min, ready for the next injection.

The selection of the sodium chloride concentration in eluents 1 and 2 was important because an imbalance in the sodium chloride concentration in two eluents made the baseline shift when column switching was operated and quantitative analysis became difficult. Therefore, eluent 1 contained 0.11 *M* and eluent 2 contained 0.094 *M* sodium chloride in this system.

The effluent was first mixed with NaOCl reagent from pump 3-1, then mixed with OPA reagent from pump 3-2. The reation products were monitored by a fluorescence detector excitation at 340 nm and emission at 450 nm).

These four process were repeated for each separation. A standard amino acid mixture was chromatographed by the above system and a chromatogram is shown in Fig. 5a. Seventeen amino acids were successively separated within 35 min. If two fluorescence detectors were used, separation in 25 min was possible. The retention time of tryptophan was very long and when this compound is present it is better to

TABLE I

OPERATION OF THE SEPARATION OF FREE AMINO ACIDS BY COLUMN SWITCHING

Inj. = injection point; I–IV = column-switching points; Ph and ODS = phenyl- and octadecyl-bonded columns; eluents 1 and 2, see text.

Parameter	*Process*				
	Inj.	*I*	*II*	*III*	*IV*
Time (min)		0	3.5	23.5	34.5
Separation column	Ph + ODS		PH	ODS	Ph
Eluent		2	1	2	2

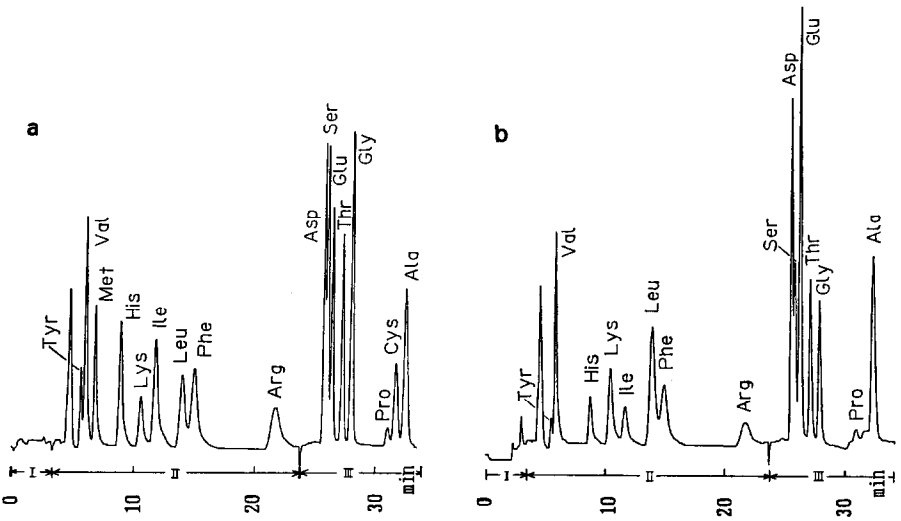

Fig. 5. Chromatograms of (a) standard amino acids and (b) amino acids in a hydrolysate of bovine serum albumin.

add one more column-switching stage in the above system. Further, this system was applied to the analysis of hydrolysate of bovine serum albumin, and a chromatogram is shown in Fig. 5b. The composition of amino acids analysed by this system was satisfactorily correlated with that calculated from the structure in the literature[22], and

TABLE II

REPRODUCIBILITIES OF RETENTION TIME AND PEAK HEIGHT

Experimental conditions: see Fig. 4. Amounts of amino acids: 100 pmol each.

Amino acid	*Variation (±%)*	
	Retention time	*Peak height*
Tyr	0.07	5.27
Val	0.06	1.77
Met	0.08	0.46
His	0.11	1.25
Lys	0.15	2.81
Ile	0.08	1.03
Leu	0.14	5.01
Phe	0.11	1.58
Arg	0.19	15.1
Asp	0.04	2.18
Ser	0.02	1.91
Glu	0.02	2.92
Thr	0.003	1.22
Gly	0.002	1.22
Pro	0.06	4.23
Cys	0.04	1.00
Ala	0.03	0.83

the correlation coefficient was 0.96. The correlation coefficient between this result and that measured by liquid chromatography[8] was 0.97.

The reproducibilities of the retention time and peak height were examined by manual five injections of a mixture of 100 pmol each of various amino acids, and the results are given in Table II. The reproducibility of the retention time was fairly good; the variation was less than ±0.2%. The peak-height variation was less than ±5%, except for arginine. The retention time of arginine was relatively long, and the integrator could not be well adjusted.

The sensitivity for amino acids having shorter retention times was 2–5 pmol and that for those having longer retention time was 3–9 pmol; that for proline was 20 pmol (signal-to-noise ratio = 3). Their calibration graphs were linear up to 500 pmol.

CONCLUSION

Seventeen free amino acids were separated within 35 min by RP-IPC using a column-switching technique. This system can be applied to the analysis of FAA in hydrolysate samples by applying post-column reaction detection with OPA–N-Ac-Cys. This study demonstrated the possibility of further development of a faster separation system by using two fluorescence detectors and packings of smaller particle size.

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