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Note

Reversed-phase ion-pair chromatography of amino acids

Application to the determination of amino acids in plasma samples and dried blood on filter papers

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High-performance liquid chromatographic separation of amino acids has been mainly performed by the use of ion-exchange chromatography [1] coupled to reaction with ninhydrin [2], fluorescamine [3] and *o*-phthalaldehyde, followed by ultraviolet absorbance or fluorescence detection. In comparison with partition chromatography, however, ion-exchange chromatography entails problems with column efficiency, the long equilibrium time until the next analysis, the high cost of columns and the necessity of a complicated stepwise gradient elution technique using different kinds of solvents for good separation.

Reversed-phase chromatography has been also used for the same purpose. But this method requires pre-column derivatization with phenylisothiocyanate [4], or 1-N,N-dimethylaminonaphthalene-5-sulfonyl chloride [5, 6].

Ion-pair chromatography has been recently used for the separation of ionic compounds as well as ion-exchange chromatography. Radjai and Hatch [7] applied this chromatographic system to separate amino acids in conjunction with post-column derivatization using *o*-phthalaldehyde—2-mercaptoethanol. Their study showed good results similar to those obtained using the ion-exchange system. With this method, however, the complicated stepwise gradient elution technique must have been applied, and its application to biological samples such as serum was not shown.

This paper deals with a simpler reversed-phase ion-pair chromatographic method for the separation of amino acids and with their determination in plasma and dried blood on filter papers using this method.

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EXPERIMENTAL

Chemicals

o-Phthalaldehyde was purchased from Tokyo Chemical Industry (Tokyo, Japan), 2-mercaptoethanol and acetonitrile were from Kanto Chemicals (Tokyo, Japan). Sodium lauryl sulfate and monochloroacetic acid were obtained from Wako Pure Chemicals (Osaka, Japan). Redistilled water was used for all reagent preparation. Other reagents and solvents were of analytical grade.

o-Phthalaldehyde–2-mercaptoethanol reagent solution was prepared as follows: 40 mg of *o*-phthalaldehyde were dissolved in 20 ml of ethanol and then 0.1 ml of 2-mercaptoethanol and 200 ml of 0.1 *M* borate buffer (pH 9.0) were added to this ethanolic solution.

Apparatus

All parts were obtained from Japan Spectroscopic (Tokyo, Japan). The high-performance liquid chromatographic system consisted of a Tri Rotor I high-performance liquid chromatograph connected with a Model GP-A30 solvent programmer and a Model FP-110 fluorescence spectrofluorometer with a Model RC strip chart recorder. A 250 × 4.0 mm stainless steel column packed with LiChrosorb RP-8 (particle size 5 μm, E. Merck, Darmstadt, G.F.R.) was used for the separation of amino acids. The eluate from the column was successively mixed with *o*-phthalaldehyde–2-mercaptoethanol reagent solution using a Model LCP 150 liquid chromatographic pump and introduced into a fluorescence detector after being passed through a mixing coil made of stainless steel tube (1 m × 0.25 mm). The operating conditions are shown in Fig. 1.

Procedure for high-performance liquid chromatographic separation of amino acids in plasma and dried blood on filter paper

The solution of amino acids (50 μl) was injected onto the column in the form of a solution prepared in the starting mobile phase solvent: 0.05 *M* sodium lauryl sulfate solution acidified with concentrated monochloroacetic acid at pH 3.0.

A 10-μl volume of human plasma was mixed with 500 μl of 70% ethanol containing γ-aminobutyric acid (0.5 mg/dl) as an internal standard. The mixture was vigorously shaken and the supernatant was evaporated to dryness after centrifugation. The residue was dissolved with 100 μl of the starting solvent and then 50 μl of the resulting solution were injected onto the column.

In the case of the dried blood on filter paper, a 3 mm diameter disk of dried blood was placed into a small test tube. A 500-μl volume of 70% ethanol containing γ-aminobutyric acid (0.5 mg/dl) was added to the tube and kept at 4°C overnight. The resulting ethanolic solution was prepared in the same manner as that of the plasma sample.

RESULTS AND DISCUSSION

Fig. 1 shows a typical high-performance liquid chromatogram of a standard mixture of amino acids, each at a concentration of 2 mg/dl. These conditions

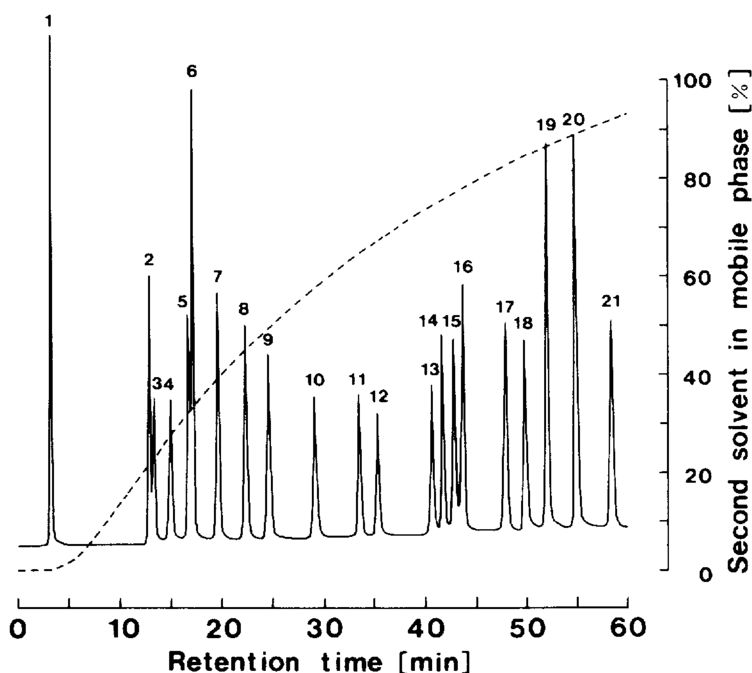

Fig. 1. High-performance liquid chromatogram of a standard mixture of amino acids (dotted line shows the percentage of the second solvent in the mobile phase). Peaks: 1 = taurine, 2 = aspartic acid, 3 = serine, 4 = glutamic acid, 5 = threonine, 6 = glycine, 7 = citrulline, 8 = alanine, 9 = γ-aminobutyric acid (internal standard, IS), 10 = tyrosine, 11 = valine, 12 = methionine, 13 = alloisoleucine, 14 = isoleucine, 15 = phenylalanine, 16 = leucine, 17 = tryptophan, 18 = histidine, 19 = ornithine, 20 = lysine, 21 = arginine. Operating conditions: column, 250 × 4.0 mm LiChrosorb RP-8 (5 μm); mobile phase first solvent, 0.05 M sodium lauryl sulfate (pH 3.0), second solvent 0.05 M sodium lauryl sulfate—acetonitrile (55:45). The gradient was prepared using a Model GP-A30 solvent programmer (Convex 1, 64 min); mobile phase flow-rate, 0.7 ml/min; reagent solution flow-rate, 1.2 ml/min; column and mixing coil temperature, room temperature; fluorescence detector, excitation 365 nm, emission 455 nm.

did not offer sufficient separation between aspartic acid and serine, and threonine and glycine. The separation of 21 amino acids was achieved within about 60 min. Flowing the first solvent for 10 min after the end of analysis made it possible to inject the next sample.

Radjai and Hatch [7] used acetic acid to adjust the pH of the starting mobile phase to 2.85 in order to inhibit the ionization of the carboxyl group of amino acids and obtain better separation. However, considering that the pK_a of acetic acid is 4.75, it might not be a suitable means of adjusting the pH of the solvent to about 2.8. Therefore, monochloroacetic acid, the pK_a value of which is 2.85, was used to adjust the pH in this study, instead of acetic acid.

Radjai and Hatch [7] also applied a four-step gradient elution technique using two kinds of counter-ion solutions to achieve separation of the amino acids. This technique is very complicated and can not easily be applied to the routine analysis of amino acids. The technique adopted for our method was the simpler gradient elution technique, whereby 0.05 M sodium lauryl sulfate—acetonitrile (55:45) is successively added to 0.05 M sodium lauryl sulfate (pH

3.0) according to the solvent program (Convex 1, 64 min). The percentage of the second solvent in mobile phase is shown by a dotted line in Fig. 1.

The temperature of the column markedly influenced the separation efficiency. Within the temperature range investigated (15—50°C), the lower the temperature the better the separation obtained. This was especially true of the separation of isoleucine, phenylalanine and leucine. On the basis of these results the column was maintained at 20°C.

Investigations of the flow-rate of mobile phase from 0.6 to 1.0 ml/min showed that the separation efficiency of amino acids hardly varied within this range. The need to protect the column from high pressure and also to shorten the analytical time led us to select a flow-rate of 0.7 ml/min.

The reagent blank from *o*-phthalaldehyde—2-mercaptoethanol reagent solution gave rise to a serious drift in the baseline. To overcome this problem, the pH of the reagent solution was adjusted to 9.0 according to Roth's [8] suggestion about the reaction conditions of *o*-phthalaldehyde with amino acids. This step suppressed the drift as shown in Fig. 1. Further, the flow-rate of the reagent solution was arranged at 1.2 ml/min for the same purpose.

Figs. 2 and 3 show the chromatograms obtained from a normal human plasma sample and from a sample from a patient with maple syrup urine disease (MSUD), respectively. On the chromatogram from the MSUD patient it can be observed that there were remarkable increases in the amounts of the branched-chain amino acids valine, isoleucine and leucine. Also, a peak corresponding to alloisoleucine was detected, which could not be found on the chromatogram from normal plasma.

The analytical results of this method are summarized in Table I. The recovery and reproducibility of aspartic acid, serine, glycine and threonine were relatively low, compared with other amino acids.These results might be due to inferior separations.

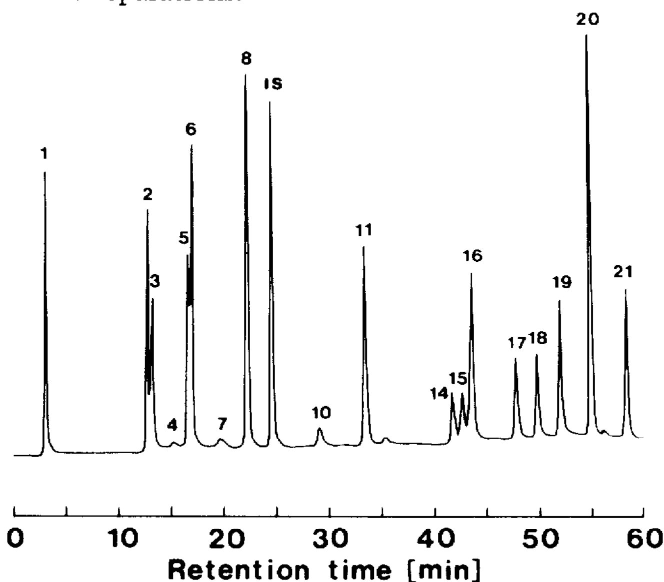

Fig. 2. High-performance liquid chromatogram obtained from a normal human plasma sample. Peak numbering and operating conditions as in Fig. 1.

TABLE I

RECOVERY TEST OF AMINO ACIDS FROM HUMAN PLASMA

Values are given in mg/dl.

Sample		Tau	Asp Ser	Glu	Thr	Gly	Cit	Ala	Tyr
Spiked plasma	1	2.67	15.43	4.94	4.69	2.18	2.50	7.86	3.56
	2	2.63	15.43	5.41	4.69	2.04	2.50	7.72	3.56
	3	2.56	16.29	5.18	4.41	2.04	2.50	7.86	3.33
	4	2.63	16.14	5.18	4.28	1.98	2.50	7.65	3.33
	5	2.74	14.00	5.65	4.55	2.11	2.63	8.21	3.56
	6	2.49	12.71	5.18	3.86	1.98	2.50	7.72	3.33
	7	2.58	13.86	4.94	3.72	2.04	2.50	7.58	3.33
Mean		2.61	14.84	5.21	4.31	2.05	2.52	7.80	3.43
C.V. (%)		3.1	9.0	4.8	9.0	3.5	1.9	2.7	3.6
Plasma blank		0.51	9.28	0.71	1.80	0.85	0.44	3.69	1.33
Added		2.00	2.00 2.00	4.00	4.00	2.00	2.00	4.00	2.00
Recovery (%)		105	139	113	63	60	104	103	105

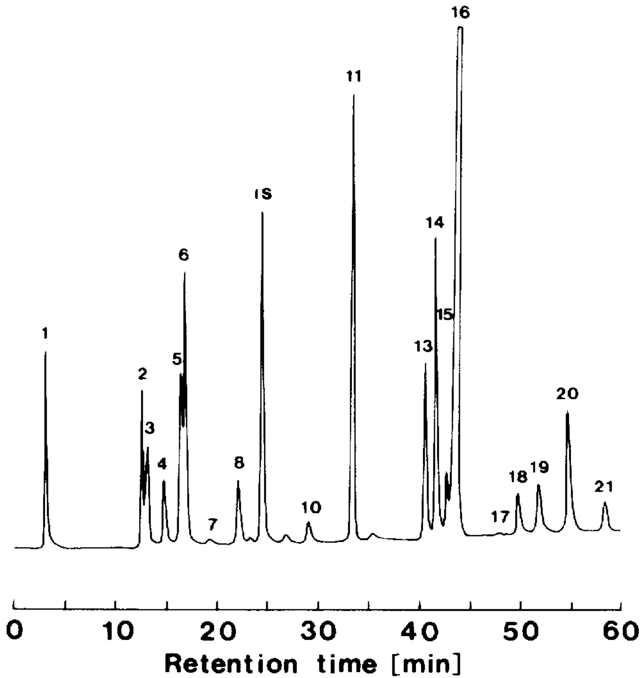

Fig. 3. High-performance liquid chromatogram obtained from the plasma sample of a MSUD patient. Peak numbering and operating conditions as in Fig. 1.

Dried blood on filter paper has been widely used for screening newborns throughout the world [9, 10]. Our method was applied to the analysis of amino acids in dried blood samples. Fig 4 shows a chromatogram obtained from a 3 mm diameter disk of dried blood from a patient with phenyl-

Val	alloIle	Ile	Phe	Leu	Try	His	Orn	Lys	Arg
7.00	2.20	3.18	2.88	3.57	2.89	3.38	2.68	6.92	5.87
6.88	2.20	3.06	3.13	3.71	3.00	3.25	2.75	6.79	6.04
6.75	2.20	3.06	2.88	3.64	3.00	3.50	2.79	7.24	5.96
6.63	2.00	2.94	2.88	3.43	2.78	3.25	2.75	7.06	5.96
7.25	2.20	3.18	3.00	3.79	3.22	3.50	2.98	7.64	6.13
6.75	2.20	3.06	3.00	3.64	3.00	3.38	3.02	7.60	6.13
6.50	2.20	3.06	3.00	3.64	3.00	3.38	3.06	7.78	6.40
6.82	2.17	3.08	2.97	3.63	3.00	3.38	2.86	7.29	6.07
3.6	3.5	2.7	3.2	3.1	4.7	3.0	5.4	5.3	2.9
2.94	—	1.06	1.07	1.75	1.00	1.25	0.72	2.92	1.83
4.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	4.00	4.00
99	109	101	95	94	100	107	107	109	106

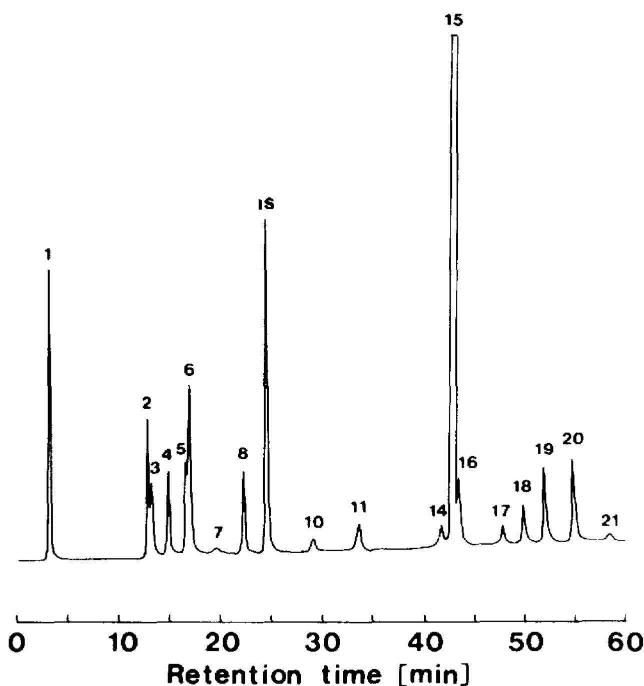

Fig. 4. High-performance liquid chromatogram obtained from 3 mm disk of dried blood of a patient with phenylketonuria. Peak numbering and operating conditions as in Fig. 1.

ketonuria. The results of the determination agree well with those obtained by means of a conventional amino acid analyzer. These data suggest that one 3 mm disk of dried blood is sufficient to analyze the amino acids.

CONCLUSION

This study has demonstrated that the reversed-phase ion-pair chromatographic method can be effectively used for the determination of amino acids in plasma samples and dried blood on filter paper. Relatively good recovery and reproducibility were obtained from the investigations using plasma samples. This method appears to be superior to conventional ion-exchange chromatography in terms of the equilibrium time until the next sample injection, the cost of the column and the simplicity of the elution technique, etc. The present results suggest that this method could be used for screening newborns by means of dried blood on filter paper.

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