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RECOMMENDED METHOD FOR THE ANALYSIS OF AMINO ACIDS IN BIOLOGICAL MATERIALS

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SUMMARY

Fifty-five ninhydrin-positive compounds in physiological fluids were determined with a Hitachi Model KLA-5 amino acid analyzer by a two-column chromatographic procedure. Both columns were packed with Hitachi Custom 2618 ion-exchange resin. The total analysis time was 9.5 h.

In this procedure, particularly glucosamine, mannosamine and galactosamine were separated completely from normal "protein" amino acids, and N^G-monomethylarginine, N^G,-N^G-dimethylarginine and N^G,N'^G-dimethylarginine, which were present in the myelin basic protein of several species and excreted in human urine, were separated from other basic amino acids. The method is useful for various applications with biological materials.

INTRODUCTION

Amino acid analysis has been applied to a wide variety of hydrolyzates of protein or glycoprotein, physiological fluids and tissue extracts. In such applications a combination of high resolution and speed of analysis is essential.

There are a large number of procedures available for the quantitative analysis of amino acids in physiological fluids [1—9]. Perry et al. [3] reported a versatile lithium buffer elution system for single-column chromatography, and 65 ninhydrin-positive substances were analyzed by this method. Although five steps with lithium buffers were used, all of the substances were not separated completely and the time of analysis was 21 h. On the other hand, Benson [8] reported a more rapid two-column chromatographic method for the analysis of amino acids in physiological fluids. He used two buffers for each analysis and 41 ninhydrin-positive compounds could be analyzed in 7.5 h. When the amino acid analysis was performed by this method using a different type of analyzer, unsatisfactory results were obtained. It was clear that the resolution of amino acids depends on the characteristic properties of the resin such as the degree of cross-linkage and the particle diameter.

We have used a Hitachi amino acid analyzer and have developed a two-column chromatographic procedure which gives a high resolution and is also widely appliciable.

MATERIALS AND METHODS

Reagents

Amino acid standard calibration mixture was a product of Takara Kosan Co., Tokyo, Japan. Other ninhydrin-positive compounds were purchased from Sigma (St. Louis, Mo., U.S.A.), Calbiochem (Los Angeles, Calif., U.S.A.), Fluka (Buchs, Switzerland), Wako (Osaka, Japan) and Tokyo Chemical Industry Co. (Tokyo, Japan). N^G-Monomethylarginine, N^G,N^G-dimethylarginine and N^G,N'^G-dimethylarginine were products of Calbiochem and N-ϵ-lysine was a product of Sigma. β-(2-Thienyl-DL-serine and S-β-(4-pyridylethyl)cysteine used as internal standards were purchased from Pierce (Rockford, Ill., U.S.A.).

Sample preparation

Synthetic mixture of ninhydrin-positive compounds. A standard solution wad made by diluting with 0.01 *N* HCl to give concentrations of 0.1 μmole/ml of ninhydrin-positive compounds, except for 0.04 μmole/ml of cystathionine, 0.2 μmole/ml of proline and anserine, 1.0 μmole/ml of urea and 5.0 μmole/ml of creatinine.

Deproteinized human plasma. Normal human plasma was deproteinized using sulphosalicylic acid [10].

Deammoniated human urine. Normal human urine was deammoniated by the method of Benson and Patterson [1].

Hydrolysis of human plasma and urine. Deproteinized human plasma (2 ml) and deammoniated human urine (4 ml) were hydrolyzed with an equal volume of concentrated HCl for 24 h at 110°. The hydrolyzates were dried with a rotary evaporator at 55° and dissolved in 0.01 *N* HCl.

Apparatus

A Hitachi Model KLA-5 automatic amino acid analyzer was used consisting of the main instrument, a tape-controlled programmer, an automatic sample injector and an integrator.

Column and resin

A 40 × 0.9 cm I.D. glass column was used for the separation of the acidic and neutral amino acids, and a 25 × 0.9 cm I.D. glass column for the separation of the basic amino acids. Both columns were filled with Hitachi Custom Ion-Exchange Resin 2618, which is sulphonated with a 10% degree of cross-linking. The particle diameter is 11.5 ± 2 μm with an exchange capacity of 4.5 mequiv./g.

Buffers

Two lithium citrate buffers were used for acidic and neutral amino acid analyses and two sodium citrate buffers for basic amino acid analysis (Table I). The pH of the eluting buffers must be controlled to within ±0.005 because the separation of the amino acids is sensitive to small differences in pH.

TABLE I

COMPOSITION OF BUFFERS

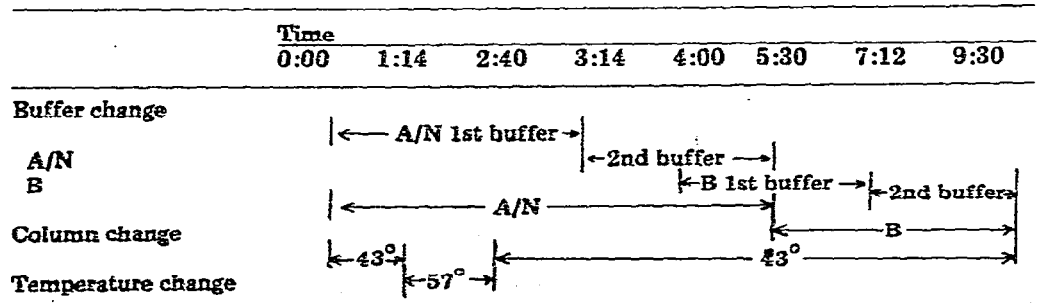
	Acidic and neutral amino acid analysis		Basic amino acid analysis	
	1st	2nd	1st	2nd*
Cation concentration				
Li (*N*)	0.25	0.25		
Na (*N*)			0.38	0.50
pH	2.90	4.30	4.10	6.09
Citric acid (g)	95.8	57.5	—	—
Lithium citrate (g)	25.2	80.1	—	—
Lithium chloride (g)	96.3	70.1	—	—
Sodium citrate (g)	—	—	373.0	490.0
Conc. HCl (ml)	—	—	150.0	25.0
Ethanol (ml)	300.0	—	300.0	—
Methanol (ml)	—	—	—	800.0
25% Brij-35 (ml)	40.0	40.0	40.0	40.0
Thiodiglycol (ml)	25.0	25.0	—	—
n-Caprylic acid (ml)	1.0	1.0	1.0	1.0
Final volume (l)	10.0	10.0	10.0	10.0

*This buffer was used for the rapid determination of glucosamine and galactosamine according to our previous paper [11].

TABLE II

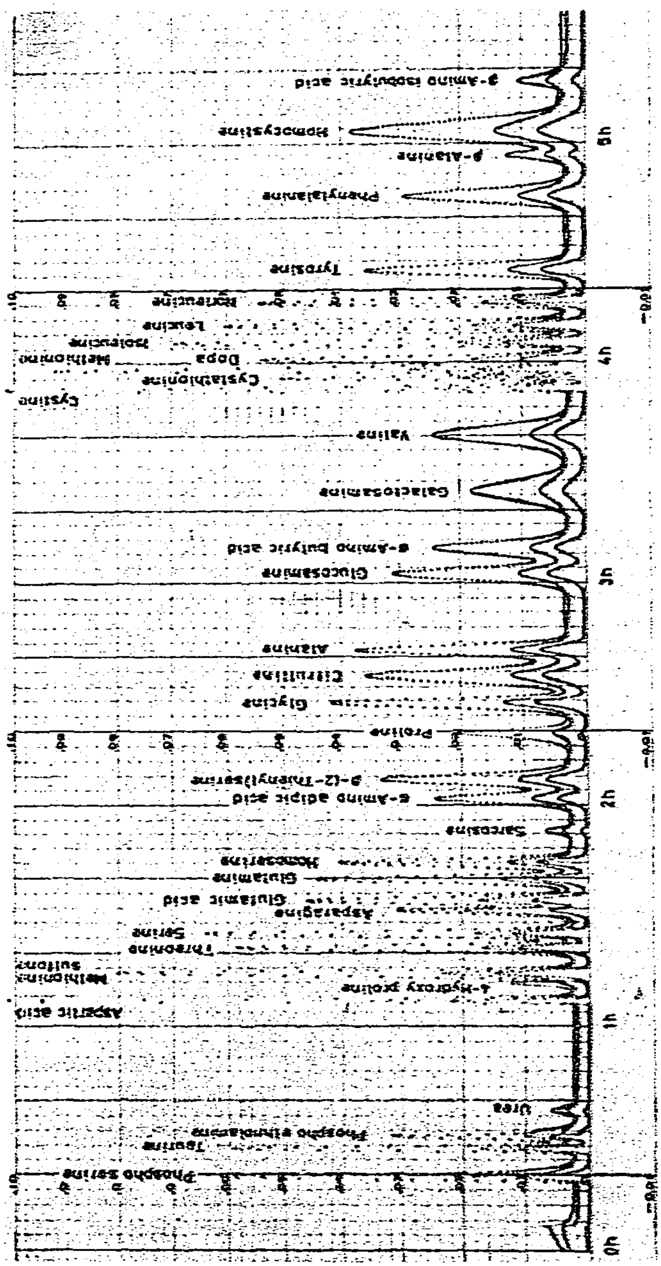
ELUTION PROGRAMME

A/N = Acidic and neutral amino acid analysis; B = basic amino acid analysis



Chromatographic conditions

The eluting buffer and ninhydrin solution were pumped at flow-rates of 60 and 30 ml/h, respectively. The operating back-pressure was 15 ± 1 kg/cm² for acidic and neutral amino acid analyses and 10 ± 1 kg/cm² for basic amino acid analysis. The elution programme is illustrated in Table II, and the column was operated at 43° and 57°, as shown in Table II.



Phospho serine
Taurine
Phospho ethanolamine
Urea
Aspartic acid
Methionine sulfone
4-Hydroxy proline
Threonine
Serine
Asparagine
Glutamic acid
Glutamine
Homoserine
Sarcosine
α-Amino adipic acid
β-(2-Thienyl)serine
Proline
Glycine
Citrulline
Alanine
Glucosamine
α-Amino butyric acid
Galactosamine
Valine
Cystine
Cystathionine
Methionine
Dopa
Isoleucine
Leucine
Norleucine
Tyrosine
Phenylalanine
β-Alanine
Homocystine
β-Amino isobutyric acid
0h
1h
2h
3h
4h
5h

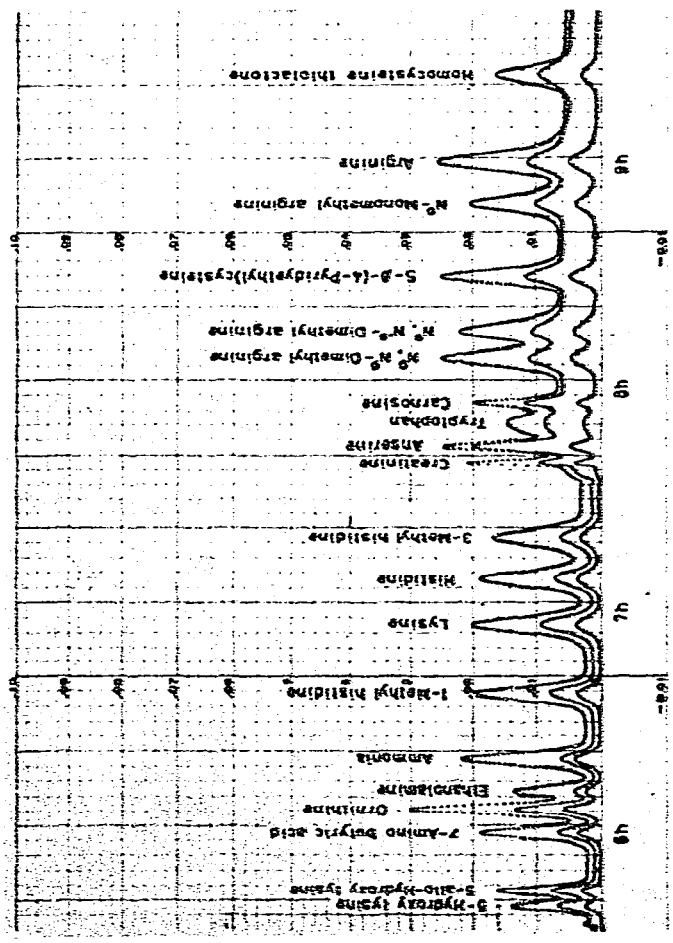


Fig. 1. Chromatogram from 0.5 ml of a synthetic mixture of 55 ninhydrin-positive compounds. The amount of each compound analyzed was 0.05 μmole. The following amino acids and their amounts were used: cystathionine (0.02 μmole), proline and anserine (0.1 μmole), urea (0.5 μmole) and creatinine (2.5 μmole).

TABLE III

RETENTION TIMES OF 79 NINHYDRIN-POSITIVE COMPOUNDS

Ninhydrin-positive compounds	Retention time (min)
Acidic and neutral amino acid analysis	
Cystic acid	17
Homocysteic acid	17
Phosphoserine	18
Taurine	25
Phosphoethanolamine	28
Laevulinic acid	28*
Urea	36
Aspartic acid	65
S-CMC	65
Glutathione (reduced)	66
Hydroxyproline	69
Methionine sulphone	73
Methionine sulphoxides	75, 78**
Threonine	80
Serine	83
Asparagine	90
Glutamic acid	93
Glutathione (oxidized)	97
Glutamine	98
Homoserine	102
Sarcosine	111
α-Aminoadipic acid	120
β-(2-Thienyl)-DL-serine	125
Proline	138
Glycine	146
Hippuric acid	146
Citrulline	153
Alanine	160
Glucosamine	181
α-Aminobutyric acid	188
Mannosamine	190
Galactosamine	204
Valine	219
Cystine	231
Norvaline	231
Pipecolic acid	232
Homocitrulline	234
Cystathionine	234
Methionine	236
Lanthionine	237
DOPA	239
Isoleucine	244
Leucine	248
Norleucine	255
β-(2-Thienyl)-DL-alanine	259
Tyrosine	264
Phenylalanine	284
β-Alanine	295
Homocystine	302
β-Aminoisobutyric acid	315

TABLE III *(continued)*

Ninhydrin-positive compounds	Retention time (min)
Basic amino acid analysis	
5-Hydroxylysine	100
allo-Hydroxylysine	104
δ-Aminolaevulinic acid	105
γ-Aminobutyric acid	120
Ornithine	126
Ethanolamine	131
Ammonia	140
Kynurenine	155
1-Methylhistidine	157
Lysine	175
N-ε-Methyllysine	176
Histidine	188
3-Methylhistidine	199
Creatinine	220
Creatine	221
Anserine	224*
Tryptophan	230
α-Amino-β-guanidinopropionic acid	232
Monoiodotyrosine	233
ε-Aminocaproic acid	234
Carnosine	236
Homocarnosine	236
Canavanine	239
N^G,N^G-Dimethylarginine	247
N^G,N'^G-Dimethylarginine	255
S-β-(4-Pyridylethyl)cysteine	268
N^G-Monomethylarginine	288
Arginine	300
Diiodotyrosine	318
Homocysteine thiolactone	322

*Ratio of 440 to 570 nm for laevulinic acid and anserine was higher than that of other amino acids and related compounds except for proline and hydroxyproline; for the former the ratio was 1.25 and for the latter 0.84.
**Methionine sulphoxide gave two peaks.

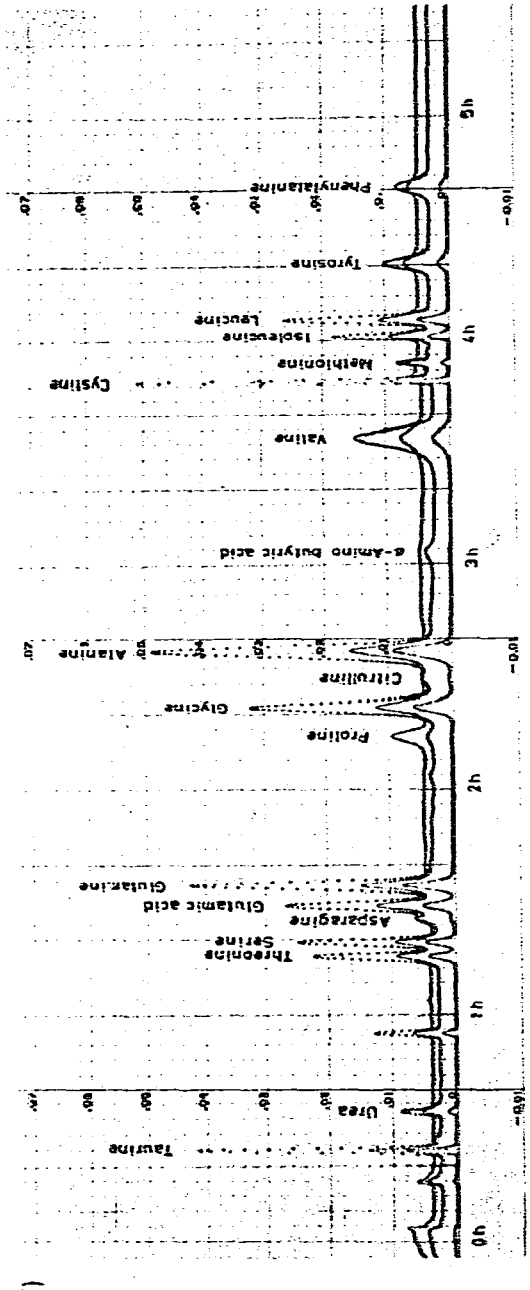
RESULTS

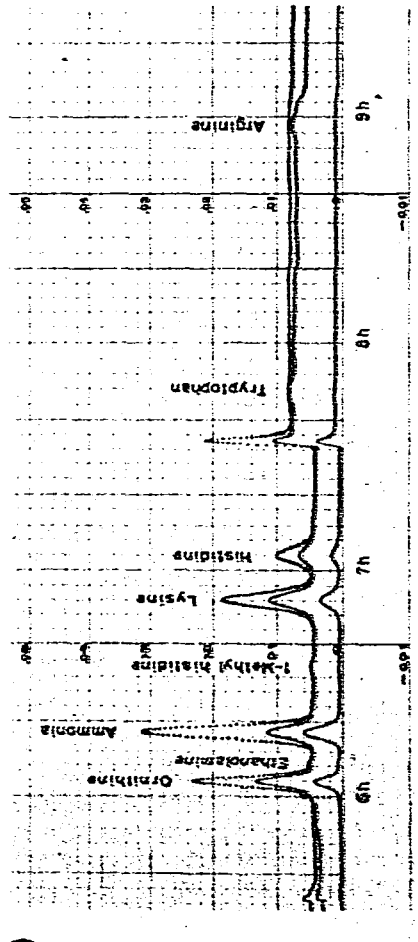
Analysis of a synthetic mixture

The results of the chromatography of a synthetic mixture containing 55 ninhydrin-positive compounds are shown in Fig. 1. A high resolution of all of these compounds was obtained and the total analysis time was 9.5 h.

A further 24 ninhydrin-positive compounds were investigated under the same conditions, and Table III lists the retention times of the 79 substances studied. It can be seen that not all of the latter group were separated from the first 55 ninhydrin-positive compounds.

The acidic and neutral amino acid analysis was improved so that 36 compounds containing methionine sulphone, homoserine, homocystine, DOPA, glu-


(B')
Taurine
Urea
Threonine
Serine
Asparagine
Glutamic acid
Glutamine
Proline
Glycine
Citrulline
Alanine
α-Amino butyric acid
Valine
Cystine
Methionine
Isoleucine
Leucine
Tyrosine
Phenylalanine
0h
1h
2h
3h
4h
5h


(B")
Ornithine
Ethanolamine
Ammonia
1-Methyl histidine
Lysine
Histidine
Tryptophan
Arginine
6h
7h
8h
9h

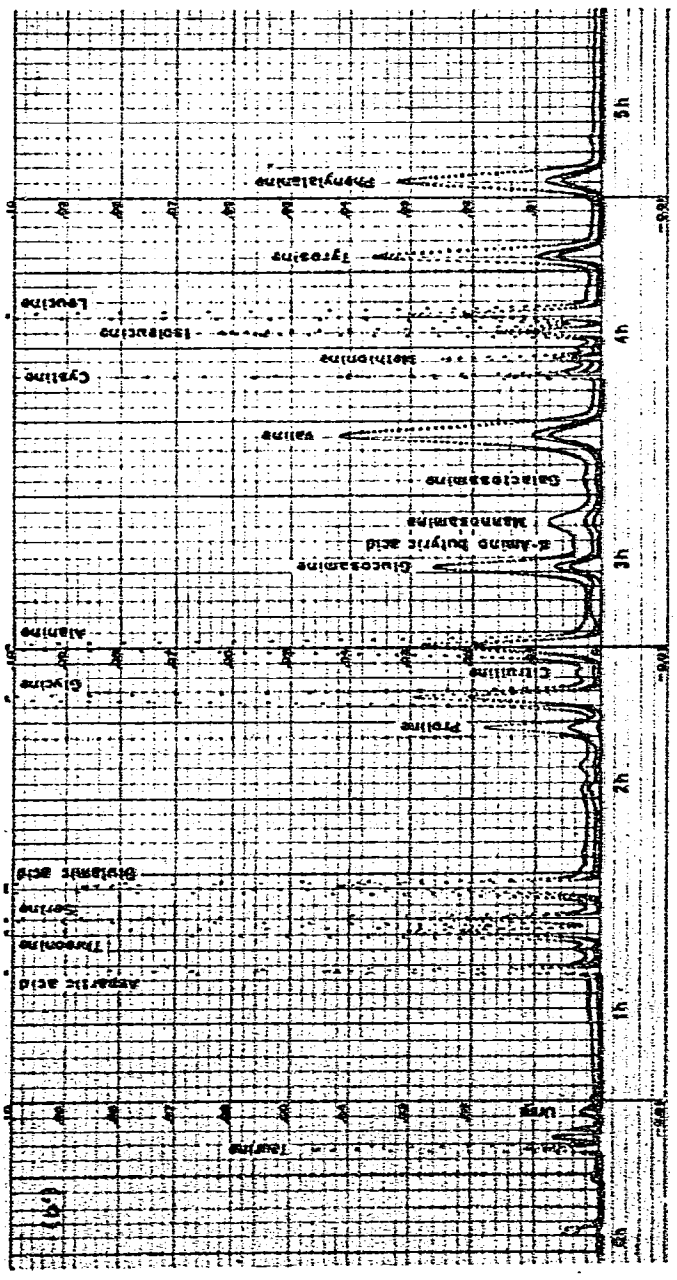


Fig. 2.

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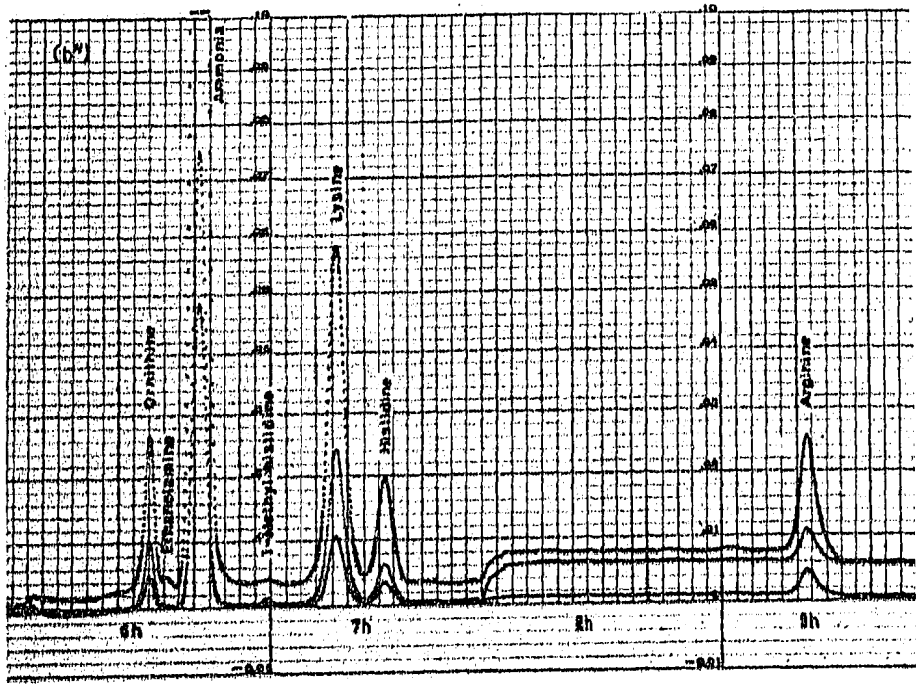

Fig. 2. (a) Chromatogram from 0.24 ml of normal human plasma; (b) chromatogram from 0.24 ml of hydrolyzate of normal human plasma.

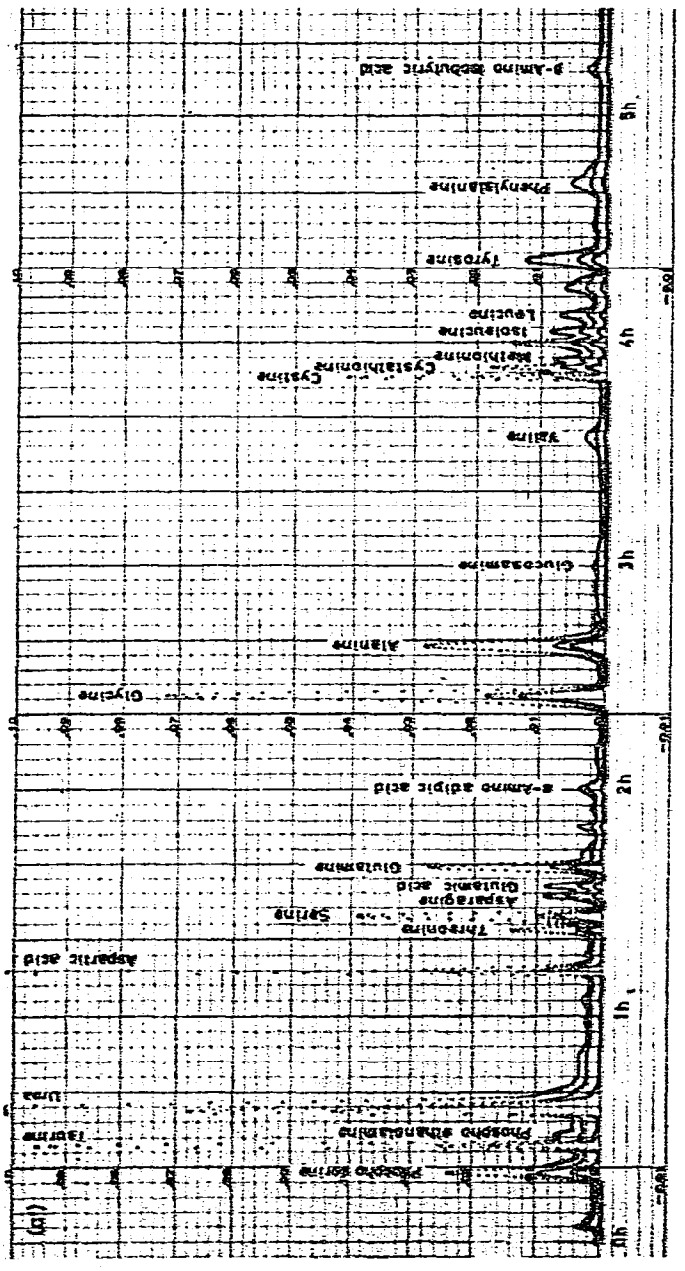

Fig. 3.

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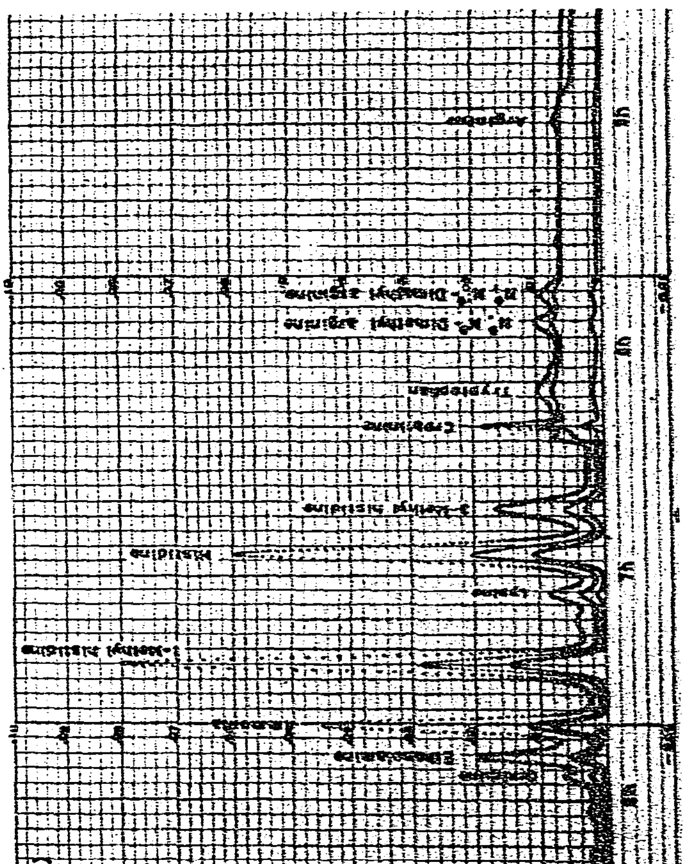

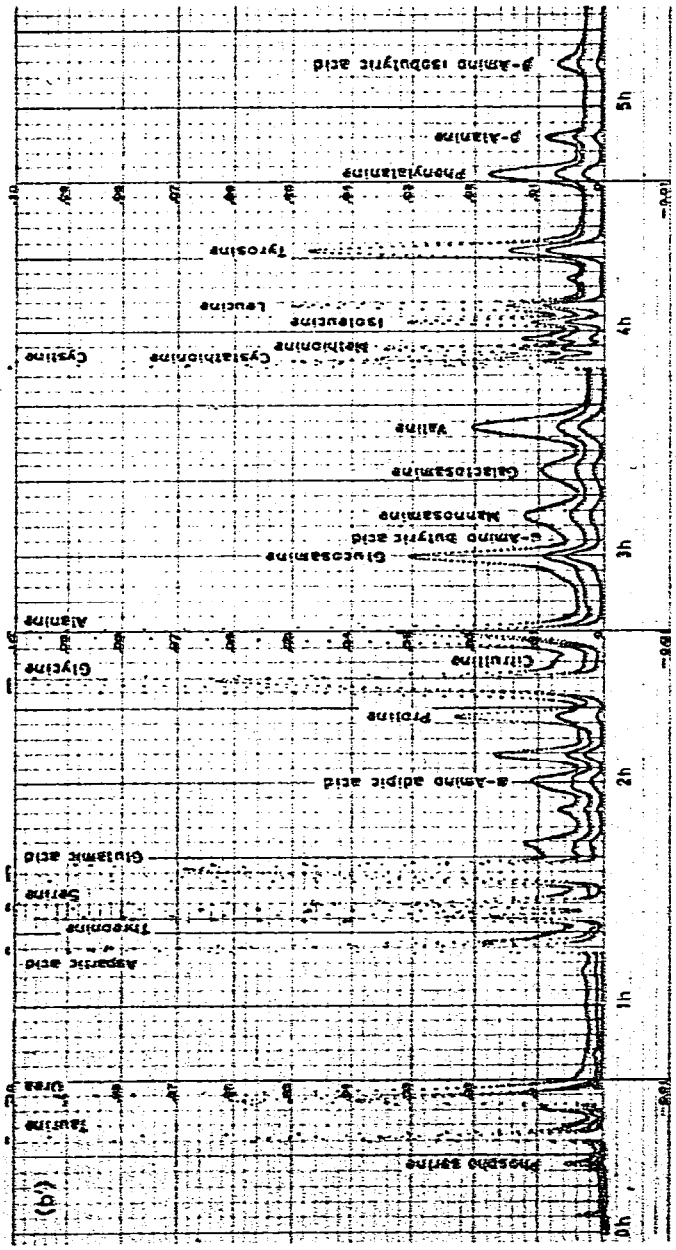

Fig. 3.

(Continued on p. 150)

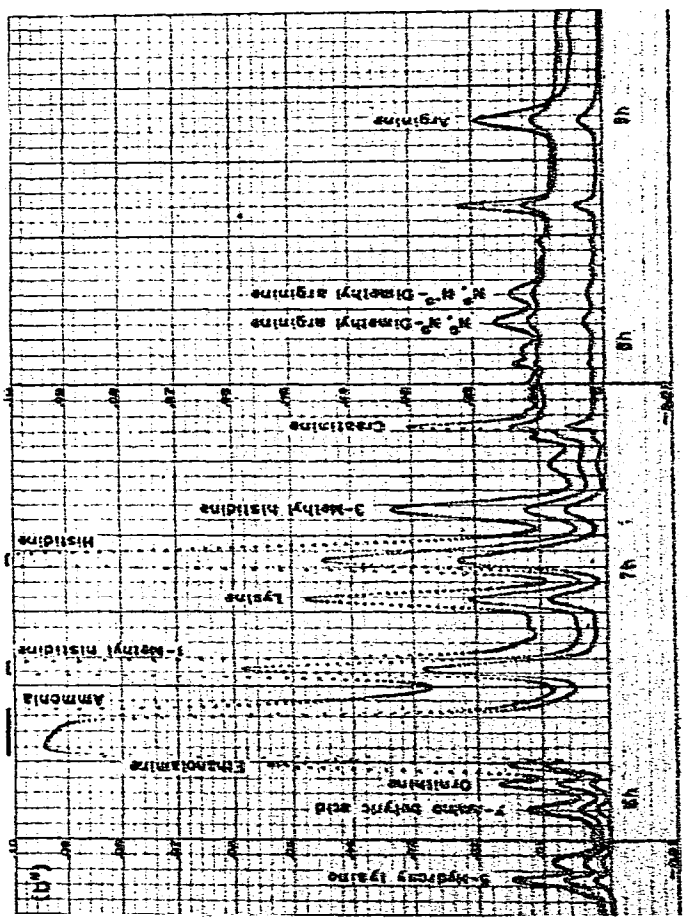

Fig. 3. (a) Chromatogram from 0.25 ml of normal human urine; (b) chromatogram from 0.5 ml of hydrolyzate of normal urine.

cosamine, galactosamine and β-(2-thienyl)serine were resolved by using two steps with lithium buffer for 5.5 h. Mannosamine occurred as a shoulder on the α-aminobutyric acid peak. Glucosamine, mannosamine and galactosamine were completely resolved from each other in the absence of α-aminobutyric acid. In order to separate citrulline from glycine and alanine, the column temperature was increased to 57° for 1 h 26 min.

In the basic amino acid analysis, the resolution of ornithine, γ-aminobutyric acid, ethanolamine and ammonia was very difficult. The column temperature and the concentration of alcohol in the buffer affected the chromatographic behaviour of these compounds; the optimal column temperature was 43° (isothermal) and 3% of ethanol was added to the basic first buffer. The most serious problem was to separate creatinine, anserine, tryptophan and carnosine completely from each other. In this instance, it was necessary to change the buffer at the correct moment; when the buffer was changed too late, anserine overlapped with creatinine and when it was changed too soon, carnosine was eluted simultaneously with tryptophan. We have also achieved the determination of methylated basic amino acids. In comparison with Deibler and Martenson's method [12], N^G,N^G-dimethyl-, N^G,N'^G-dimethyl-, and N^G-monomethylarginine were rapidly and completely separated. However, N-ε-methyllysine occurred as a shoulder on the lysine peak.

Analysis of normal human plasma

Fig. 2a is a chromatogram of deproteinized normal human plasma. Urea, glutamine, glycine and alanine were always present in relatively high concentrations. Although our method gave a very high resolution, two unknown peaks were evident, one of which was eluted immediately before aspartic acid and the other was eluted after the buffer change in the basic amino acid chromatogram. The latter was almost always present in plasma and could be confused with creatinine. The hydrolysis of deproteinized plasma was carried out in order to investigate whether these compounds were amino acid derivatives or peptides. It can be seen in Fig. 2b that these peaks disappeared in the hydrolyzate of deproteinized plasma and glutamine was also converted into glutamic acid. At the same time, the total amino acid content increased about 3-fold before hydrolysis. In addition, glucosamine and mannosamine occurred in relatively large amounts and galactosamine was observed in trace amounts. These results suggest that the two unknown peaks were peptides or possibly mucopolysaccharidepeptides.

Analysis of normal human urine

Fig. 3 shows the resolution of ninhydrin-positive compounds in normal human urine and the hydrolyzate of urine. As shown in Fig. 3a, N^G,N^G-dimethyl- and N^G,N'^G-dimethylarginine were identified in human urine. The normal occurrence of these methylarginines in human urine has been reported by Kakimoto and Akazawa [13]. The total amino acid contents in the hydrolyzate of urine were increased about 2-fold and amino sugars occurred at the same level as in the hydrolyzate of plasma. It can be concluded that glucosamine and galactosamine were derived from mucopolysaccharides that are a family of chondroitin sulphates characterized by a varying sulphate content [14].

DISCUSSION

Although a single-column system for the analysis of amino acids in physiological fluids has high resolution, it is time consuming and requires five or six different buffers [3, 4], and it is difficult to obtain reproducible chromatograms simply by such a method. The reproducibility and rapidity of analysis are greater with the two-column system described here.

In order to separate efficiently a large number of ninhydrin-positive substances, it is important to select the most suitable resin. Benson [8] recommended a 7% cross-linked polymer for this purpose, but we have found that a 10% linked polymer (2618) is more suitable than a 7% cross-linked polymer (2614). When the latter resin was used for acidic and neutral amino acid analyses, proline was not separated from α-aminoadipic acid.

The particle size of the resin also affected the resolution of amino acids. When a smaller particle size was used, a higher resolution was obtained on the chromatogram. In this instance, a higher pressure was generated in buffer pump, frequently resulting in various problems. We have overcome these problems by using a shorter column (40 cm) under an operating back-pressure of 15 kg/cm^2. The optimal particle size of the resin was 11.5 ± 2 μm (2618). In addition, uniformity of the particle size increased the resolution and also lowered the column pressure.

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