CHROM. 3851

A NEW SYSTEM OF AUTOMATIC AMINO ACID ANALYSIS

PART II

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SUMMARY

A method for the analysis of amino acids and related compounds, found in biological fluids, by means of a new two-channel automatic apparatus for ion exchange chromatography previously described, is reported. A crushed Amberlite IR 120 resin has been employed. The elution of both columns with lithium citrate buffers has allowed the separation of glutamine and asparagine and has considerably reduced the analysis time of the basic amino acids. The total analysis (two columns) takes less than 220 minutes and the peaks of the chromatogram can be evaluated simply by measuring their heights above the base-line.

INTRODUCTION

The need for a rapid, but highly precise and accurate automatic system for the chromatographic determination of the amino acids commonly present in protein hydrolysates, and whose chromatograms were easily evaluated, gave rise to the project by which the apparatus described in Part I of this paper[1] was realized.

The present paper deals with the application of this new system to the analysis of amino acids and related compounds found in biological fluids and demonstrates that the same advantages, as were found for the protein hydrolysates, can be attained. The peaks are evaluated, as before, simply by measuring their heights in terms of millimeters above the base-line.

In order to achieve the separation of glutamine and asparagine without sacrificing resolution of other amino acids, lithium citrate buffers were used. This has already been mentioned by MOORE AND STEIN[2] and more recently by BENSON, GORDON AND PATTERSON[3]; the latter authors achieved the separation of the acidic and neutral amino acids by using a spherical Beckman Custom Research Resin Type PA-28 on a Beckman 120 C Model Amino Acid Analyzer.

It has also been possible to use in our apparatus an ordinary crushed Amberlite IR 120 resin, prepared and conditioned in our laboratory. This resin when eluted

with lithium citrate buffers has given very good resolution of the acidic and neutral amino acids and related compounds in a very short analysis time (less than 220 min); beta-aminoisobutyric acid (BAIBA) included.

Furthermore, the conditions for eluting the basic amino acids and related compounds from the short column, employing crushed Amberlite IR 120 resin in the lithium cycle, have been determined; in this way the separation of gamma-aminobutyric acid (GABA), tryptophan, creatinine, ornithine, lysine, ammonia, 1-methylhistidine, 3-methylhistidine and arginine can be obtained in 120 min. Such a separation in such a short time is not possible on Amberlite IR 120 resin when it is eluted with sodium citrate buffer. If analysis is started on the second column analysis after the proline peak has been recorded on the first channel, the chromatogram of the basic amino acids and related compounds and the chromatogram of the acidic and neutral amino acids and related compounds are complete at the same time. The total analysis time is therefore less than 220 min.

Moreover, it should be noted that, owing to the high sensitivity of the system, less than 0.5 ml of serum or urine are needed for the analysis on both columns.

MATERIAL AND METHODS

Sample preparation

Synthetic mixture of amino acids and related compounds. Amino acids obtained from British Drug House (London) and Calbiochem (Los Angeles) were dissolved in pH 2.20, 0.30 *N* lithium citrate buffer. One ml of this solution contains 1 μmole of each amino acid or related compound except in the cases below where the concentrations were 4 μmoles of urea; 0.5 μmoles of cystine; 2 μmoles of beta-aminoisobutyric acid; and 0.5 μmoles of gamma-aminobutyric acid. Samples of 0.2 ml, 0.1 ml and 0.05 ml of this solution were loaded onto each column in order to obtain concentration levels of 200, 100 and 50 nmoles, respectively.

Human blood serum. 50 ml from a pool of human blood sera, obtained from the clinical chemistry laboratory of the local hospital, were divided into small portions which were kept frozen. It has been observed that if serum is kept at 4° only, asparagine and glutamine undergo hydrolysis, probably due to some hydrolytic enzymes present in the serum.

As far as the deproteination is concerned, it is advisable to use methods in which the serum is not diluted. It has been observed that very good reproducibility of the elution times of the acidic amino acids is attained, in comparison with the calibration mixture, by precipitating the serum with the least possible quantity of solid sulfosalicylic acid. This was carried out by preparing an 6% aqueous solution of sulfosalicylic acid and placing 0.5 ml of this solution in each of a number of centrifuge test tubes (Servall No. 201 "Wasserman"). After freezing they are lyophilized and kept on silica gel. Each tube will contain 30 mg of solid sulfosalicylic acid, and is ready to receive 1 ml of serum. After addition of the serum, the tube is shaken for a few seconds and then centrifuged at 10,000 r.p.m. for 10 min at 0° in order to avoid any possible hydrolysis of glutamine and asparagine. 0.2 or 0.1 ml of supernatant liquid is then loaded on each column.

Resin and columns

A crushed Amberlite IR 120 resin was used; it was prepared in our laboratory as previously described[1] starting from industrial grade and mesh. After grinding, the 25–35 μ fraction obtained by hydraulic regradation was washed on a suction funnel, first with 6 N nitric acid and then with 4 N hydrochloric acid and finally with boiling 4 N sodium hydroxyde. The resin in sodium form, after several washings with water, is converted to the lithium form, while still on the suction funnel, by passing 20 bed volumes of 2% lithium hydroxide through it; it is then washed with water and suspended in 5 bed volumes of lithium hydroxide, 0.30 N. This slurry is used to fill both the long and short column. Packing is made by sections, pumping 0.30 N lithium hydroxide at 1.4 ml per minute. Care must be taken to stop pumping and to refill the column with resin slurry before the preceding section is completely packed. After packing, the columns are equilibrated with the starting buffers; it is necessary to pass at least 100 ml of buffer through the resin before starting the analysis.

The column for the separation of the acidic and neutral amino acids has a total length of 50 cm, a diameter of 1.0 cm and a resin bed height of 42 cm, *i.e.* an apparent volume of 33 ml of resin. The column for the separation of the basic amino acids has a total length of 40 cm, a diameter of 0.7 cm and a resin bed height of 27 cm, thus an apparent volume of 10 ml of resin.

Each column is thermostated by a water bath; on each bath the temperature can be held at two different temperatures, the switch being automatically operated by a presettable timer. The temperature of the long column is held at 37.5° for 103 min and then switched to 55° till the end of the chromatogram; the temperature of the short column is held at 53° for 40 min and then switched to 65° till the end of the chromatogram.

TABLE I

LITHIUM CITRATE BUFFERS

pH	*2.20*	*2.80*	*4.15*	*4.50*
Lithium concentration (N)	0.30	0.30	0.30	0.60
Citrate concentration (M)	0.10	0.053	0.10	0.20
Lithium citrate·4 H_2O (g)	28.20	15.05	28.20	56.40
Lithium chloride (g)	—	5.95	—	—
Concentrated HCl (ml)	26.00	11.00	12.00	17.50
Thiodiglycol (ml)	—	2.50	2.50	—
10% Brij solution (ml)	—	2.00	2.00	2.00
Phenol (g)	1.00	1.00	1.00	1.00
Final volume (l)	1.00	1.00	1.00	1.00

Buffers and reagents

Lithium citrate buffers. The composition of the buffers used for the analysis of the acidic and neutral amino acids, as well as the basic amino acids and related compounds, is reported in Table I. The volumes of concentrated hydrochloric acid indicated are lower than the theoretical amounts; in this way the correct pH can be reached by adding only hydrochloric acid without changing the ionic strengtht of

lithium, as would happen if lithium hydroxide were added for the adjustment of the pH. Thiodiglycol and Brij must be added just before using the buffers.

Ninhydrin reagent. This was prepared and controlled according to the method described in Part I of this paper[1]. The methylcellosolve employed is obtained by distillation of a commercial product after the addition of 6 ml per l of 65% phosphoric acid. The fraction distilling at 125–127° is collected and stabilized by adding 5 ml per l of thiodiglycol.

Buffer, pH 5.5, for the color reagent. This was prepared according to the method of SPACKAM, STEIN AND MOORE[4].

Equipment

The same equipment as that described in Part I was employed in this investigation and operated in the same way. The pumps are filled as indicated in Table II, which also provides the relative flow-rates.

TABLE II

PUMP FILLING AND SETTING SCHEDULE

Pump No.	*Filling*	*Flow rate (ml/min)*
1	Color reagent	0.7
2	Buffer, pH 2.80	1.4
3	Buffer, pH 4.15	1.4
4	0.30 *N* LiOH	1.4
5	Color reagent	0.7
6	Buffer, pH 4.50	1.4

Pump No. 2 is preset to pump 190 ml of buffer, pH 2.80 and then to stop after having started pump No. 3. Pump No. 3 is preset to pump 100 ml of buffer, pH 4.15 and then to stop after having started pump No. 4, which will pump 50 ml of 0.30 *N* lithium hydroxide.

Two sensitivity range expansions have been added to both the colorimeters, which now can read at 0.125, 0.25, 0.50, 1.0 and 2.0 O.D. units at full scale deflection, and always give a linearized signal recorded on two solid-line recorders. Three cuvettes of 10 mm light path were employed. Colorimeter No. 1, which reads the eluate of the acidic and neutral amino acid column at 570 mμ and records on recorder No. 1, was set at 1 O.D. full scale deflection sensitivity; colorimeter No. 2 was set at 0.25 O.D. full scale deflection sensitivity when reading the same eluate at 440 mμ. Just after the proline peak has been recorded on both recorders, the 440 mμ filter in colorimeter No. 2 is substituted by a 570 mμ filter, and the cuvette of the second channel is slid into the light beam; at this point the sensitivity of the second colorimeter is switched to 1 O.D. full scale deflection for reading the basic amino acids.

RESULTS

To determine the accuracy and precision of the system, six analyses were performed loading the columns with 0.2 ml of the calibration mixture, six with 0.1 ml

TABLE III

RESULTS EXPRESSED IN TERMS OF PEAK HEIGHTS IN MILLIMETERS OF STANDARD CHROMATOGRAMS RUN AT 3

Amino acid	*Chromatogram No.*										
	1	*2*	*3*	*4*	*5*	*6*	*M*[a]	*S.D.*[a]	*V.C.*[a]	*7*	*8*
	0.2 ml of the calibration mixture									*0.1 ml of*	
Phosphoserine	146.5	145.0	145.5	146.0	144.0	143.0	145.0	± 1.30	± 0.89	73.0	72.0
Taurine	164.5	162.0	166.5	163.0	163.0	165.0	164.0	± 1.60	± 1.00	81.0	82.5
Phosphoethanolamine	168.0	165.0	170.0	167.0	165.0	168.0	167.0	± 1.94	± 1.16	82.5	81.7
Urea	30.0	29.5	30.0	30.5	29.6	30.0	29.9	± 0.36	± 1.19	14.7	14.5
Aspartic acid	129.0	128.0	129.5	127.0	130.0	129.0	129.0	± 1.10	± 0.84	64.5	65.0
Hydroxyproline	60.0	59.0	59.0	60.5	59.5	60.0	59.7	± 0.60	± 1.01	29.5	30.0
Threonine	91.0	89.0	90.0	90.5	89.0	90.0	89.9	± 0.80	± 0.89	44.7	45.0
Serine	100.5	99.0	101.0	100.0	99.0	99.5	99.8	± 0.82	± 0.82	49.7	50.5
Asparagine	134.5	133.0	134.0	132.0	135.0	132.0	133.0	± 1.30	± 0.98	66.0	66.5
Glutamic acid	92.0	91.0	91.0	93.0	91.5	93.0	92.0	± 0.92	± 0.99	46.2	46.0
Glutamine	54.0	53.5	53.0	54.0	53.5	53.0	53.5	± 0.45	± 0.84	27.2	27.0
Sarcosine	21.0	21.7	21.5	21.0	21.5	21.0	21.3	± 0.32	± 1.48	10.7	10.5
Proline	50.5	50.0	51.0	50.7	51.0	50.0	50.5	± 0.45	± 0.89	25.2	25.0
Glycine	64.0	63.5	63.0	64.0	63.7	63.0	63.5	± 0.45	± 0.70	32.3	31.7
Alanine	54.5	54.0	55.0	54.2	53.7	53.5	54.2	± 0.55	± 1.01	27.3	26.7
Citrulline	66.0	65.0	65.5	65.0	66.0	65.7	65.5	± 0.45	± 0.70	33.3	32.5
AABA	69.0	68.0	67.7	68.5	68.0	69.0	68.4	± 0.55	± 0.81	34.0	34.5
Valine	42.5	43.0	42.5	43.2	43.2	43.0	42.9	± 0.32	± 0.75	21.8	21.3
Cystine	112.5	110.0	112.0	111.0	111.5	112.0	111.5	± 0.89	± 0.80	55.6	56.0
Methionine	155.5	154.0	157.0	156.2	155.0	153.0	155.1	± 1.46	± 0.93	78.0	77.2
Isoleucine	127.5	128.0	126.0	125.7	125.2	125.0	126.2	± 1.24	± 0.97	63.5	62.2
Leucine	125.0	127.0	124.5	124.0	125.0	123.5	124.8	± 1.21	± 0.97	63.0	62.2
Tyrosine	80.0	79.0	79.0	78.5	79.0	80.0	79.3	± 0.61	± 0.77	40.0	40.5
Phenylalanine	69.5	69.7	70.0	70.2	69.0	70.5	68.8	± 0.53	± 0.76	34.8	34.5
Beta-alanine	65.5	65.0	66.2	64.7	65.0	64.0	65.2	± 0.62	± 0.94	33.0	33.3
BAIBA	36.5	37.2	37.0	37.5	37.2	37.2	37.1	± 0.33	± 0.90	18.5	18.7
GABA	123.0	122.0	120.5	122.0	121.7	121.0	121.7	± 0.87	± 0.72	61.5	61.0
Tryptophan	44.0	43.7	44.0	43.5	43.0	43.5	43.6	± 0.38	± 0.86	22.0	22.4
Ornithine	84.0	83.2	82.7	84.0	83.5	82.5	83.3	± 0.64	± 0.76	41.8	41.0
Lysine	75.5	75.0	74.5	74.0	75.0	74.0	74.6	± 0.60	± 0.81	37.0	37.3
Histidine	78.0	77.0	76.7	76.5	77.0	78.0	77.2	± 0.65	± 0.84	38.5	38.7
1-Methylhistidine	73.5	74.5	75.0	74.5	74.0	73.5	74.2	± 0.61	± 0.82	36.8	37.3
3-Methylhistidine	69.0	68.5	68.0	69.0	67.7	67.5	68.3	± 0.65	± 0.95	34.5	34.8
Arginine	40.5	40.0	40.3	41.0	40.5	40.7	40.5	± 0.35	± 0.85	20.5	20.8

[a] M = mean; S.D. = standard deviation; V.C. = coefficient of variation.

of the calibration mixture plus 0.1 ml of buffer, pH 2.20 and six with 0.05 ml of the calibration mixture plus 0.15 ml of buffer, pH 2.20. The results of these analyses are shown in Table III and are expressed in terms of peak heights *H* measured with a ruler in mm above the base-line. The standard deviation and the variation coefficient were calculated for each amino acid at each concentration level, and are reported in Table III.

As far as the linearity is concerned, it has not seemed necessary to introduce a statistical evaluation. The results of the three concentration levels (the mean values) were plotted graphically, as illustrated in Fig. 2, in order to show the strict obedience of this system to Beer's law. This also confirms that this apparatus with the resin in a lithium cycle is suitable for the evaluation of the amino acids in biological fluids by

CONCENTRATION LEVELS

9	10	11	12	M	S.D.	V.C.	13	14	15	16	17	18	M	S.D.	V.C.
the calibration mixture							*0.05 ml of the calibration mixture*								
71.9	73.0	73.0	71.5	72.4	± 0.68	± 0.92	36.2	36.5	36.0	36.5	36.2	36.0	36.20	± 0.22	± 0.62
82.0	80.7	81.0	82.5	81.5	± 0.88	± 1.09	41.0	41.6	41.5	40.8	41.0	41.5	41.20	± 0.33	± 0.82
82.0	83.5	83.0	81.5	82.4	± 0.78	± 0.95	42.0	42.5	42.5	42.2	41.7	42.0	42.10	± 0.81	± 0.74
15.0	15.1	15.0	15.0	14.9	± 0.23	± 1.55	7.5	7.5	7.4	7.5	7.7	7.5	7.51	± 0.09	± 1.30
64.0	64.2	63.7	64.0	64.2	± 0.46	± 0.72	32.5	32.8	32.7	32.5	32.2	32.0	32.40	± 0.30	± 0.92
30.2	30.0	30.0	29.5	29.9	± 0.29	± 0.98	15.0	15.2	15.0	14.8	15.0	15.2	15.00	± 0.15	± 0.99
44.0	45.2	44.5	45.0	44.7	± 0.43	± 0.97	22.5	22.8	22.0	22.5	22.5	22.6	22.48	± 0.26	± 1.17
50.0	49.5	50.0	49.5	49.8	± 0.38	± 0.80	25.0	25.4	25.2	24.8	25.0	25.0	25.00	± 0.20	± 0.82
67.0	66.0	66.2	67.2	66.5	± 0.51	± 0.77	33.2	33.5	33.2	33.0	33.0	33.5	33.20	± 0.22	± 0.67
45.5	45.7	45.0	46.0	45.7	± 0.44	± 0.95	23.0	23.2	23.2	22.7	22.7	23.0	22.90	± 0.22	± 0.97
26.8	26.5	27.2	27.2	27.0	± 0.29	± 1.05	13.4	13.5	13.2	13.2	13.4	13.5	13.36	± 0.13	± 1.04
10.5	10.2	10.2	10.5	10.4	± 0.19	± 1.88	5.3	5.3	5.2	5.2	5.4	5.2	5.26	± 0.08	± 1.51
24.7	25.2	24.8	25.5	25.0	± 0.29	± 1.17	12.6	12.8	12.5	12.4	12.5	12.6	12.56	± 0.14	± 1.08
32.0	31.6	32.3	32.0	32.0	± 0.29	± 0.91	15.8	16.0	15.8	15.5	15.8	15.7	15.76	± 0.16	± 1.03
27.0	27.0	26.7	27.2	27.0	± 0.25	± 0.90	13.5	13.5	13.4	13.7	13.5	13.6	13.53	± 0.10	± 0.76
33.0	33.2	33.0	33.3	33.0	± 0.30	± 0.91	16.4	16.8	16.2	16.4	16.5	16.4	16.45	± 0.20	± 1.20
34.2	34.0	34.2	34.7	34.2	± 0.28	± 0.81	17.1	17.0	17.0	17.4	16.8	17.0	17.00	± 0.19	± 1.15
21.5	21.2	21.6	21.2	21.4	± 0.24	± 1.12	10.7	10.5	10.8	10.6	10.6	10.7	10.65	± 0.10	± 0.98
56.5	55.5	55.5	56.2	55.9	± 0.42	± 0.74	27.8	28.0	28.0	27.5	27.8	28.0	27.85	± 0.20	± 0.70
77.5	78.0	76.8	78.2	77.6	± 0.54	± 0.70	38.7	39.0	39.2	38.5	39.0	38.7	38.80	± 0.25	± 0.66
62.5	63.0	63.5	63.0	62.9	± 0.52	± 0.83	31.5	31.5	31.2	31.6	31.6	31.7	31.50	± 0.17	± 0.54
62.5	62.5	63.0	62.0	62.5	± 0.41	± 0.65	31.2	31.4	31.2	31.2	31.0	31.0	31.16	± 0.15	± 0.50
39.8	39.5	40.2	40.5	40.0	± 0.39	± 0.98	19.8	20.0	20.0	19.6	19.8	19.6	19.80	± 0.17	± 0.90
34.4	34.2	34.5	34.0	34.4	± 0.28	± 0.80	17.2	17.5	17.0	17.2	17.2	17.0	17.20	± 0.18	± 1.06
32.5	33.2	33.0	33.0	33.0	± 0.27	± 0.83	16.3	16.5	16.2	16.5	16.3	16.3	16.35	± 0.12	± 0.80
18.5	18.2	18.5	18.3	18.5	± 0.18	± 0.95	9.3	9.5	9.3	9.1	9.3	9.2	9.28	± 0.13	± 1.42
61.0	61.0	60.7	60.2	61.0	± 0.49	± 0.81	30.4	30.0	30.8	30.0	30.0	30.5	30.30	± 0.33	± 1.11
21.8	22.2	21.8	22.0	22.0	± 0.22	± 0.98	10.9	10.6	11.0	10.8	10.6	11.0	10.80	± 0.18	± 1.69
41.5	42.0	41.5	41.6	41.6	± 0.34	± 0.81	20.8	21.2	20.9	20.5	20.7	20.8	20.80	± 0.23	± 1.09
36.5	37.0	37.3	37.0	37.0	± 0.29	± 0.79	18.6	18.4	19.0	18.5	18.6	18.8	18.65	± 0.22	± 1.16
38.0	38.2	38.5	38.7	38.4	± 0.28	± 0.72	19.3	20.0	19.5	19.3	19.3	19.3	19.40	± 0.28	± 1.44
37.1	37.6	37.0	37.0	37.1	± 0.28	± 0.75	18.5	18.5	19.0	18.2	18.2	19.0	18.60	± 0.32	± 1.71
34.0	34.2	34.8	34.5	34.5	± 0.32	± 0.92	17.0	17.4	16.7	17.0	17.0	16.7	16.96	± 0.26	± 1.52
20.2	20.5	20.5	20.8	20.5	± 0.23	± 1.69	10.1	10.0	10.2	10.0	10.0	10.3	10.10	± 0.13	± 1.25

simply measuring the height of the amino acid peaks in terms of mm above the base-line.

Fig. 1 illustrates a chromatogram obtained with 0.2 ml of the standard solution. As can be seen, it is convenient to read not only hydroxyproline and proline at 440 mμ, but also asparagine.

In addition, six analyses of the same pool of human sera were run. Each sample was deproteinized separately as previously indicated and Table IV shows the results of each chromatogram expressed in terms of the peak heights in mm and the corresponding values in mg per 100 ml of serum. The standard deviation and coefficient of variation were calculated and reported for each amino acid and related compound. The number of mg per 100 ml of serum of each amino acid was obtained from the

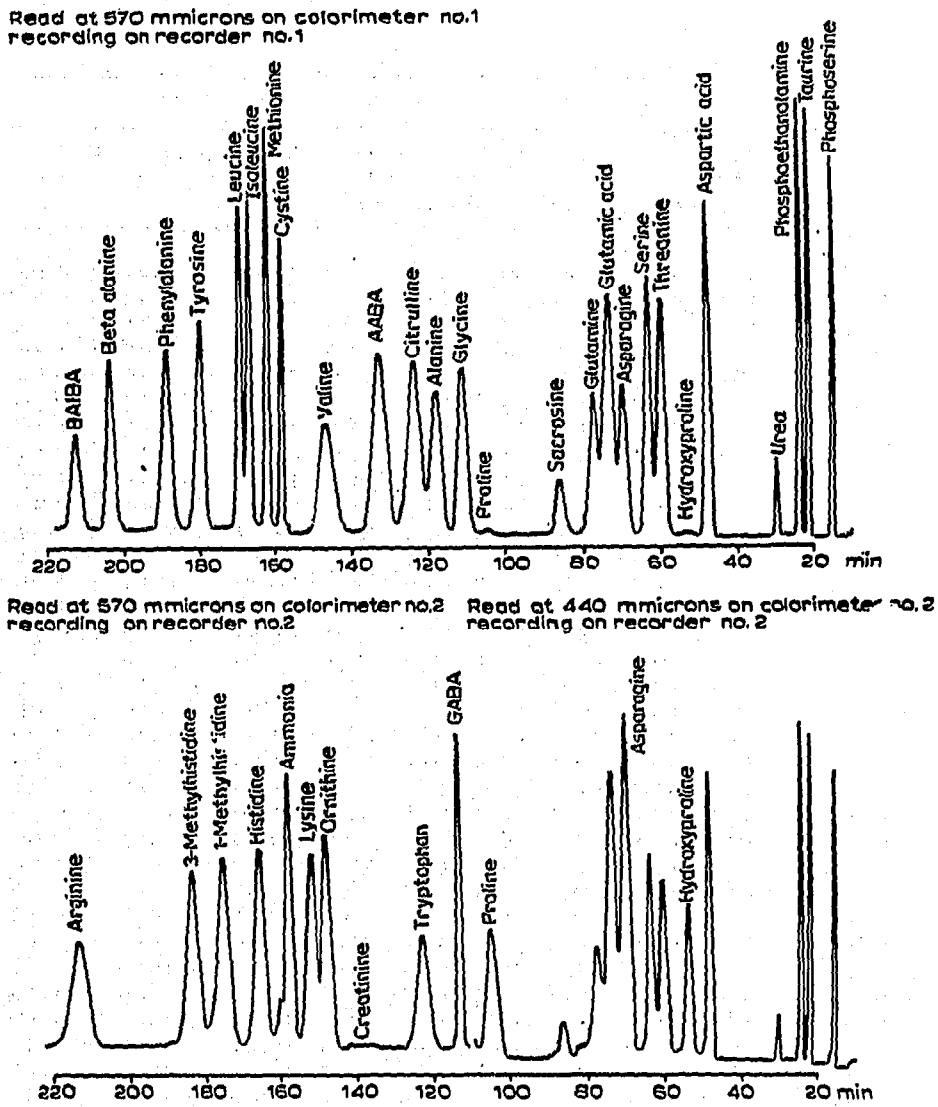

Fig. 1. Recording of the chromatogram of a calibration mixture containing 200 nmoles of each amino acid; 800 nmoles of urea, 100 nmoles of cystine; 400 nmoles of β-aminoisobutyric acid (BAIBA) and 100 nmoles of γ-aminobutyric acid (GABA). Cuvette light paths: 10 mm. Sensitivities: 1 O.D. full scale at 570 mmicrons; 0.25 O.D. full scale at 440 mmicrons. Chart speed: 3 in./h. The basic amino acids and related compounds have been separated during the run of the acidic and neutral ones, so that the total analysis time is 220 min. Operating pressures: 16 $\pm$ 2 kg/cm^2 for the long column and 15 $\pm$ 2 kg/cm^2 for the short one.

following equation:

$$\text{mg \% ml of serum} = \frac{H \cdot nM \cdot \text{MW}}{H_S \cdot V_s \cdot 10^4}$$

in which

H = peak height in mm of the amino acid on the chromatogram

nM = number of nmoles of the amino acid, which have given H_S on the standard chromatogram

TABLE IV

RESULTS EXPRESSED IN TERMS OF PEAK HEIGHTS IN MILLIMETERS OF SIX CHROMATOGRAMS OBTAINED BY ANALYZING 0.2 ML OF A SAME POOL OF HUMAN SERA SIX TIMES

Amino acid	*Chromatogram No.*									
	1	*2*	*3*	*4*	*5*	*6*	*M*[a]	*S.D.*[a]	*V.C.*[a]	*mg%*[a]
Phosphoserine	4.0	4.1	4.0	4.0	4.1	3.9	4.01	± 0.07	± 1.86	0.21
Taurine	51.2	51.0	50.7	50.8	50.5	50.0	51.1	± 0.61	± 1.21	1.57
Urea	5.9	5.8	5.9	5.7	5.8	5.8	5.84	± 0.69	± 1.18	19.50
Aspartic acid	20.8	20.8	20.5	20.5	21.0	20.7	20.78	± 0.21	± 1.02	0.86
Hydroxyproline	traces									
Threonine	33.8	33.0	33.5	34.0	33.5	33.5	33.6	± 0.34	± 1.01	1.80
Serine	40.5	41.0	41.2	40.0	40.5	41.0	40.7	± 0.45	± 1.09	1.72
Asparagine	12.5	12.8	12.2	12.6	12.5	12.6	12.5	± 0.20	± 1.56	0.50
Glutamic acid	28.9	28.5	29.5	28.5	29.0	29.0	28.9	± 0.37	± 1.29	1.86
Glutamine	46.5	46.0	47.0	46.0	46.2	46.5	46.4	± 0.38	± 0.82	2.97
Proline	23.0	23.5	23.0	22.7	23.5	23.0	23.1	± 0.32	± 1.37	0.10
Glycine	48.5	48.0	49.2	47.8	48.0	47.5	48.2	± 0.60	± 1.25	2.26
Alanine	40.4	41.0	40.0	41.4	41.5	41.0	40.8	± 0.58	± 1.42	2.69
Citrulline	4.7	4.5	4.7	4.7	4.5	4.5	4.6	± 0.11	± 2.38	0.49
AABA	2.8	2.8	2.5	2.7	2.8	2.5	2.7	± 0.15	± 5.47	0.16
Valine	22.0	22.5	21.8	22.2	22.5	22.2	22.2	± 0.28	± 1.24	2.43
Cystine	traces									
Methionine	16.0	15.8	15.8	16.2	16.0	15.8	15.9	± 0.16	± 1.02	0.61
Isoleucine	30.5	29.8	30.5	30.0	30.0	30.5	30.2	± 0.32	± 1.05	1.26
Leucine	56.0	55.0	55.0	56.2	56.8	55.0	55.7	± 0.77	± 1.39	2.34
Tyrosine	11.1	11.0	11.5	11.0	11.2	11.5	11.2	± 0.23	± 2.06	1.05
Phenylalanine	10.8	11.4	11.0	10.7	11.4	11.4	11.1	± 0.33	± 2.80	1.07
Tryptophan	9.0	8.8	9.3	9.0	9.2	9.0	9.04	± 0.17	± 1.91	1.97
Ornithine	37.0	36.0	35.8	36.5	36.0	36.5	36.3	± 0.44	± 1.23	2.31
Lysine	65.0	64.0	63.5	64.2	65.0	64.0	64.25	± 0.61	± 0.95	5.10
Histidine	19.2	19.0	19.5	19.0	19.0	19.5	19.2	± 0.24	± 1.27	1.55
Arginine	11.5	11.2	11.5	11.0	11.0	11.2	11.2	± 0.22	± 2.00	1.90

[a] M = mean; S.D. = standard deviation; V.C. = coefficient of variation; mg% = mg per 100 ml of serum.

MW = molecular weight
H_S = peak height in mm of the amino acid on the standard chromatogram
V_s = volume of serum loaded on the column (ml).

DISCUSSION

It was observed that in the lithium cycle the operation of the columns becomes more critical than in the sodium cycle, so that all parameters and operations must be carefully standardized when a high degree of accuracy and precision is desired. Very small variations of ionic strength can produce drastic changes in both the relative and absolute peak positions; consequently, it is necessary to prepare the buffers in large quantities because the specifications and analyses of lithium salts are not as constant, from batch to batch, as those of the corresponding sodium salts. The grade of purity obtainable is also much lower and for this reason the resin should be equilibrated with the least possible quantity of the starting buffer, thus reducing the quantity of metal ions and ammonia which can be introduced onto the column.

Furthermore, this operation must be performed at the same temperature as that at which the chromatogram commences; otherwise a shift of the elution times in the first part of the chromatogram may take place with a consequent loss of accuracy. As this effect is not noticed in the sodium cycle, it can be presumed that in the lithium cycle the exchange capacity of Amberlite IR 120 resin is influenced much more by temperature than in the sodium cycle, so that the levels of lithium ions which can be adsorbed by the resin are more strictly dependent on the temperature. Consequently, when the first chromatogram is finished, the temperature must be brought down to 37.5° before starting regeneration; this is done by means of tap water running through a refrigerating coil immersed in the thermostating baths. Furthermore, a more strict control of the thermostating is necessary than is the case in the sodium cycle, since it has been observed that variations of even a few tenths of a degree in the circulating water may greatly affect the elution times of several amino acids.

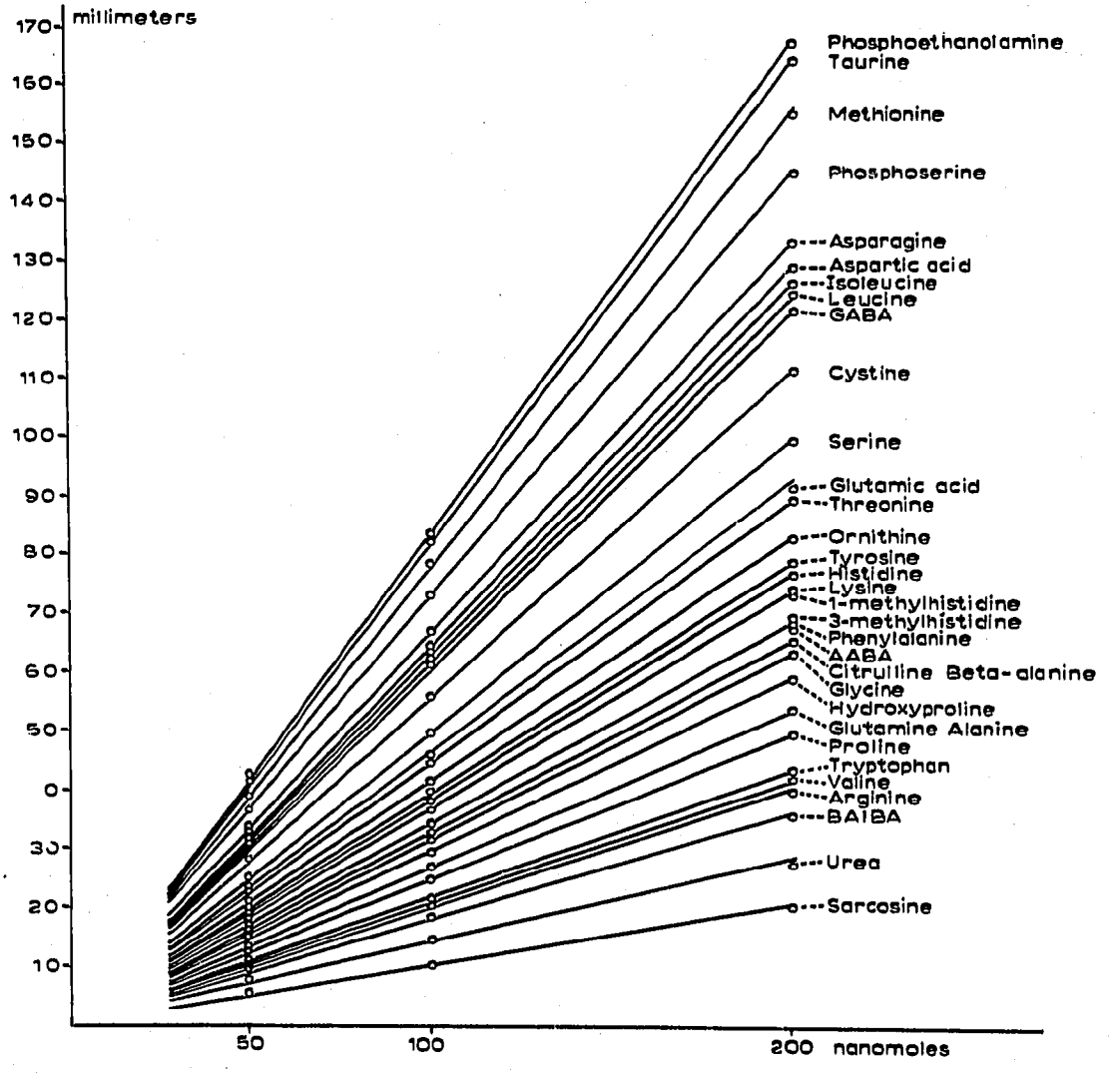

Fig. 2. Plot of the mean values at 3 concentration levels of all the amino acids and related compounds, showing a strict linearity for all of them.

It has been observed that there is no difference of reproducibility if the standard sample is dissolved in either 0.1 *N* HCl, 0.01 *N* HCl or buffer, pH 2.20, provided the volume loaded does not exceed 0.2 ml. Since biological samples often have a pH and ionic strength very different from those of both the calibration mixture and the first eluting buffer, the chromatographic equilibrium may be altered so that it affects the elution times and the shape of peaks of the first amino acids. These alterations can be almost completely avoided if, again, the sample volume does not exceed 0.2 ml.

With regard to the method of serum deproteination which has been described, it must be stressed that it does not interfere with the chromatographic elution of the amino acids and it needs very small quantities of serum, so that it is to be recommended when repetitive analyses on the same subject have to be made[5]. Furthermore in such cases there is no problem about the possibility of partial losses of some amino acids[6], since relative values only are compared. When absolute values are needed, methods like ultracentrifugation[3,6] and ultrafiltration are preferable; but with these methods the pH of serum has to be adjusted to a value which does not disturb the chromatographic equilibrium of the long column; consequently the quantities of serum to be handled must be larger. The method of ultrafiltration described by VAN STEKELENBURG[7], which does not anticipate an adjustment of pH, has been tried with very bad results, consisting in a dramatic foreshortening of the elution times of all the amino acids eluted by the pH 2.80 buffer, with a total loss of resolution for the threonine–serine doublet and the asparagine–glutamic acid–glutamine triplet.

No extensive study on the sample preparation of urine was performed. It can be said, however, that it is not advisable to introduce untreated urine into the column because the high ammonia content interferes with the resolution of ornithine, lysine, histidine and 1-methylhistidine. We tried to remove ammonia by addition of 20% NaOH to the urine in an amount sufficient to bring it to pH 10. After filtration, in order to separate the precipitate of calcium and magnesium polyphosphates, 1 ml of the filtrate was evaporated to dryness under vacuum and then reconstituted with 1 ml of water. This operation was repeated 7 times. Finally 1 ml of buffer, pH 2.2, was added to the dry residue and 0.2 ml of this solution loaded on the columns. In this way the ammonia content is reduced to values which do not give any trouble with the amino acid resolution, but it has been observed that after 6–8 runs, the columns loose resolving power and the resin must be reconditioned with 4 *N* HCl. Therefore it seems advisable to treat the urine by a good desalting procedure prior to analysis.

In agreement with BENSON *et al.*[3], it can be stated that the degree of effective cross-linking in the resin does not affect the amino acid separations in the lithium cycle as much as it does in the sodium cycle. These authors reported that AA-15 and PA-28 Beckman spherical resins give almost identical peak elution times and resolutions when employed in the lithium cycle. The Amberlite IR 120 resin employed in this study has a higher degree of cross-linking, even so the best results have been obtained when using buffers of the same pH and ionic strength as those employed for the Beckman resins by BENSON *et al.*; furthermore almost identical resolution patterns and elution volumes have been obtained for the acidic and neutral amino acids.

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