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Note

Amino acid analysis of physiological fluids by a single-column programme based on stepwise elution with lithium citrate

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In recent years, the gradient elution of amino acids with lithium buffers has become the predominant technique used in automatic amino acid analysis[1]. Nevertheless, step-change analysers are still in use for routine work and require programmes which combine a satisfactory resolution of amino acids with an appropriate elution time. This is particularly true for the analysis of free amino acids from physiological fluids.

Recently, useful systems for the stepwise elution of free amino acids from a single column with lithium buffers have been described by Oulevey and Heitefuss[2] and Young and Yamamoto[3]. Based on the investigations of Lorenz[4], the former work includes a sodium citrate–lithium citrate buffer combination for the separation of the basic amino acids. The buffers employed by Young and Yamamoto, however, contain only lithium citrate. This principle is also utilized in the programme presented in this paper. In comparison with the system of Young and Yamamoto, the pH range is extended by the use of five buffers, and the elution time is shortened to about 8 h. The method was developed for the analysis of physiological fluids and has successfully been applied to the separation of free amino acids of different plant origin[5–7].

EXPERIMENTAL

The analyser used is a Biotronik LC-6000 instrument. The 60 × 0.9 cm column is filled with Durrum DC-1 A ion-exchange resin up to a bed height of 55 cm. In the course of a complete analysis the back-pressure does not exceed 28–30 kp/cm^2 in the buffer pump and 7 kp/cm^2 in the ninhydrin pump. The flow-rates are 100 ml/h for the buffers and 50 ml/h for ninhydrin. The effluents are monitored at 570 and 440 nm and recorded with a dual-pen recorder. The compositions and pH values of the buffers are given in Table I. After the preparation of the buffer solutions, the pH is adjusted to the value given in Table I by adding 37% hydrochloric acid or solid lithium hydroxide.

TABLE I

COMPOSITION AND pH OF THE LITHIUM BUFFERS

Parameter	*Buffer*				
	A	*B*	*C*	*D*	*E*
pH	2.71	2.90	3.04	4.56	3.45
Lithium concentration (*N*)	0.20	0.30	0.45	1.00	1.40
Components per 1000 ml:					
Lithium citrate·$4H_2O$ (g)	18.8	18.8	18.8	18.8	18.8
Lithium chloride (g)	—	4.2	12.7	33.9	50.9
37% HCl (ml)	14.0	14.0	12.5	4.5	9.2
Thiodiglycol (ml)	2.0	2.0	—	—	—
Phenol (g)	1.0	1.0	1.0	1.0	1.0
30% Brij-35 (ml)	3.0	3.0	3.0	3.0	3.0

The programme for the stepwise elution of the amino acids is given in Table II.

Regeneration and equilibration of the resin are performed during steps 8 and 9/10, respectively.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a calibration standard (Hamilton, Reno, Nev., U.S.A.) containing 41 amino acids. The buffer changes and the elution shifts caused by the time interval between automatic change and recording (45 min) are additionally noted in Fig. 1. Unless marked on the elution profile, 125 nmole per amino acid were

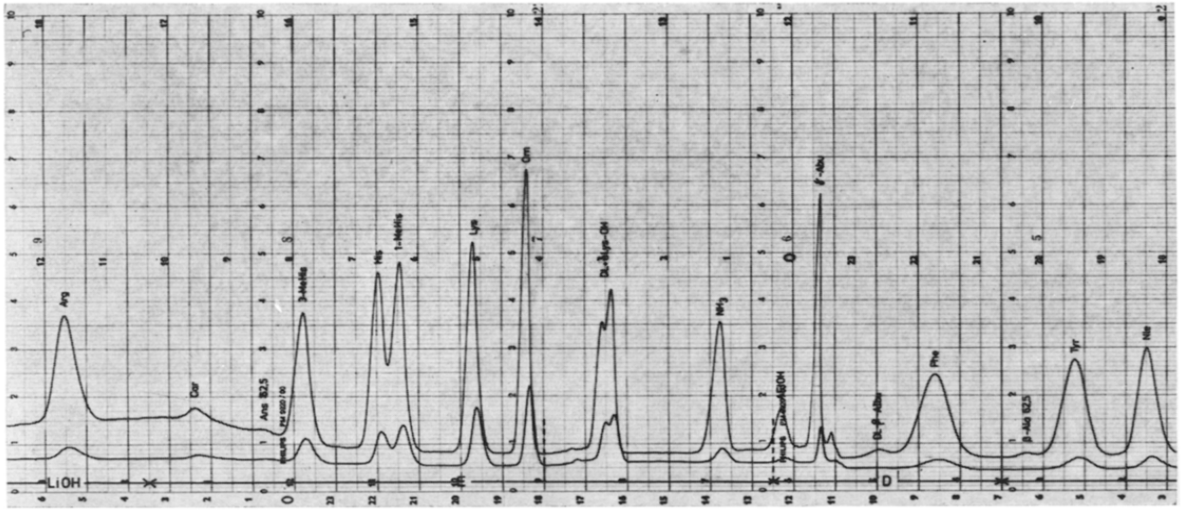


Fig. 1. Separation of a reference mixture of amino acids for the analysis of physiological fluids. The short vertical broken lines indicate the beginning of the elution by a certain buffer after an automatic buffer change. Symbols for less common amino acids: A = amino; I = iso; P = phospho; Me = methyl; OH = hydroxy; a = allo; ad = adipic acid; bu = butyric acid; EtOH = ethanol; Citr = citrulline; Cyst = cystathionine.

TABLE II

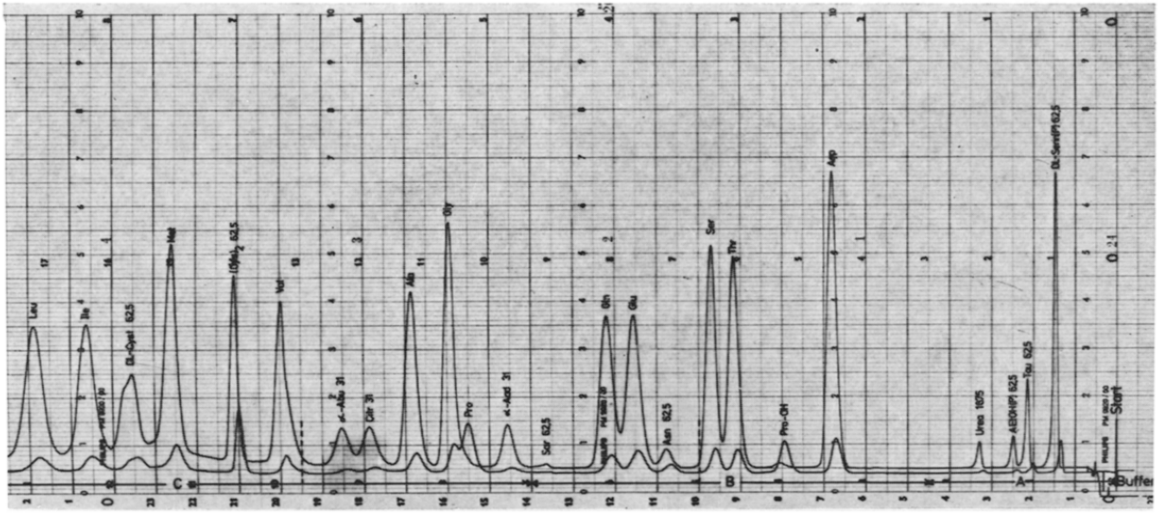
PROGRAMME FOR THE STEPWISE ELUTION OF AMINO ACIDS

Step	*Buffer*	*Column temperature (°C)*	*Time (min)*
1	A	37	35
2	B	37	75
3	C	37	45
4	C	61	90
5	D	61	45
6	E	61	70
7	E	70	50
8	0.4 *N* LiOH	70	40
9	A	70	10
10	A	37	40

applied to the column. The reference mixture and the samples were dissolved in a dilution buffer (pH 2.2) the lithium concentration of which corresponds to that of buffer A.

The overall feature of the chromatogram is the good separation in all areas, including the critical ones such as the asparagine–glutamic acid–glutamine group and the proline–glycine and alanine–citrulline pairs. Moreover, additional work effected some improvement of the resolution of particular amino acids, as follows.

The peak between β-aminoisobutyric acid and γ-aminobutyric acid is due to a buffer effect, which was eliminated by the use of distilled water instead of deionized water for the preparation of the buffers. When organic solvents such as propanol,



methanol or methylcellosolve are added to buffer B at a concentration of 2–3%, proline is resolved up to the base-line on both sides of the peak. Among the basic amino acids, a better separation is achieved between 1-methylhistidine and histidine by using a lower pH of buffer E. Reducing the pH of this buffer by 0.3 results in base-line peaks for the whole lysine–1-methylhistidine–histidine group. By this treatment, the elution of anserine and carnosine is delayed so that carnosine appears before arginine. Arginine itself is eluted later but can be recorded by prolonging step 7 for 20 min. Tryptophan, which is absent from the standard in Fig. 1, appears between ornithine and lysine but is poorly separated under the conditions summarized in Table II. However, as the position of tryptophan is strongly pH dependent, the alteration of the pH of buffer E as described above effects the elution of tryptophan between 3-methylhistidine and anserine. Thus, a better separation is obtained than before. Further work utilizing the sensitivity of the position of the tryptophan peak to temperature is necessary in order to achieve its satisfactory resolution.

In principle, the elution sequence agrees with that of the step-change system described by Young and Yamamoto[3]. However, many less common amino compounds are absent from our reference mixture. As demonstrated by Young and Yamamoto, these substances will seriously overlap with certain well known amino acids or cause crowding of the peaks in particular zones of the chromatogram, for instance in the cystine–leucine group.

Further experiments must be carried out in order to test the ability of the programme to resolve minor compounds, including amino sugars. Except for this problem, the system described here provides a sensitive and reproducible method for the analysis of the common amino acids.

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