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A VERSATILE LITHIUM BUFFER ELUTION SYSTEM FOR SINGLE COLUMN AUTOMATIC AMINO ACID CHROMATOGRAPHY

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SUMMARY

A new chromatographic system employing lithium citrate buffers is described for use on a single 120 cm resin column of the Technicon amino acid analyzer. With this system, adequate resolution of a large number of amino acids up to and including arginine can be achieved in a chromatographic run of 21 hours. The amides asparagine and glutamine are well separated from other amino acids. This chromatographic procedure should prove useful when it is necessary to quantitate each of a large number of amino acids and related compounds in physiological fluids or tissue homogenates.

INTRODUCTION

Most standard techniques for the column chromatographic determination of amino acids fail to separate glutamine, asparagine, threonine, and serine from one another, when all four amino acids are present in a mixture. In addition, some buffer elution systems commonly used on automatic amino acid analyzers fail to separate homocystine, β-alanine, γ-aminobutyric acid and ethanolamine from ammonia. These can be serious handicaps if one is examining physiological fluids or homogenates of tissues, and wishes to determine quantitatively all of the amino acids and related compounds that are present.

BENSON *et al.*[1] and PETERS *et al.*[2] have recently described the separation of asparagine and glutamine from other amino acids by substitution of lithium citrate buffers for the usual sodium citrate buffers. Their techniques were employed for separation of the acidic and neutral amino acids only, using the Beckman/Spinco model 120C amino acid analyzer.

We wish to describe the successful application of lithium buffers to a one-column separation of amino acids on the Technicon amino acid analyzer. With this system it is possible to achieve on a single chromatogram adequate separation of the great majority of acidic, neutral, and basic amino acids likely to be encountered in tissues or physiological fluids, including the amides asparagine and glutamine, as well as several small peptides.

EXPERIMENTAL

Apparatus

The version of the Technicon automatic amino acid analyzer available in 1965 was used in this study. This analyzer utilizes the PIEZ AND MORRIS system[3] of a single chromatographic column, and a continuous buffer gradient. The only modification made to the apparatus was the addition of a second thermoregulator to the recirculating heating bath for the column jackets. This was connected with a reset interval

TABLE I

COMPOSITION AND PREPARATION OF LITHIUM BUFFERS

pH	*Molarity of lithium*	*Preparation*
2.80	0.2	14.09 g $Li_3C_6H_5O_7 \cdot 4H_2O$ + 25 ml 2 *M* LiOH + 10 ml Brij 35 solution[a] + 5 ml thiodiglycol + 900 ml water[b]. Titrate with 6 *N* HCl to pH 2.80 on an accurate pH meter, make up to 1000 ml with water, and make final adjustment of pH.
3.80	0.2	Same as pH 2.80 buffer, except solution adjusted to pH 3.80.
6.10	1.2	14.09 g $Li_3C_6H_5O_7 \cdot 4H_2O$ + 25 ml 2 *M* LiOH + 42.09 g LiCl + 10 ml Brij 35 solution + 900 ml water. Titrate with 6 *N* HCl to pH 6.10, make up to 1000 ml with water, and make final adjustment of pH.

[a] 100 g Brij 35 dissolved in 200 ml water.
[b] Glass-distilled water demineralized by passage through column of Dowex 50 x 12.

TABLE II

BUFFER ELUTION GRADIENT FOR 21-HOURS CHROMATOGRAM

Autograd chamber	*Methanol*	*pH 2.80, 0.2 M Li^+ buffer*	*pH 3.80, 0.2 M Li^+ buffer*	*pH 6.10, 1.2 M Li^+ buffer*
1	5 ml	70 ml		
2	3 ml	72 ml		
3		75 ml		
4			75 ml	
5			75 ml	
6				75 ml
7				75 ml
8				75 ml
9				75 ml

timer and a double-pole, double-throw relay, so that the columns could be programmed to operate at two different temperatures. A nine-chambered Autograd was used to supply the gradient elution buffer. Except for the use of the lithium buffers described below, and for the modifications to the recirculating heating bath, the Technicon analyzer was operated in the recommended manner[4].

Buffers

Lithium citrate buffers were prepared as shown in Table I. The amounts of each buffer placed in the 9 chambers of the Autograd are shown in Table II.

Resin columns

The 140 × 0.6 cm glass columns were filled at room temperature with Technicon Chromobeads Type B resin (17μ spherical particles). The resin column when poured and packed and in operation was about 120 cm in length. Before columns were poured, the resin was carefully washed successively with acetone, water, 6 *N* nitric acid, water, 2 *M* lithium hydroxide, and water. After the resin columns were poured, and after each chromatographic run, the resin in the column was regenerated by pumping 0.2 *M* lithium hydroxide through it for 30 min, followed by pH 2.80, 0.2 *M* lithium buffer for a further 90 min. Columns were regenerated at 70°.

TABLE III

ELUTION TIMES OF AMINO ACIDS AND RELATED COMPOUNDS

Compound	*Elution time of peak (min)*[a]	*Compound*	*Elution time of peak (min)*[a]
Cysteic acid	45	Cystathionine[c]	548
Cysteine sulfinic acid	46	3,4-Dihydroxyphenylalanine	552
Homocysteic acid	46	Isoleucine	561
Phosphothreonine	48	Selenomethionine	570
Taurine	72	Glucosamine	571
Homotaurine	72	Leucine	575
Phosphoethanolamine	81	Cysteine-homocysteine mixed disulfide	590
2-Aminoethylphosphonic acid	96	Norleucine	593
Urea	98	Galactosamine	615
Hypotaurine	100	Tyrosine	615
Aspartic acid	190	Phenylalanine	645
Glutathione (reduced)	198	Homocystine	686
Hydroxyproline	201	β-Alanine	696
Methionine sulfoxide[b]	226, 236	β-Aminoisobutyric acid	714
Threonine	234	5-Hydroxytryptophan	753
Serine	249	Δ-Aminolevulinic acid	757
Asparagine	275	Kynurenine	759
Glutamic acid	288	γ-Aminobutyric acid	795
Homoserine	300	Tryptophan	863
Glutamine	305	S-Adenosylhomocysteine	879
Sarcosine	328	Ethanolamine	912
Proline	367	Ammonia	950
α-Aminoadipic acid	377	Hydroxylysine	957
Glutathione (oxidized)	394	Ornithine	1007
Glycine	403	Lysine	1023
Alanine	419	Histidine	1064
Citrulline	433	1-Methylhistidine	1085
α-Amino-n-butyric acid	443	Anserine	1098
Valine	466	3-Methylhistidine	1102
Cystine	493	Carnosine	1111
Homocitrulline	513	Homocarnosine	1114
Methionine	523	Arginine	1247
Allo-isoleucine	540		

[a] Elution times listed refer to the number of minutes after the chromatogram was started before each peak appeared on the recorder chart. The mean effluent volume in ml at which each compound emerged from the bottom of the ion exchange column can be calculated by subtracting 16 from each figure listed, and dividing the resulting number by 2.

[b] Two peaks are produced by DL-methionine-*dl*-sulfoxide.

[c] Time refers to peak of authentic L-cystathionine. DL- and allo-cystathionine gives a double peak.

Operation of columns

The eluting buffer was pumped through the resin column at a rate of 30 ml/h, for 21 h. The column was operated at 35° for the first 6½ h, and at 70° for the remainder of the chromatogram, the temperature of the circulating heating bath being automatically changed when the reset timer activated the 70° thermoregulator. Maximum operating presssures were 325–375 p.s.i. when the column was operated at 35°, and 175–200 p.s.i. at 70°. The running time of 21 h for each chromatogram, and regeneration time of 2 h, allowed a new sample to be applied to the column each day.

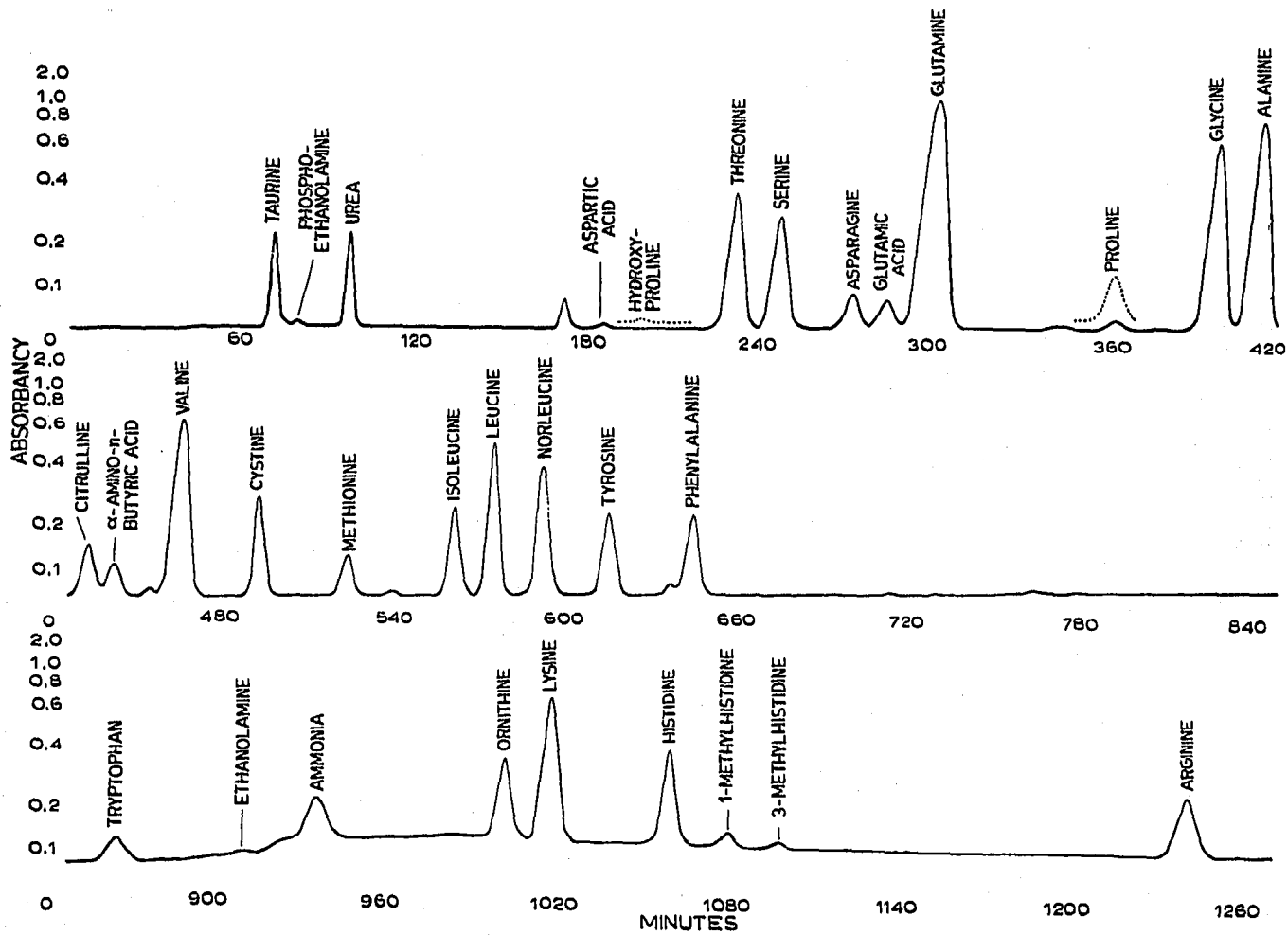

Fig. 1. Tracing of a chromatogram of 1.0 ml of deproteinized fasting human blood plasma. Solid line indicates optical density at 570 mμ. Broken line indicates optical density at 440 mμ. Internal standard = 0.1 μmole of norleucine. Figures below tracings indicate elution time in minutes.

RESULTS AND DISCUSSION

Table III lists the mean elution times of a number of authentic amino acids and related compounds when chromatographed on a 120 cm resin column by the technique described. Table III includes all the amino acids normally found in physiological fluids and tissues, as well as a number of less common amino acids of biological interest. Times listed are those at which the peak optical density for each compound appeared

on the chromatographic chart. With the amino acid analyzer that we used, each compound actually emerged from the bottom of the resin column approximately 16 min earlier than recorded on the chromatogram, and shown in Table III. Slight variations in lengths of resin columns, and in buffer pump rates, resulted in minor variations in the absolute elution times for the amino acids listed, but their relative elution times were unaltered.

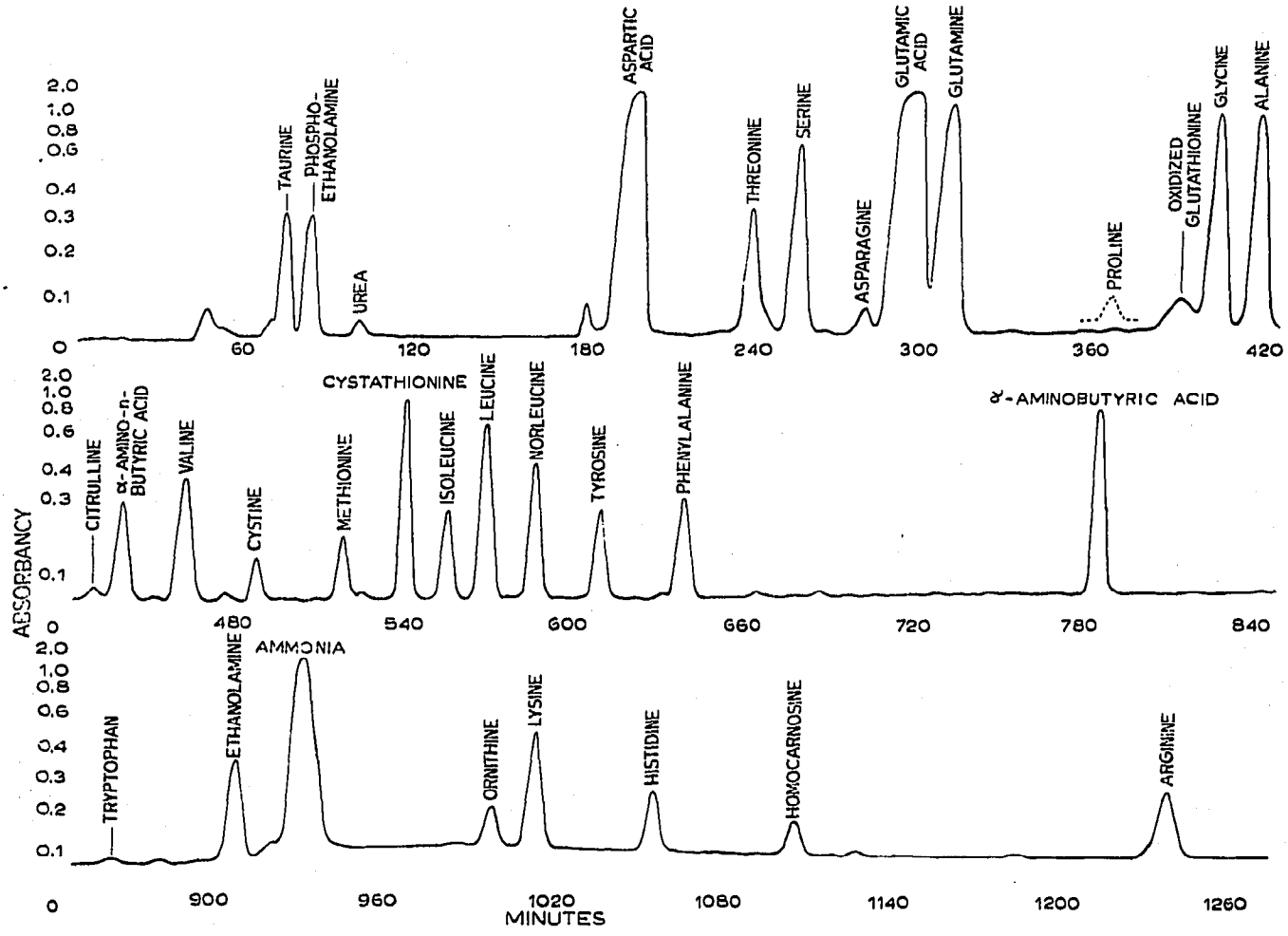

Fig. 2. Tracing of a chromatogram of a deproteinized homogenate of 0.2 g of human frontal cortex. Solid line indicates optical density at 570 mμ. Broken line indicates optical density at 440 mμ. Internal standard = 0.1 μmole of norleucine. Figures below tracings indicate elution time in minutes.

Fig. 1 is a tracing of a chromatogram obtained by this technique from 1.0 ml of fasting human blood plasma deproteinized with sulfosalicylic acid. Thirty mg of solid sulfosalicylic acid was added for each ml of fresh plasma in a stoppered centrifuge tube, the tube was shaken to mix, and the clear supernatant was separated from the denatured protein by centrifugation. Fig. 2 is a tracing of a chromatogram prepared from an aqueous homogenate of 0.2 g of human frontal cortex, similarly deproteinized with sulfosalicylic acid. It can be seen in both figures that the separation of the identified amino acids is sufficiently good to make possible accurate quantitation of almost all of them. The chromatogram of deproteinized brain homogenate was deliberately

over-loaded with respect to certain amino acids (aspartic acid, glutamic acid, glutamine), in order to illustrate some of the lesser components present. Separation would have been improved had only ¼–½ as much brain homogenate been applied to the resin column.

The amides, asparagine and glutamine, can be readily separated with this system from threonine, serine, and glutamic acid. At the same time, a good separation is achieved for almost all the other major ninhydrin-positive components likely to be encountered in physiological fluids and tissue homogenates. An advantage of our procedure as compared to the lithium buffer systems previously reported[1,2] is that acidic, neutral, and basic amino acids are all separated on a single column in one chromatographic run.

Column temperature is maintained at 35° for the first 6½ h, primarily to ensure accurate determination of glutamine, which is easily cyclized to the ninhydrin-negative compound ammonium pyrrolidone carboxylate at higher temperatures[5]. Additional reasons for maintaining a low operating temperature this long are to avoid possible cyclization of the γ-glutamyl peptide glutathione[5] and to improve the separation between alanine, citrulline, and α-amino-*n*-butyric acid. In determining the ratio of the ninhydrin colour yield of the amino acid used as an internal standard to that of glutamine, it is important to select the same column temperature that is to be used in analyzing unknown samples. We found that appreciably less colour is produced when the same amount of authentic glutamine is chromatographed at 45° instead of at 35°. The latter part of each chromatogram is run at 70°, partly to speed up the elution of the basic amino acids, but chiefly in order to elute tryptophan well before ammonia. As pointed out by HAMILTON[6], the speed of elution of tryptophan is unusually sensitive to the temperature at which the ion exchange column is operated.

A minor disadvantage to the use of lithium in place of sodium buffers is that columns of Technicon Chromobeads B resin seem to pack more tightly with lithium buffers. Excessive packing of the column decreases the excellence of resolution of amino acids. We have found several practical steps which help avoid undue column packing. Columns should not be operated longer than necessary at 35°, since operating pressure is higher at the low temperature, and they should be regenerated at 70°. The molarity of the final buffer in respect to lithium should not be increased beyond 1.2. When we used a pH 6.10 buffer 2.4 *M* in lithium, the basic amino acids were eluted from the column sooner, but resolution was poor after a few runs. It is particularly important not to apply the sample to the column dissolved in sucrose solution, as is recommended by Technicon for use with sodium citrate buffer systems[4]. When the sample is blown into the resin column with nitrogen pressure, rather than layering it above the top of the column in sucrose solution, many more good chromatograms can be obtained before it is necessary to repour the column. Regular removal of the top few millimeters of the resin in the column when it becomes discoloured with pump packing, or installation of a resin filter in the high pressure line, also increases the useful life of the column.

Inspection of Table III shows several limitations of our technique. The first 4 amino acids listed are eluted with the buffer front, and are not separable. One of the peaks of the methionine sulfoxides is eluted at virtually the same point as threonine, so that threonine cannot be quantitated accurately if a physiological fluid such as plasma or urine also contains methionine sulfoxide. In measuring amounts of the

unusual sulfur-containing amino acids in the physiological fluids of patients with homocystinuria, one must use an internal standard other than norleucine, since the mixed disulfide of cysteine and homocysteine present in the plasma and urine of such patients is eluted from the column simultaneously with norleucine. Finally, the slight separation of homocarnosine from carnosine is inadequate to obtain accurate quantitation of these dipeptides if both are present in the same specimen. In actual practice, most of the unusual amino acids listed in Table III are either absent from physiological fluids, or are present in such small amounts that they do not interfere with quantitation of the more common amino acids emerging from the ion exchange column at similar effluent volumes.

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