CHROM. 5719

Single column amino acid analysis using lithium buffers and an ammonia filtration system

Two major problems associated with column chromatography of amino acids have been accurate resolution of the amides asparagine and glutamine and the elimination of baseline artifacts due to ammonia contamination. The advent of lithium citrate buffer systems has permitted the resolution of glutamine and asparagine without sacrificing the resolution of other amino acids[1–5]. Two-stage lithium buffer systems described by BENSON *et al.*[1] and PETERS *et al.*[2] failed to offer the simultaneous elution of the basic amino acids. Other methods[3–5] utilizing gradients or sequences of lithium buffers in addition to column temperature changes, achieved complete separation of most common amino acids but required longer analysis time or excessive and costly technical updating. In addition, the persistent release of ammonia from the column during elution with the more basic buffers has not been eliminated from these latter systems.

We wish to describe the successful application of a three-stage, single column, lithium buffer procedure on a Beckman/Spinco Model 120B amino acid analyzer. We have modified the pH and lithium molarity of the initial and second buffers in order to improve the separation of the acidic and neutral amino acids up to phenylalanine. Furthermore, by introducing a third lithium citrate buffer and an ammonia filtration system we achieved the separation of most basic amino acids and eliminated baseline irregularities in that portion of the chromatogram.

Experimental

Buffers and reagents. The lithium citrate buffers were prepared as described by PERRY *et al.*[3]. The final pH adjustments were made to pH 2.85, 3.80 and 4.30 for the first, second and third buffer respectively, and 2.20 for the diluting buffer. Ninhydrin solution was prepared as described in the Beckman 120B handbook. The calibration mixtures of eighteen amino acids were obtained from Sigma Chemical Co. and Beckman Instrument Inc. Other amino acids used in this study were obtained from Calbiochem and added to the calibration mixture prior to analysis. Norleucine was routinely used as an internal standard.

Equipment and procedure. A Beckman analyzer Model 120B was modified to include 6.6 mm cuvettes and two 69 × 0.90 cm columns packed with Beckman Custom Research Resin, AA-15. The resin column height was 55 cm when packed and in operation. A 10 × $1^1/_2$ in. glass column containing 200 g of a special ammonia filtration resin, Hi-Rez, Type DC-3 (Pierce Chemical Company) was introduced in each buffer line between the buffer container and the pump. The regeneration of this resin was accomplished according to the manufacturer's instructions except for the substitution of 5 % lithium hydroxide for sodium hydroxide. A second moto-valve (Beckman part No. 313373) and buffer change timer (Beckman part No. 327446) were introduced to permit fully automatic change from the second to the third buffer (Fig. 1). A buffer flow rate of 60 ml/h and a ninhydrin flow rate of 30 ml/h were used. Regeneration of the AA-15 resin was performed after each chromatographic run by pumping 0.2 *M* lithium hydroxide for 15 min, followed by lithium citrate buffer

pH 2.85 for 45 min at 55°. A separate system, with an independent pump, permits the regeneration of one column while the other is in operation. This system connects the lithium hydroxide and starting buffer through a manually operated valve.

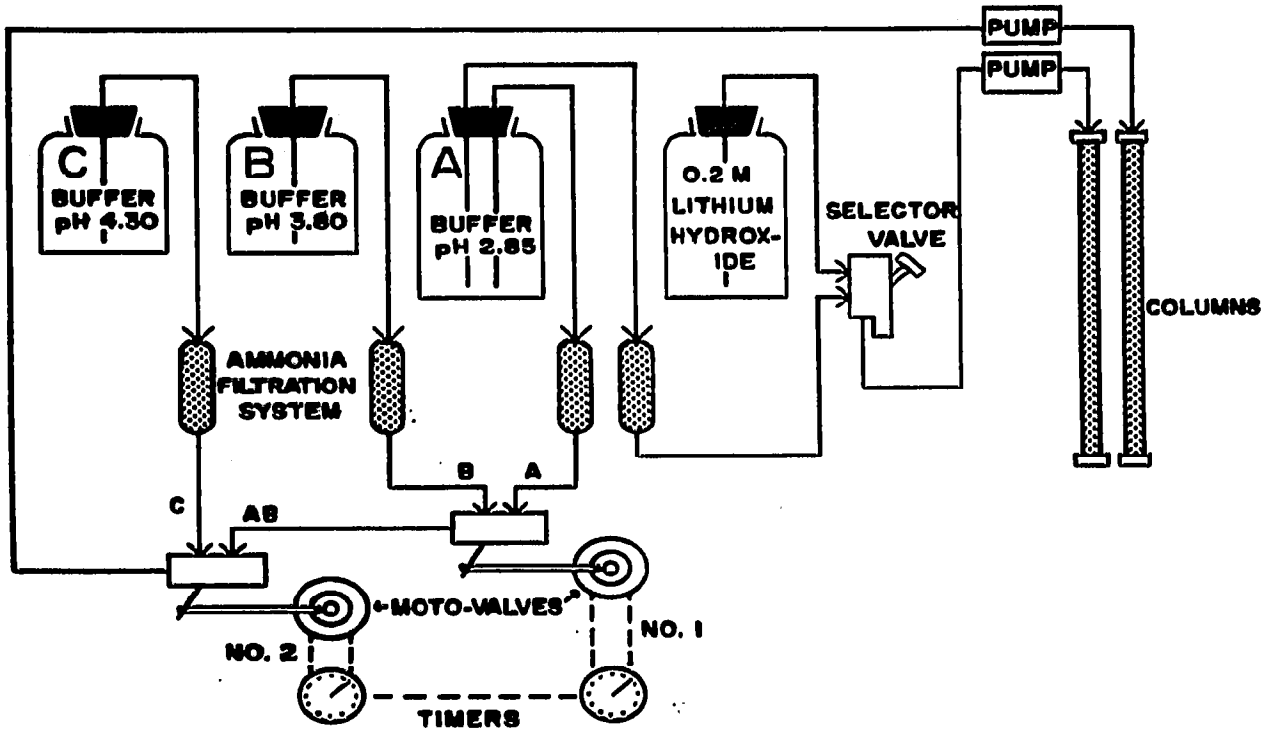

Fig. 1. Illustration of the buffer selection and ammonia filtration system. At 245 min moto-valve 1 is activated and switches from buffer A to buffer B. At 390 min moto-valve 2 is activated and switches to buffer C. The selector valve is manually operated for the regeneration of the column.

Column temperature was maintained at 37° for 150 min and automatically switched to 55° for the remainder of the analysis. Maximum operating pressures were 250–375 p.s.i. at 37° and 150–200 p.s.i. at 55°. The optimal time for buffer changes from the first to the second and the second to the third were 245 min and 390 min, respectively. Total run time through the arginine peak was $11^1/_2$ h thus permitting two chromatographic analyses a day. The samples were prepared in pH 2.20 lithium citrate buffer and applied to the column with nitrogen pressure.

Results and discussion

Table I lists the mean elution time of authentic amino acids and related compounds as observed under our conditions. Minute variations in buffer change times did not result in any significant variation in the relative elution time of the amino acids. Integration constants estimated under the current conditions do not differ significantly from previously reported values using comparable systems[1,2,5]. Fig. 2 is a tracing of chromatograms prepared from a mixture of authentic amino acids encountered in most physiological fluids. The upper third of the chromatogram

TABLE I

MEAN ELUTION TIME OF AUTHENTIC AMINO ACIDS AND RELATED COMPOUNDS FROM THE START OF THE CHROMATOGRAM

Compound	*Time (min)*	*Compound*	*Time (min)*
Cysteic acid	18	Methionine	315
Phosphoserine	21	*allo*-Isoleucine	317
Glycerophosphoethanolamine	26	Cystathionine	321
Taurine	30	Isoleucine	329
Phosphoethanolamine	33	Leucine	336
Urea	43	Norleucine	346
Aspartic acid	79	Tyrosine	358
Hydroxyproline	88	Phenylalanine	382
Threonine	101	β-Alanine	420
Serine	106	β-Aminoisobutyric acid	426
Asparagine	115	γ-Aminobutyric acid	447
Glutamic acid	122	Ethanolamine	473
Glutamine	130	Ammonia	496
Sarcosine	144	Ornithine	511
α-Aminoadipic acid	167	Lysine	520
Proline	177	Tryptophan	538
Glycine	183	Histidine	564
Alanine	200	1-Methylhistidine	567
Citrulline	213	Carnosine	576
α-Aminobutyric acid	232	Homocarnosine	581
Valine	265	3-Methylhistidine	597
Cystine	310	Arginine	685

demonstrates an optimal separation of all acidic amino acids including asparagine and glutamine. At the same time, the cystine to phenylalanine area provides greater physical separation without undue spreading or crowding of the peaks as compared to previous reports using a comparable system[1,2]. The mid and lower portions of Fig. 2 illustrate the effect of the ammonia filtration system on the baseline stability and peak individuality in the basic portion of the chromatogram. The disappearance of the ammonia baseline drift results in a much improved separation of the ammonia to tryptophan portion of the chromatogram, permitting an accurate integration of their peak areas. The introduction of the third buffer immediately after elution of phenylalanine eliminates the undue spreading of β-alanine and β-aminoisobutyric acid observed when these amino acids are eluted with the second buffer[2]. We have found that a reduction in the lithium molarity of the third buffer from 1.2 *M* to 0.8 *M* does not further improve the overall separation of the basic amino acids[6]. The increase in column temperature from 37° to 55° just after elution of glutamine results in a significant reduction in the operating back pressure and packing of the resin column. When this rise in column temperature is delayed until after the elution of

glycine, the valine peak is unduly retarded. At 60° the cystathionine peak is brought under methionine and at 70° tryptophan is eluted with ornithine.

This system has been successfully applied to the measurement of plasma and urinary amino acids. In addition, it has proved useful for analysis of protein hydrolysates and human tissue homogenates. Although interference by small peptides which may occasionally occur in biological material is not eliminated, our system facilitates accurate and accelerated measurement of the majority of the amino acids of interest in clinical investigation.

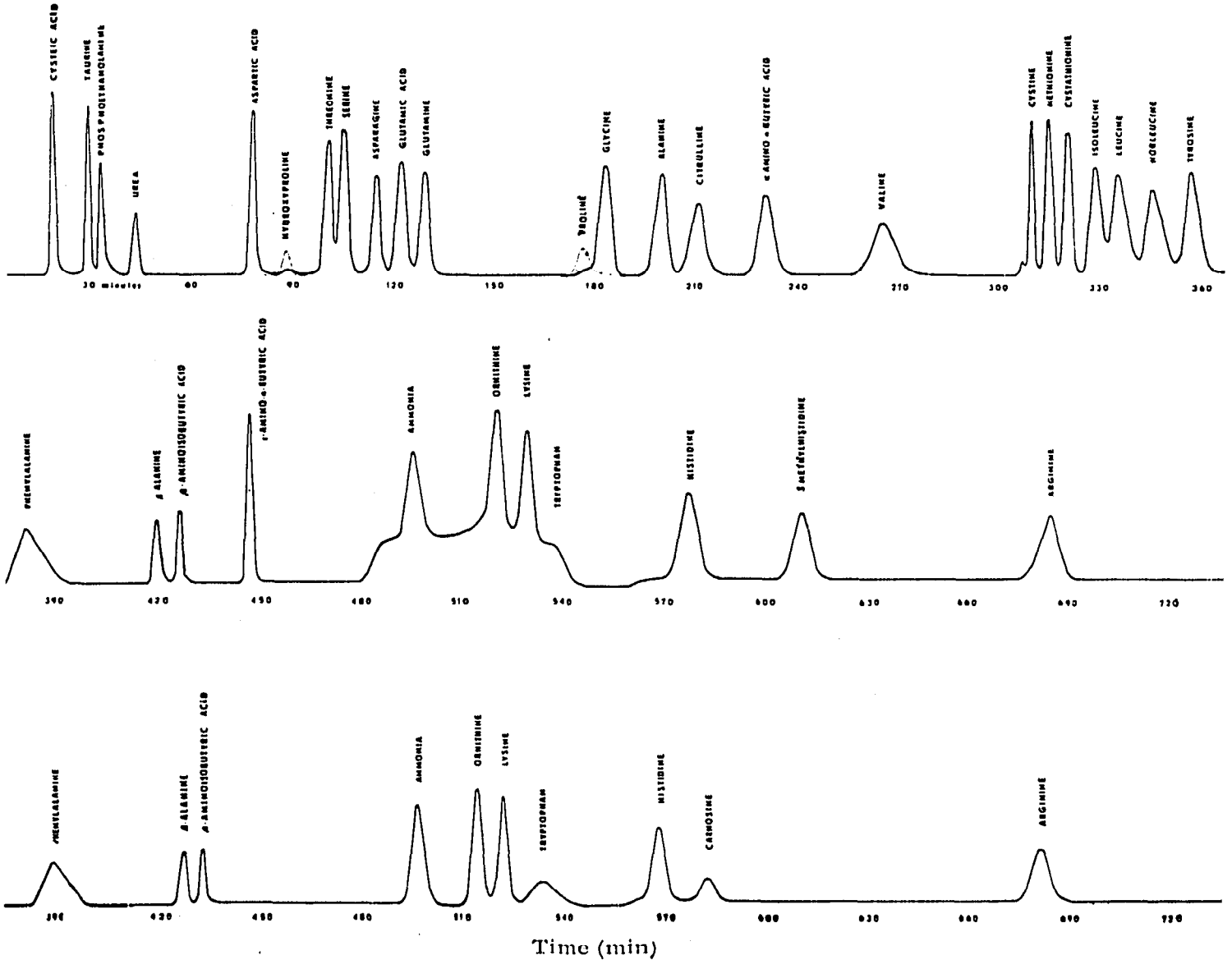

Fig. 2. The upper and lower tracings reproduce a chromatogram prepared from a mixture of authentic amino acids in equimolar amount except for urea (30 ×) and tryptophan (2 ×). The mid portion of the illustration was recorded before the introduction of the ammonia filtration system.

This study was supported by a grant from the Chicago Community Trust. S. B. MELANÇON is supported by The Quebec Medical Research Council. The authors wish to thank Drs. H. L. NADLER and G. J. S. RAO for their encouragement and suggestions.

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Received September 20th, 1971