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Note

Modification of a single column amino acid analyser to improve resolution and sensitivity

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In a quantitative study of the free amino acids in plasma and cerebrospinal fluid (CSF) a Technicon Model 1969 single column amino acid analyser using Chromobeads A (Technicon, Tarrytown, N.Y., U.S.A.) and continuous elution with sodium citrate buffers of pH range 2.875–5.000 (ref. 1) was found to be unsatisfactory. Poor resolution of some of the amino acids at the acidic end of the chromatogram occurred and threonine, serine, asparagine, glutamic acid and glutamine were eluted as a compound peak. Glutamine is a major component of CSF and occurs in a concentration equal to or greater than that in plasma. The concentrations of most of the other amino acids found in CSF are approximately 1/10 the plasma concentrations. The failure to separate glutamine was therefore particularly serious with regard to CSF, where the low concentrations of the other amino acids were at the lower limit of the sensitivity of the apparatus and produced small peaks on the chromatogram making accurate integration of their areas difficult. The analyser had to deal with a range of concentrations from less than 10 μM to greater than 1500 μM.

Changing the ion-exchange resin, alteration of the column lower fitting and analytical stream, temperature programming and the use of lithium buffers solved the problems. Adequate separation of taller sharper peaks which could be satisfactorily integrated was achieved. We should like to report these modifications as they may be helpful to other workers in a similar situation.

METHODS

Column modifications

The resolving power of an ion-exchange resin improves as the mean particle diameter becomes smaller. Aminex A-7 (particle diameter 7–11 μm, Bio-Rad Labs., Richmond, Calif., U.S.A.) resin was substituted for the Chromobeads A (particle diameter 21–23 μm). In a 140 × 0.6 cm column the Aminex A-7 produced unacceptably high operating pressures of greater than 1000 p.s.i. By shortening the glass column to 60 cm and packing it with 50 cm of resin the pressure was reduced to 600 p.s.i. at 35° without jeopardising the separation. Approximately 35 g of resin were needed for a 50 × 0.6 cm column. To prevent leakage of the smaller resin particles which would block the column outlet to the analytical stream, a piece of nylon sheet of pore

size 7 μm (manufactured in Switzerland and marketed by Pronk, Davis and Rusby, London, Great Britain), was inserted as shown in Fig. 1. The PTFE sintered plug was placed on a stainless-steel grid of mesh size 10 μm to provide greater support at the higher running pressures. Discs of this 7-μm pore nylon mesh were fitted over the ends of the regenerating lithium hydroxide and starting buffer lines and held in place by the standard glass weights.

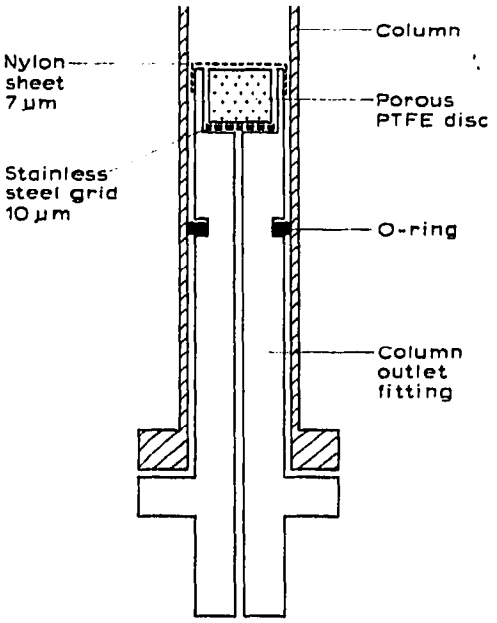

Fig. 1. Modification to amino acid column bottom-fitting showing the extra nylon sheet and stainless-steel grid.

A second thermoregulator was added to the recirculating water-bath for the column jacket and both were linked to a 24-h timer and could be interchanged through a double-pole relay switch. The column could then be programmed to operate at two different temperatures.

0.2 *M* lithium citrate buffers of pH 2.80 and 3.80 and a 1.2 *M* lithium citrate–lithium chloride buffer of pH 6.10 were substituted for the sodium buffers. The composition and volume of each buffer added to the Autograd were as described by Perry *et al.*[2] except that Brij 35 was omitted and lithium fluoride (130 mg/l) and *n*-octanoic acid (0.1 ml/l) were added during their preparation[3]. The column was maintained at 35° for the first 6½ h of the run and at 70° for the remainder.

Analytical modifications

The eluate from the column was fed directly into a hydrazine stream segmented by nitrogen using an A10 T piece (Technicon) instead of being taken through the several feet of transmission tubing necessary with a 140-cm column before joining the segmented hydrazine–ninhydrin stream. The original and modified assemblies are illustrated in Fig. 2 and it will be noted that the column flow is no longer drawn by

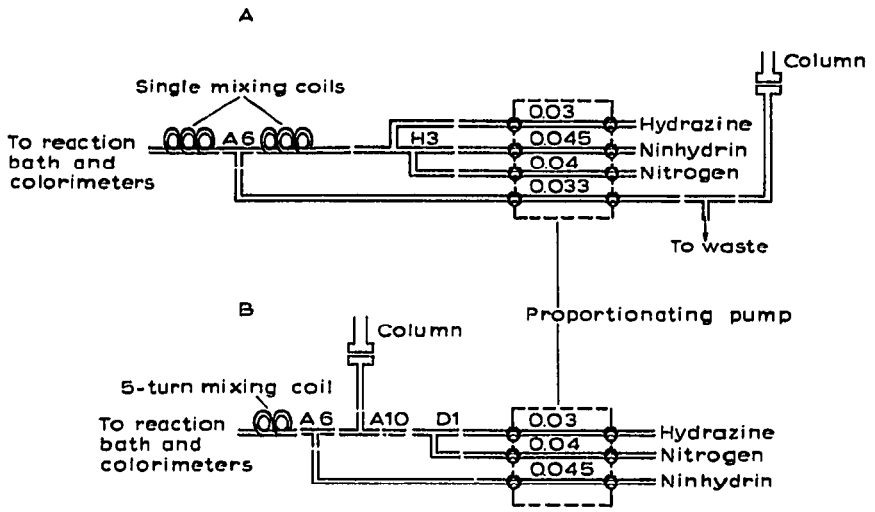

Fig. 2. Comparison of standard (A) and modified (B) amino acid analytical system. Connectors (*e.g.* A6, H3) are standard Technicon AutoAnalyser components. Tube diameters (inches) are the internal diameters of standard Technicon manifold tubing.

the proportioning pump into the analytical stream but is pumped by the column micropump. Only a single 5-turn mixing coil is included in the system after the T piece at which the ninhydrin is introduced. In the original system approximately 80% of the column eluate entered the analytical stream, the remaining 20% going to waste or to a fraction collector as desired. With the modification described the whole column flow enters the colorimeter system. For qualitative analysis to identify unknown peaks, splitting of the column effluent stream can be re-introduced by inserting a T piece (PT2, Technicon) between the column outlet and the A10. The T piece leads to a fraction collector through a variable speed peristaltic pump ("Varioperpex", LKB, Stockholm, Sweden). The rate at which this pump is set relative to the column flow determines the percentage entering the fraction collector.

In all other respects standard amino acid analyser operating procedure was followed[1].

RESULTS AND DISCUSSION

The overall effect of the modifications described is to improve markedly the separation of the acidic amino acids (Fig. 3). Aspartic acid, threonine, serine and asparagine are clearly resolved and, although glutamine sometimes separates completely from glutamic acid, a limitation of the system is that this cannot be achieved on every run. The difficulty in resolving the acidic end of the chromatogram is greater for CSF than plasma possibly because of the disproportionately high glutamine concentration in the former. The remainder of the profile is satisfactory for the most part but the resolution of citrulline from 2-aminobutyric acid and cystine from methionine is variable.

The average running time of 18 h for a complete chromatogram remains a drawback. It has not been possible to reduce this by increasing the flow-rate or alter-

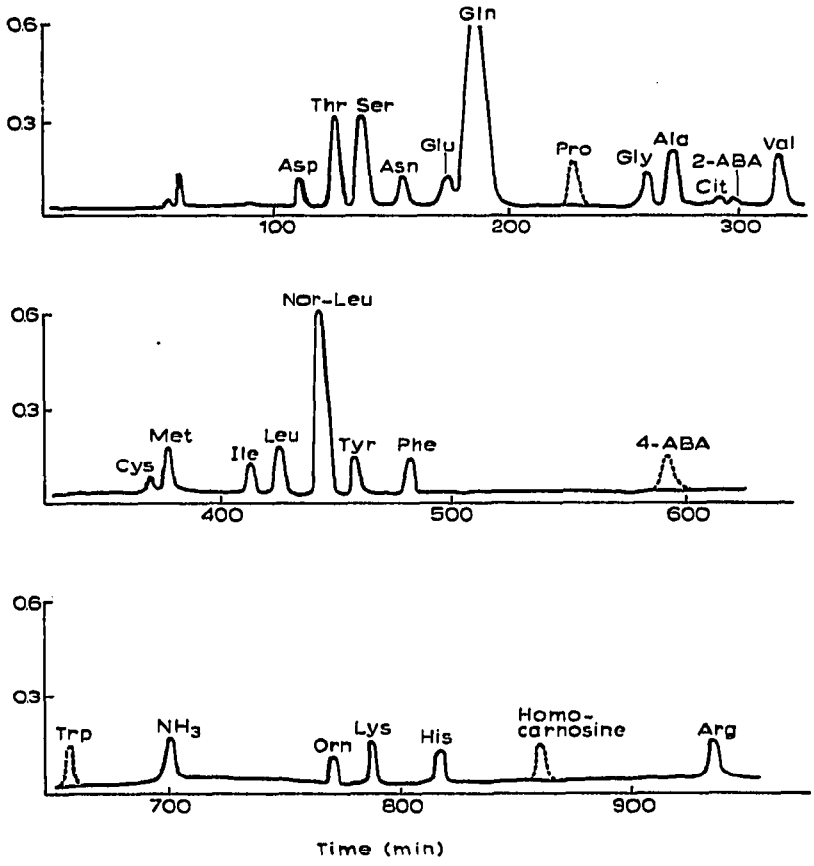

Fig. 3. Diagrammatic representation of CSF amino acid separation. Vertical axis: absorbance at 570 nm. Horizontal axis: retention time (min). Peaks shown as broken lines are amino acids present in standard mixtures, but not normally found in CSF. ABA = aminobutyric acid.

ing buffer pH without impairing resolution or raising operating pressures. Variation of buffer pH was found to alter the elution times of the acidic, neutral and basic amino acid groups but had little effect on the individual amino acids within a group. We have observed similar changes in the group elution times with the finally modified system. As these may be as much as 1 h it is difficult to enumerate the mean elution times of the individual amino acids although their separation remains satisfactory. These fluctuations are inherent in the Autograd buffer system which is susceptible to minor alterations in the eluting gradient.

Taking the total column effluent directly into a segmented stream produces taller sharper peaks in addition to improving resolution. This enables more accurate integration of most of the peaks in 2 ml of unconcentrated deproteinised CSF. We have dispensed with the 570-nm 8-mm flow-cell colorimeter in CSF analyses and also find that it is usually superfluous for plasma. The only peak likely to be off scale is glutamine and this can be quantitated satisfactorily from the 440-nm trace.

With the original Technicon system the operating pressure rose as the column

aged and the amino acid peaks broadened so that the Chromobeads required cleaning and repacking every two to three months. This occurred after much shorter periods with the Aminex A-7 resin so that the column needed to be repacked every 10–14 days. This problem can be alleviated without detriment to the amino acid separation by omission of the 50% aqueous Brij solution, which on standing becomes contaminated by a slow growing septate fungus[3]. The Brij is added to the colour reagents (0.5 ml/l) which pass through in-line filters (Gradko Scientific, Yonkers, N.Y., U.S.A.) before entering the colorimeter system. Lithium fluoride and *n*-octanoic acid inhibit fungal and microbial growth in the buffer solutions. If the modified column lower fitting is carefully removed and cleaned at weekly intervals, we only find it necessary to repack the column every two months.

CONCLUSION

The modifications described above have increased the resolution and sensitivity of a Technicon single column amino acid analyser and we feel that they provide a relatively inexpensive means of improving the analyser's performance in a laboratory where purchase of a new expensive commercial analyser is impossible. The alterations are simple and do not require elaborate equipment.

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