Note

Amino acid analyses using isocratic and gradient elution modes on Kontron AS-70 (7 μm) resin

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(Received December 18th, 1981)

The cation-exchange separation of amino acids was first described by Moore and Stein[1,2] and subsequently by Spackman *et al.*[3]. Analyses were performed at constant temperature on a two-column system, each requiring a separate sample injection, the neutral and acidic amino acids being separated using two buffers on one of the columns and the basic amino acids on the second column with a single elution buffer. The system utilized different resins for each column and ninhydrin reagent[4] for detection, and required both large amounts (micromoles per amino acid) and long analysis times (days).

As the requirements for faster and more sensitive analyses arose, a number of instrumental improvements were made: (a) microporous resins with improved flow characteristics and exchange properties became available (see Benson[5] for a review); (b) the "liquid" end of the system was reduced through the utilization of narrow-bore columns and tubing of smaller internal diameter throughout; (c) units for automatic sample application were introduced; (d) improvements in both flow-cell design and electronic stabilization of the detectors reduced the signal-to-noise ratio; and (e) integrators/computers were introduced for data handling. In many instances a microprocessor or computer was employed not only to operate the instrument but also to carry out a series of built-in diagnostic tests (pressure limits, buffer volumes, etc.). Thus, instruments have developed to the point where a number are currently available that are capable of carrying out routine subnanomole-range amino acid analyses (see Hare[6] for a related review) within 60–90 min.

As the methods used for both synthesis and particle sizing have improved, microporous resins have become available that no longer require the high pressures, resulting from increased linear flow velocities of the eluent, to achieve separations within a reasonable period. Characteristics inherent to the resin itself (particle size, size distribution and degree of cross-linking[5]) result in elevated pressures and thus the necessity for designing an instrument capable of maintaining a performance level typical of present-day high-performance liquid chromatographic (HPLC) units. In this paper we illustrate the usefulness of Kontron AS-70 resin (7 μm) for amino acid

analysis on an HPLC-based instrument[7] at relatively low pressures ($\leqslant 6.9$ MPa) using either a three-buffer isocratic elution programme (60 min run time) or a two-buffer gradient system (55 or 90 min run time). The advantages and application possibilities of each system are discussed.

EXPERIMENTAL

The HPLC-based instrument has been described previously in detail by Hughes *et al.*[7]. It consisted of two microprocessor-controlled pumps, an autosampler, a fluorimeter and a water-bath system capable of quickly changing temperatures three times during a chromatographic separation. When the three-buffer "standard" elution system was used, an eight-port valve system (operated via the microprocessor) delivered the buffers/regeneration solution to one of the pumps for elution of the column while the second delivered *o*-phthalaldehyde (OPA)[8,9] for post-column fluorescence detection. For gradient elution the microprocessor was used to control the two buffer pumps. An additional one was introduced for pumping the OPA detection solution.

The three Na$^+$ buffers for isocratic separations were prepared in the following manner (amounts per litre):

Buffer A (0.2 M Na$^+$, pH 3.20): 19.6 g of sodium citrate, *ca.* 24 ml of 6 M hydrochloric acid, 50 ml of methyl Cellosolve and 2 ml of Brij.

Buffer B (0.2 M Na$^+$, pH 4.25): 19.6 g of sodium citrate, *ca.* 18 ml of 6 M hydrochloric acid and 2 ml of Brij.

Buffer C (1.1 M Na$^+$, pH 7.9): 40.0 g of sodium citrate, 41.0 g of sodium chloride and 2 ml of Brij.

Column regeneration was carried out with 0.2 M sodium hydroxide solution; the sample application buffer and the borate detection buffer (pH 10.3) were prepared as described previously[7]. During buffer preparation all pH adjustments were made prior to addition of detergent. For the separation of the amino acids arising from collagen hydrolysates, notably 4-hydroxyproline, hydroxylysine and carboxymethylcysteine (from alkylation of cysteine with iodoacetic acid), an additional buffer was introduced (prior to buffer A, above) with a pH of 2.8. Normal hydrolysate analyses were performed utilizing buffer changes at 21 min (A → B), 32 min (B → C), 45 min (C → sodium hydroxide solution) and 49 min (sodium hydroxide solution → A); the total run time was 60 min. Temperature changes were made at 13 min (37 → 68°C) and 28 min (68 → 75°C); at 49 min rapid cooling by an external flow of water through a coil in the water bath was started (75 → 37°C). See Hughes *et al.*[7] for a more detailed description of the entire HPLC-based instrument.

Buffers for gradient separations were prepared as follows (to a final volume of 500 ml):

Buffer A (0.2 M Na$^+$, pH 3.0): 100 ml of 1 M sodium hydroxide solution, 67 ml of 0.5 M citric acid solution, 13 ml of 6 M hydrochloric acid, 50 ml of methyl Cellosolve and 1.5 ml of Brij.

Buffer B (0.2 M Na$^+$, pH 10.2): 100 ml of 1 M sodium hydroxide solution, 7 g of boric acid and 1.5 ml of Brij.

Packing of the columns (purchased from Kontron, Zürich, Switzerland) was carried out at 70°C in buffer C used for isocratic elution at flow-rates of 1.0 and 2.0

ml/min for the 3.2 and 4.6 mm I.D. columns, respectively. The back-pressure during packing was not allowed to exceed 17.2 MPa. The chemicals used were purchased from the following sources: Brij 35 and amino acid standard H from Pierce (Rockford, IL, U.S.A.); methyl Cellosolve from Calbiochem (San Diego, CA, U.S.A.); ornithine, norleucine and OPA from Sigma (St. Louis, MO, U.S.A.); all other substances were obtained from Merck (Darmstadt, G.F.R.). Water was doubly distilled in quartz; 6 M hydrochloric acid was distilled in glass prior to use.

RESULTS AND DISCUSSION

Amino acid analysis is widely employed and is the method of choice for determining the concentrations of free amino acids in both natural and hydrolysed samples. The results are often expressed as amino acid concentrations per weight or volume of sample or, as in the case of proteins/peptides, the integral number of residues per polypeptide chain. In all instances the accuracy of an analysis depends on sample injection, amino acid separation, the detection reaction and peak integration. A variation in only one of these factors can (and often does) introduce errors such that the results are no longer usable. Thus, any improvements in either the instrumentation for carrying out sample injection, elution, integration, etc., or the chemistry of resin production, sizing, elution buffers or detection would enhance the accuracy of an analysis.

In an earlier paper[7] we indicated how commercially available HPLC instrumentation can be utilized for amino acid analysis. Determinations in the picomole–nanomole ranges were carried out on Durrum DC-4A resin with run times (injection
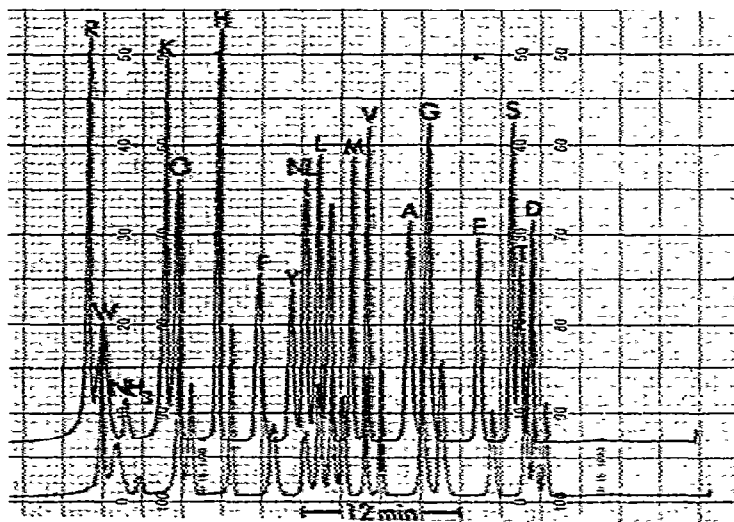

Fig. 1. Isocratic chromatography of free amino acids on Kontron AS-70 resin. The column (15 × 0.32 cm I.D.) was eluted at 0.3 ml/min with Na$^+$ buffers using a three-temperature programme (see Experimental); OPA was introduced at 0.3 ml/min. A 10 nmol standard was applied. The single-letter code for the amino acids is as recommended by the Commission on Biochemical Literature (exceptions: NL, norleucine; O, ornithine).
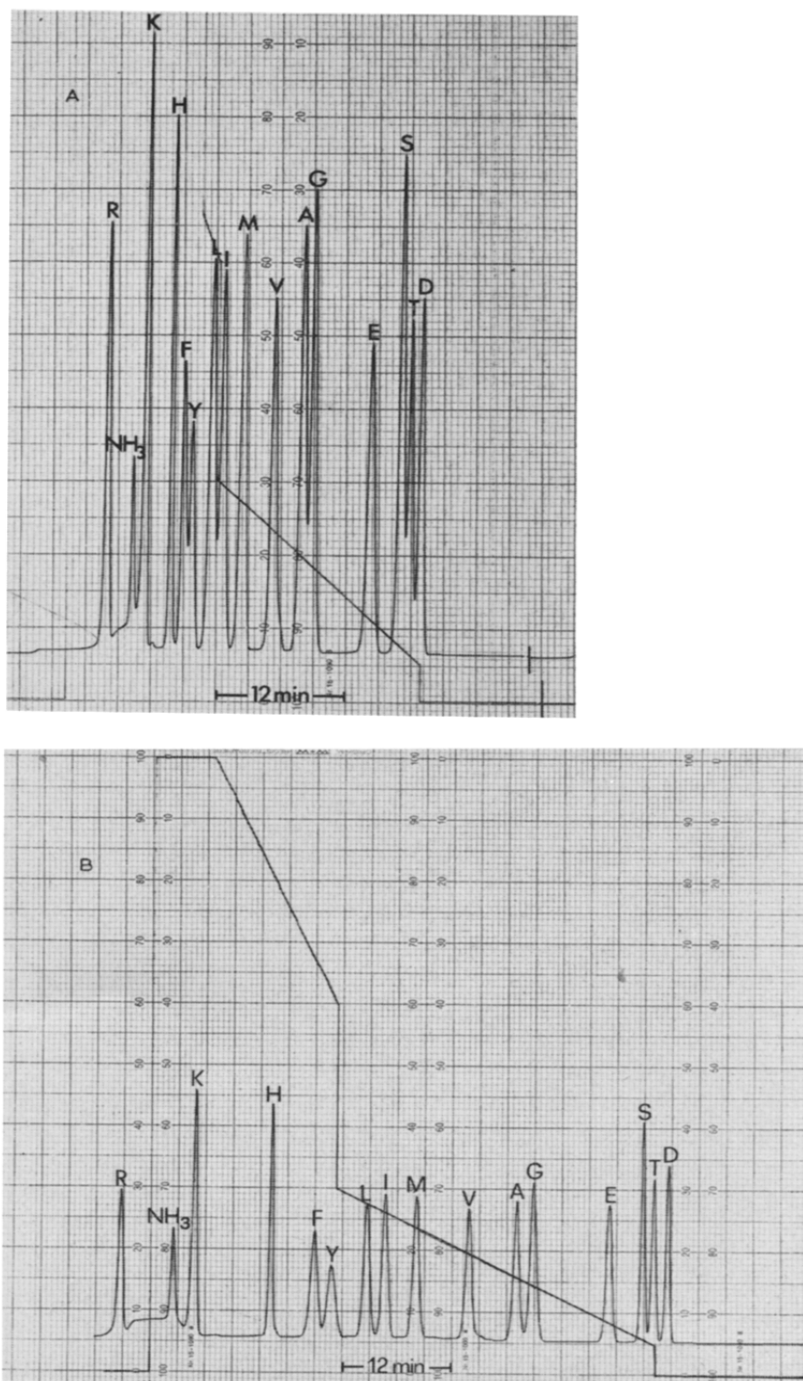
Fig. 2. Amino acid separation on Kontron AS-70 resin using gradient elution conditions. (A) The column (8 × 0.46 cm I.D.) was developed at 0.6 ml/min with the two-buffer system (see Experimental) at a constant temperature of 50°C. (B) A 15 × 0.32 cm I.D. column was eluted at 0.3 ml/min under the same conditions as in A. For both chromatograms 10 nmol amino acid standards were chromatographed; the abbreviations are as given in Fig. 1. The solid straight lines in each chromatogram indicate the gradient expressed as a percentage of buffer B in A + B. The OPA detection solution was introduced at flow-rates equal to those of the buffer pumps, i.e., 0.6 and 0.3 ml/min for A and B, respectively.

to injection) of ca. 60 min when sodium formate/citrate elution buffers were used. One disadvantage of the resin used in that study was the degree of separation of some of the amino acids (Thr–Ser, Gly–Ala, Ile–Leu).

Kontron AS-70 (7 μm) resin, has recently become available as a cation-exchange support, and, when tested in the same HPLC-based instrument using a three-buffer isocratic elution system and three column temperatures, gave results (Fig. 1) as good as, if not better than, those previously reported[7]. The effective back-pressure on the column remained under 6.9 MPa throughout the run and, with the exception of Trp–Arg, the resolution of all peaks was improved.

Through conversion of the instrument into a gradient elution mode (the introduction of an additional pump for buffer B) and utilization of the microprocessor capabilities for designing gradients, it was possible to achieve reasonably good separations at pressures of <6.9 MPa within ca. 45 min (Fig. 2A) or baseline separations within ca. 75 min (Fig. 2B). As only two buffers are required, the necessity for having a buffer selection system was eliminated. Similarly, the high pH of the second buffer allows one to carry out multiple analyses without the necessity for regeneration with sodium hydroxide solution following each separation run. The performance of the analyses at a constant temperature of 50°C, rather than using three temperatures as in Fig. 1 and in the previous work[7], eliminates the need for an elaborate water-bath system capable of multiple temperature increases and rapid cooling via an external water source.

The purpose of our original work[7], and a series of papers on peptide/protein separations[10–14], was to show that an HPLC apparatus could be used extensively as an analytical and preparative tool in protein chemistry. This paper illustrates how such an instrument, through the addition of only an extra pump, can perform amino acid analyses with an efficiency and versatility exceeding that expected from commerically built analysers.

ACKNOWLEDGEMENTS

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