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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS AND PEPTIDE-HORMONE HYDROLYSATES IN THE PICOMOLE RANGE*

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SUMMARY

A high-pressure liquid chromatographic system for the separation of amino acids and peptide-hormone hydrolysates is described. A modified high-pressure liquid chromatograph (Hewlett-Packard, model 1010B) equipped with an automatic gradient-generating system, and the applications of fluorescamine and ion-exchange resins of particle size $8 \pm 2\ \mu m$ permit the detection of amino acids in the picomole range with good resolution. Compared with conventional amino acid analyzers, the times for separation are reduced by a factor of 3–4.

INTRODUCTION

In modern biochemical separation techniques, automatic amino acid analysis has played an important role since its introduction by Spackman *et al.*[1]. Since then, several workers have attempted to improve the sensitivity and reduce the separation times; however, the detection reagent used (ninhydrin) and the available ion-exchange resins have prevented much progress in this direction. On the other hand, many problems can be solved only with lower detection limits, *e.g.*, the trace analysis of body fluids or elucidation of the structure of failure sequences[2] resulting from solid-phase peptide synthesis[3]. The development of high-performance liquid chromatographs and stable resins of small particle size have permitted considerable reduction in separation times, and, by using fluorescamine[4–6] (4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione) as reagent, primary amines can be detected fluorimetrically in the picomole range. As shown in Fig. 1, fluorescamine, a non-fluorescent compound, reacts with amines at pH 9 to 10 and room temperature within milliseconds[6], and the excess of reagent is hydrolyzed within seconds to water-soluble non-fluorescent products. This reagent was first used in a conventional automatic recording apparatus for the analysis of amino acids by Stein *et al.*[7]. In this communication, a method is described for determining amino acids with use of resins of small particle size, high pressure and fluorescamine as the detection reagent.

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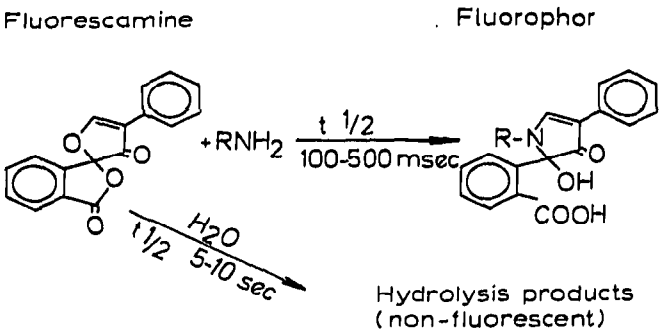

Fig. 1. Formation of the fluorophore from fluorescamine and primary amines.

EXPERIMENTAL

Apparatus

A modified high-performance liquid chromatograph (Hewlett-Packard, model 1010B) with an automatic gradient-generating system (option 005 and 008) is used. Fig. 2 shows a functional diagram of the analyzer. Constant flow-rates of 0.05 ml/min can be achieved with the gradient-generating system only if the buffer mixture is

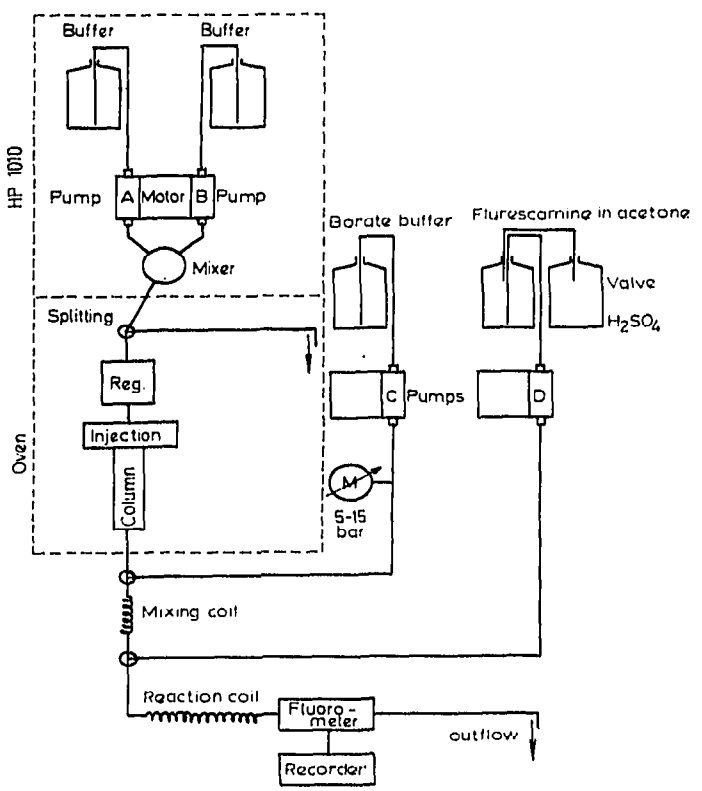

Fig. 2. Functional diagram of the automatic amino acid analyzer.

pumped through a splitting system to a stainless-steel separation column (250 × 3 mm) and a reference column (100 × 4 mm), each filled with the same spherical strongly acid cation-exchange resin (particle size 8 ± 2 μm; 4% cross-linkage) (Durrum, Palo Alto, Calif., U.S.A.; DC-4A resin). Several polystyrene–divinylbenzene resins were tested by us, but the material supplied by Durrum was the most suitable for our purpose.

The gradient-generating system of the HP 1010 B apparatus permits application of the single-column gradient-elution method (with only two buffer solutions) for separation of the amino acids. The flexibility of the gradient generator allows optimum separation conditions to be established rapidly, which proved to be of great value in the investigation of biological fluids. Two citrate buffer solutions (pH 2.6 and pH 6.3; 0.2 and 1.2 *N* in sodium, respectively) are used for the separations described here. After each run, the column is regenerated with 0.2 *N* sodium hydroxide; a constant volume (2.2 ml) of solvent for regeneration is automatically pumped through a stainless-steel coil on to the separation column.

Borate buffer is mixed with the column effluent in a common tee-piece to adjust the pH to 9 and thereby ensure rapid reaction with fluorescamine; this borate buffer is supplied via a Dosapro micro-pump (Milton Roy, Philadelphia, Pa., U.S.A.). The buffered column effluent then passes through a stainless-steel capillary (3 m long; 0.25 mm I.D.), fitted with a pressure gauge. Fluorescamine solution (15–25 mg per 100 ml) in absolute acetone is admitted through a second mixing tee-piece by a Labotron pump to the column effluent already adjusted to pH 9. A stainless-steel capillary (4 m long; 0.3 mm I.D.) serves as a reaction coil. For fluorimetric detection, a Hewlett-Packard 1033 A fluorimeter with a 10-μl flow-through cell is used; constant pressure (12–15 bar) in the detection system is provided by connecting this cell with a coil of length 50 m and I.D. 0.25 mm.

Preparation of the column

A slurry of 4 g of ion-exchange resin in 0.2 *N* hydrochloric acid is filtered and washed with deionized water until it is free from acid. The resin is then placed in 0.2 *N* sodium hydroxide for 30 min, filtered off, washed with citrate buffer solution of pH 2.6 (0.2 *N* in sodium), suspended for 12 h in citrate buffer solution of pH 3.28 (0.2 *N* in sodium) and finally packed into the separation column by means of a slurry-packing apparatus[8]. The same buffer as was used to suspend the resin is pumped through the column for 3 h.

RESULTS AND DISCUSSION

Chromatographic separations

All separations are performed with the column described above, the temperature of the oven in which the columns are installed being kept at 53°. By using an initial flow-rate of 0.75 ml/min and a final one of 1.25 ml/min, separation of 16 of the most common amino acids can be achieved in less than 1 h. After each separation, the column is regenerated by passage of 2.2 ml of 0.2 *N* sodium hydroxide in 3 min and then equilibrated for 15 min with citrate buffer solution of pH 3.28 (0.2 *N* in sodium).

The borate buffer solution is prepared from 0.16 *M* boric acid, the pH of which

is adjusted with 50% sodium hydroxide solution to 9.6; this buffer solution is pumped into the column effluent at about 0.5 ml/min to raise the pH to 9. The fluorescamine solution (25 mg per 100 ml) in acetone is mixed at 8 ml/h with the buffered effluent.

Fig. 3 shows a typical chromatogram of an amino acid mixture and also shows the buffer flow-rates through pumps A and B; it can be seen that the over-all flow-rate changes from 0.75 to 1.25 ml/min.

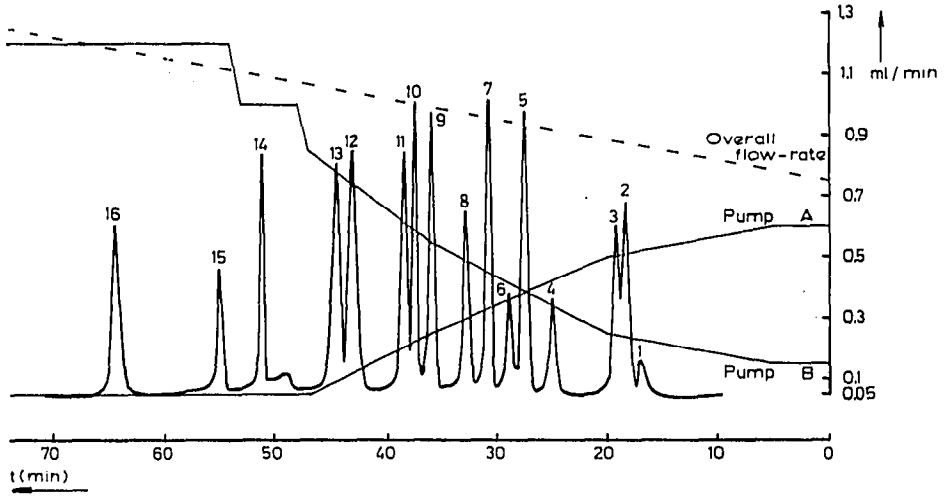

Fig. 3. Chromatogram of an amino acid mixture, with flow-rate diagrams. 1 = Asp, 2 = Thr, 3 = Ser, 4 = Glu, 5 = Gly, 6 = Ala, 7 = Cys, 8 = Val, 9 = Met, 10 = Ile, 11 = Leu, 12 = Tyr, 13 = Phe, 14 = Lys, 15 = His, 16 = Arg.

Relationship of peak area to amino acid concentration

The analyzer is tested with a standard amino acid solution (Beckman Instruments). No fluorescence is obtained with ammonia or proline: proline, being a secondary amine, does not react with fluorescamine, and, according to Stein *et al.*[7], less than 1% of ammonia cannot be detected. The peak areas (in mm^2) and the corresponding constants are shown in Table I; the constants are related to the glycine peak. Each value is calculated from 3 to 5 chromatograms of amino acid mixtures containing each component in the picomole range. The coefficient of variation is calculated to be approximately 4%.

Our experiments show that, for the reaction of fluorescamine with amino acids, linearity of response is achieved in the range 100 picomoles to 5 nanomoles. To test our system, a mixture of threonine, leucine and histidine is used. Fig. 4 shows the variation of fluorescence with amino acid concentration for the reaction with fluorescamine.

Comparison of different methods for amino acid separations and concluding remarks

A heptapeptide amide with the natural sequence of physalaemin, synthesized by the solid-phase method[9], is used to compare the amino acid separation of a conventional amino acid analyzer with that of our system. The peptide amide is

TABLE I

AMINO ACID PEAK AREAS AND CONSTANT FACTORS

Amino acid	*Peak area (mm^2)*	*Constant factor*
Aspartic acid	179	0.62
Threonine	215	0.72
Serine	233	0.81
Glutamic acid	197	0.68
Glycine	288	1.0
Alanine	215	0.75
Cysteine	248	0.86
Valine	281	0.98
Methionine	275	0.95
Isoleucine	340	1.18
Leucine	358	1.24
Tyrosine	314	1.09
Phenylalanine	320	1.11
Lysine	379	1.32
Histidine	203	0.70
Arginine	225	0.78

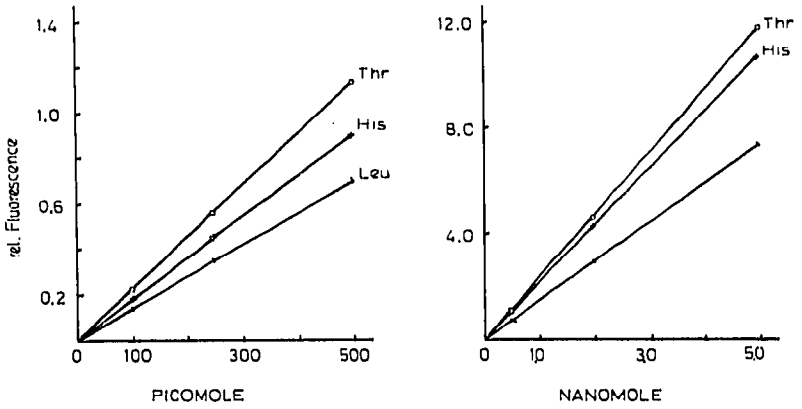

Fig. 4. Relationships between fluorescence and amino acid concentrations for threonine, histidine and leucine in the range 100 picomoles to 5 nanomoles for the reaction with fluorescamine.

hydrolyzed with 6 *N* hydrochloric acid, and 100 nanomoles per component are injected into a Beckman Unichrom amino acid analyzer; 500 picomoles of each amino acid are used for the separation with our system. The two chromatograms are shown in Figs. 5a and 5b.

Comparison of the separations by the two different systems shows that high-performance liquid chromatography in combination with fluorescamine permits separation and detection of primary amino compounds with better sensitivity and in a shorter time. In particular, the system described here will open new possibilities for biochemical analysis. The rapid reaction of fluorescamine at room temperature allows the use of a short reaction coil, and, as a consequence of lower diffusion, well-resolved peaks are obtained. A detection limit of 1 nanomole per amino acid has been reported

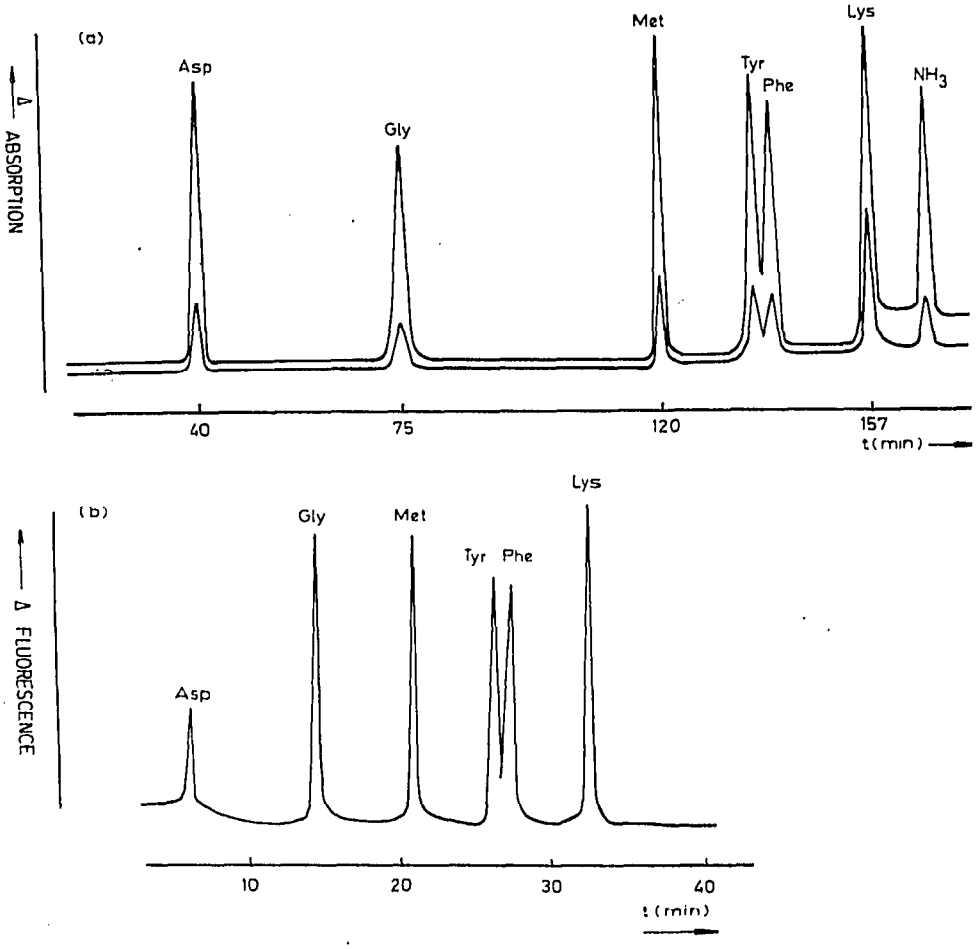

Fig. 5. Separations of a hydrolyzed solid-phase peptide amide with the natural sequence of physalaemin. (a), Chromatogram of a Beckman Unichrom amino acid analyzer after injection of 100 nanomoles of the hydrolyzed peptide amide; detection reagent, ninhydrin; (b), chromatogram of the amino acid analyzer described in this paper after injection of 500 picomoles of the hydrolyzed peptide amide; detection reagent, fluorescamine.

recently for a conventional amino acid analyzer equipped with a computer and with ninhydrin as detection reagent. Thus, by using fluorescamine, the sensitivity is improved by a factor of 100 to 1000. The use of spherical ion-exchange resins of extremely small particle size also considerably improves the resolution of the peaks and shortens the separation time by a factor of 3 to 4.

As fluorescamine is added to the column effluent, the apparatus described permits the separation of amino acids, peptides and proteins on the preparative scale (with use of a splitting system) and is thus advantageous in methods in which dansyl chloride[10], pyridoxal[11] or phthaldehyde[12] is used for detection. As fluorescamine gives little fluorescence with ammonia, our system is especially suitable for investigations on biological fluids; after separation of the proteins, the samples may be directly injected on to the column.

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