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Note

Identification of amino acid phenylthiohydantoins by gradient, high-performance liquid chromatography on Spherisorb S5-ODS

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A problem in the sequence determination of peptides and proteins by the Edman method is that of the identification of the liberated amino acids which are present as their phenylthiohydantoin derivatives (PTH-amino acids). This task has usually been solved by applying techniques such as gas and/or thin-layer chromatography, hydrolysis to the free amino acids, etc. Over the past few years an increasing number of publications have described the introduction and application of high-performance liquid chromatography (HPLC) in this field. The advantage of HPLC lies in the direct identification of the PTH-amino acids without further derivatization and in their quantitative estimation which is possible with modern equipment.

Current methods employ one- or two-column procedures with either gradient elution or isocratic development of the chromatogram[1–12]. We now report a method which allows the identification of the PTH-amino acids on a commercially available reversed-phase system in a single analysis with a gradient elution program.

MATERIALS AND METHODS

The gradient system was formed with the following solutions: A = 0.02 *M* lithiumacetate buffer, pH 5.2 (checked and adjusted at 25°), obtained by appropriate dilution of a 4 *M*, pH 5.2, stock solution (for amino acid analysis; Pierce, Rockford, Ill., U.S.A.); B = 80% acetonitrile (p.a.; E. Merck, Darmstadt, G.F.R.) and 20% 0.1 *M* lithium acetate buffer, pH 5.2. PTH-Amino acids were obtained from Fluka (Buchs, Switzerland), except for PTH-hydroxyproline which was from Sigma (St. Louis, Mo., U.S.A.). PTH-Carboxamidomethylcysteine was synthesized from carboxamidomethylcysteine by coupling with phenyl isothiocyanate according to conditions used in the manual Edman degradation[13].

Standard mixtures of PTH-amino acids were obtained by preparing 4 m*M* stock solutions of the individual derivatives in acetonitrile–water (50:50). Aliquots of the stock solution, containing 200 nmoles per PTH-amino acid, were mixed together in small vials, lyophilized and stored at 4°. Before use the samples were dissolved in 2 ml of the starting eluent (solution A containing 25% solution B). Usually 20 μl of this mixture (with 2 nmoles of each PTH-amino acid) were injected per analysis.

The column (stainless steel, 250 × 4.6 mm) was packed with Spherisorb S5-ODS (5-μm particles, bonded with octadecylsilane; Phase Separations, Queensferry, Great Britain) by the balanced-density slurry-packing method.

The liquid chromatograph was a Hewlett-Packard Model 1084 A apparatus equipped with two heatable solvent reservoirs, two independently operated pumps for gradient formation which allowed flow-rates from 0.1 to 10 ml min^{-1}, a heatable column compartment, a detector operating at a fixed wavelength of 254 nm and an automated injection mechanism. A built-in microcomputer connected to a Hewlett-Packard 79850 A Terminal allowed the programming of the liquid chromatograph as well as the registration of the chromatograms and their quantitative evaluation.

RESULTS AND DISCUSSION

A chromatogram of a typical separation of the individual PTH-amino acids of a standard mixture (see Table I) is shown in Fig. 1a (PTH-hydroxyproline was used as an internal standard especially when a quantitative evaluation was attempted). The gradient program is shown in Fig. 1b. The time axis of the two graphs is on the same scale and thus permits the elution pattern to be related to the actual chromatographic conditions. A delay of *ca.* 2 min (at a flow-rate of 1.5 ml min^{-1}) has to be taken into account which is due to the time taken by the solvent void volume of the chromatographic system to pass the detector cell.

The chromatogram is started with solvent A containing 25% solvent B. An

TABLE I

COMPOSITION OF A 23-COMPONENT STANDARD MIXTURE OF PTH-AMINO ACID DERIVATIVES WITH INDIVIDUAL RETENTION TIMES (ACCORDING TO A REPRESENTATIVE ANALYTICAL RUN)

PTH-cysteic acid appears exclusively in the aqueous phase and therefore does not interfere with the identification of PTH-Asp.

No.	*Compound*	*Retention time (min)*
1	PTH-Aspartic acid	1.98
2	PTH-Cysteic acid	(~1.95)
3	PTH-(S-carboxymethyl)cysteine (CM-C)	2.46
4	PTH-Glutamic acid	2.73
5	PTH-Asparagine	4.06
6	PTH-Serine	4.43
7	PTH-Threonine	4.80
8	PTH-Glutamine	5.11
9	PTH-Glycine	5.61
10	PTH-(S-carboxamidomethyl)cysteine (CAM-C)	5.95
11	PTH-Histidine monohydrochloride	6.73
12	PTH-Alanine	7.25
13	PTH-Hydroxyproline (HYP)	7.65
14	PTH-Tyrosine	9.11
15	PTH-Arginine	10.21
16	PTH-Valine	11.60
17	PTH-Methionine	11.75
18	PTH-Proline	12.14
19	PTH-Isoleucine	14.99
20	PTH-Tryptophan	15.28
21	PTH-Phenylalanine	15.44
22	PTH-Leucine	15.68
23	PTH-N^{ε}-Phenylthiocarbamoyllysine	16.64

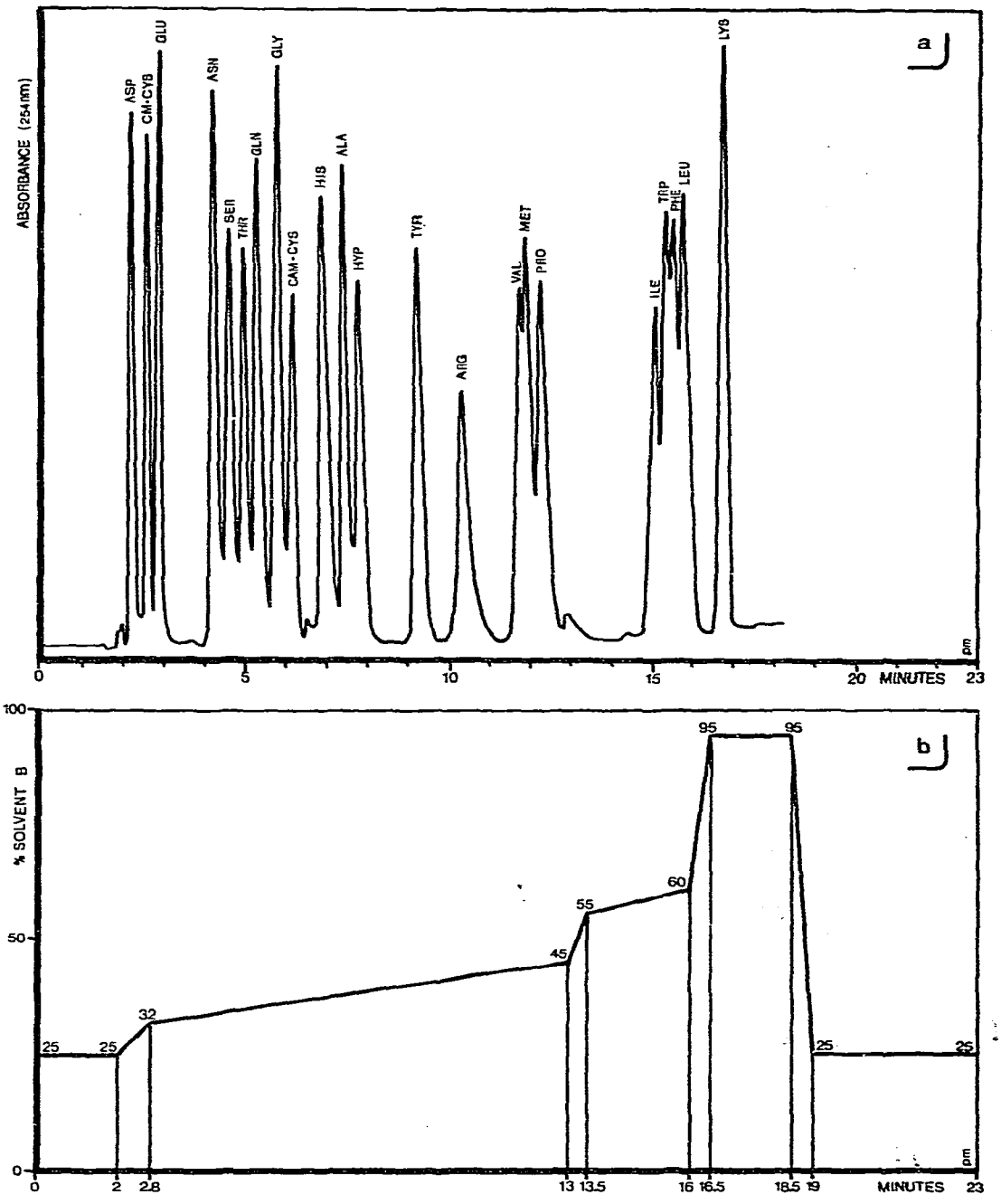

Fig. 1. (a) Typical standard chromatogram of 22 PTH-amino acids. Sample volume: 20 μl (containing *ca.* 2 nmoles per amino acid). Column: 250 × 4.6 mm, Spherisorb S5-ODS (5 μm particles). (b) Gradient program (including regeneration and equilibration). Solvents: A = 0.02 *M* lithium acetate, pH 5.2; B = 80% acetonitrile, 20% 0.1 *M* lithium acetate, pH 5.2. Flow-rate: 1.55 ml min^{-1}. Column pressure: *ca.* 130 bar; temperature, 37°.

initial isocratic phase (0–2 min) is followed by a sharp increase from 25 to 32% solvent B in order to reach the starting conditions for gradient I which leads to 45% solvent B. Under such conditions, most of the PTH-amino acids (PTH-Asp to PTH-Pro) are eluted. The relatively uniform spacing of the peaks is primarily governed by the slope of gradient I. The elution of the last group of derivatives (PTH-Ile to PTH-Lys) within acceptably short retention times requires relatively large amounts of solvent B. Its percentage is therefore raised sharply to a value of 55%. This is the starting point of gradient II (leading to 60% solvent B) the slope of which has to be kept moderate to guarantee a distinction between PTH-Ile, -Trp, -Phe and -Leu, whereas the separation of PTH-Lys is less critical. The regeneration of the column by washing with 95% solvent B is started before the last PTH-amino acid is eluted. The system is finally equilibrated with 25% solvent B.

In this way a near or even complete separation of most of the compounds tested was accomplished, except for PTH-Val and -Met, and for PTH-Ile, -Trp, -Phe and -Leu. As is evident from a number of publications, the separation of these derivatives is difficult and, depending on the analytical system used, is realized with different degrees of success. In our system the elution characteristics of PTH-Val and -Met and also of PTH-Ile, -Trp, -Phe and -Leu appear similar, yet the differences in the individual retention times, of the order of 0.2 min, are sufficient for unequivocal identification of any of these derivatives. The same elution program is also used for the identification of PTH-cysteic acid, -His and -Arg which, after conversion, are constituents of the aqueous phase.

Besides the dependence on the solvent composition, the elution pattern is further influenced by changes in pH and temperature; variations in the ionic strength of the buffer and in the flow-rate, however, have almost no effect.

Influence of pH. The peak positions of the acidic and basic amino acids are primarily affected. With decreasing pH, the retention times of PTH-cysteic acid, -Asp and -Glu are increased, as, but to a lesser extent, is also that of PTH-His. On the other hand, PTH-Arg as well as PTH-Asn and -Gln are eluted earlier and also PTH-Lys shows a drift, although less pronounced, towards shorter retention times.

Influence of temperature. With increasing temperature, the retention times are generally shortened. In addition there is a contrary tendency in terms of the separation power within two groups of PTH-amino acids. The resolution of PTH-Val and -Met decreases at elevated temperature, and PTH-Gln is shifted towards the position of PTH-Thr resulting finally in a fusion of the peaks at *ca.* 50°. On the other hand, the separation within the group of PTH-Ile, -Trp, -Phe and -Leu is generally improved by increasing temperature, except for PTH-Trp which, due to a pronounced drift towards shorter retention times, may interfere with the identification of PTH-Ile at temperatures greater than 45°. As a compromise, due to these limitations, a relatively narrow temperature interval between 35 and 39° gives optimal conditions for the entire chromatogram.

Influence of the amount of solvent B. The amount of solvent B in the eluent mixture is particularly critical for the resolution of the components within two groups of PTH-amino acids. In order to separate PTH-Val and -Met a threshold value of the percentage of solvent B should not be exceeded. On the other side, considerably higher amounts of solvent B are needed as early as possible for the elution of the group comprising PTH-Ile, -Trp, -Phe and -Leu. These requirements are considered

in the elution program by choosing a low percentage of solvent B in the starting eluent and by selecting a gentle slope of gradient I. Only after the elution of the PTH-Val/-Met group is it possible to reach the desired level of solvent B for eluting the remaining PTH-amino acids.

Fine adjustment of the elution program. The possibilities and limits are outlined in principle in the above sections. A fine adjustment is preferably achieved by one of the following measures or by a combination thereof: variation of the column temperature by *ca.* $\pm 1°$, changing the concentration of solvent B by *ca.* $\pm 5\%$ (of the concentration present), alterations in the position and height of the individual steps or gradients or a decrease in the pH in solvents A and B. The last mentioned possibility may become necessary when the elution of PTH-Arg is retarded with increasing age of the column. A slight shift to more acidic pH will push the PTH-Arg peak away from the PTH-Val/-Met/-Pro group.

The method described above has been successfully used in our laboratory for the identification of PTH-amino acids arising mainly from automated Edman degradation of fragments of model proteins with known primary structure as well as of plasminogen fragments of unknown sequence. It has also been applied with success to the identification of PTH-amino acids produced by manual Edman degradation according to Peterson *et al.*[13]. UV-absorbing impurities which may occur with this technique are also characterized in our system by defined retention times, and they normally appear in positions which do not interfere with the established elution pattern of the PTH-amino acids. In all these applications the method has proved its high resolving power and reproducibility even after close to 1000 analytical runs on the same column. Operating costs are low and the time required for one analysis (23 min including regeneration and equilibration) easily allows one to keep pace with the rhythm of the automated Edman degradation.

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