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SEPARATION OF PHENYLTHIOHYDANTOIN-AMINO ACIDS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the separation of all phenylthiohydantoin (PTH)-amino acids except PTH-arginine and PTH-histidine by high-pressure liquid chromatography on a silica column is described. Elution is performed with a concave solvent gradient from hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). A complete run is achieved in 40 min with a pressure drop of 1000 p.s.i. over the 250 mm × 2.1 mm column. Eluted peaks of 2–5 nmole are easily detected by their ultraviolet absorption at 254 nm. This method is superior to existing gas–liquid and thin-layer chromatographic techniques since all PTH-amino acids except PTH-arginine and PTH-histidine may be both separated and quantitated in a single run of 40 min.

The use of the technique in conjunction with an automated peptide sequence analyser is illustrated.

INTRODUCTION

Phenylthiohydantoin (PTH) derivatives of amino acids occupy a prime position in the sequence determination of polypeptides. Edman's degradation method for the analysis of peptides and proteins sequentially produces the amino acid residues as their phenylthiohydantoin derivatives[1]. The currently used methods for their separation, identification and quantitation are, however, not wholly satisfactory[2,3].

Thin-layer and paper chromatograms easily perform the separation and identification (although not in single solvent runs), but quantitation is difficult and development times are relatively long.

Gas–liquid chromatography (GLC) is relatively fast and gives good separation and quantitation but only for those PTH-amino acids with non-polar side-chains. Polar PTH-amino acids must be further derivatised to increase their volatility and re-chromatographed, and then quantitation may not be trustworthy. Moreover, the PTH derivatives of threonine and serine are decomposed by the high running temperatures while those of glutamine and asparagine are converted (in varying degrees) to the corresponding acids.

We present here a method for the separation and quantitation of PTH-amino acids using high-pressure liquid chromatography (HPLC) on silica columns.

All PTH-amino acids except PTH-arginine and PTH-histidine may be separated and quantitated in a single run of 40 min, without the need for derivatisation. The method is sufficiently mild that PTH-threonine, PTH-serine, PTH-asparagine and PTH-glutamine are not degraded.

EXPERIMENTAL

Solvents and standards

Hexane was the petroleum fraction with b.p. 62°–68° (Shell Mex and B.P., Hemel Hempstead, Great Britain). All other solvents were analytical grade. PTH-amino acids were purchased from Sigma (St. Louis, Mo., U.S.A.).

High-pressure liquid chromatography

A Model 830 liquid chromatograph (Du Pont de Nemours, Wilmington, Del., U.S.A.) fitted with a gradient elution accessory was employed. Separations were carried out on 8 μm silica spheres (Zorbax SIL, Du Pont de Nemours) packed in a steel tube (250 mm × 2.1 mm I.D.) kept at 40°. Samples (1–10 μl) in methanol, were injected through a perfluorelastomer septum into the high-pressure line before the column and eluted by the gradient. Except for a momentary release of pressure at the time of injection, solvents were pumped through the column at a pressure of 1000 p.s.i., giving a flow-rate of 0.6 ml/min. Eluted PTH-amino acids were detected by their ultraviolet absorption at 254 nm in an 8-μl volume flow cell.

RESULTS

Separation of PTH-amino acids

Edman's degradation method cleaves the N-terminal residue of a peptide as its anilinothiazolinone derivative which is then treated with aqueous acid to convert it to a PTH derivative. Most PTH-amino acids may then be extracted from the aqueous solution with ethyl acetate whilst PTH-arginine and PTH-histidine are retained in the aqueous phase and must be dealt with separately. The ethyl acetate phase is dried by a stream of nitrogen and re-dissolved in a small volume of methanol.

The separation of ethyl acetate-extractable PTH-amino acids by HPLC is shown in Fig. 1. The peaks illustrated were obtained with 2–5 nmole PTH-amino acids demonstrating the sensitivity of the system. The mixture is injected in methanol solution and elution is performed with a concave solvent gradient from hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). The shape of the gradient is described by $c = kt^5$, where c is the concentration of the second solvent in the first, k is a constant and t is the fraction in time of the completed gradient. The order of elution is generally in order of increasing polarity of the amino acid side-chain. Between analyses at least 10 min is allowed for equilibration with the initial solvent.

Several other solvent systems employing hexane and various alcohol mixtures also give satisfactory separations of standard mixtures but in test samples from actual sequenator runs the elution times vary (presumably affected by by-products).

As may be seen in Fig. 1, the pairs PTH-glycine plus PTH-threonine and PTH-lysine plus PTH-tyrosine co-elute from the column. However, distinction within these pairs is rarely difficult since, as shown in the lower part of Fig. 1, both

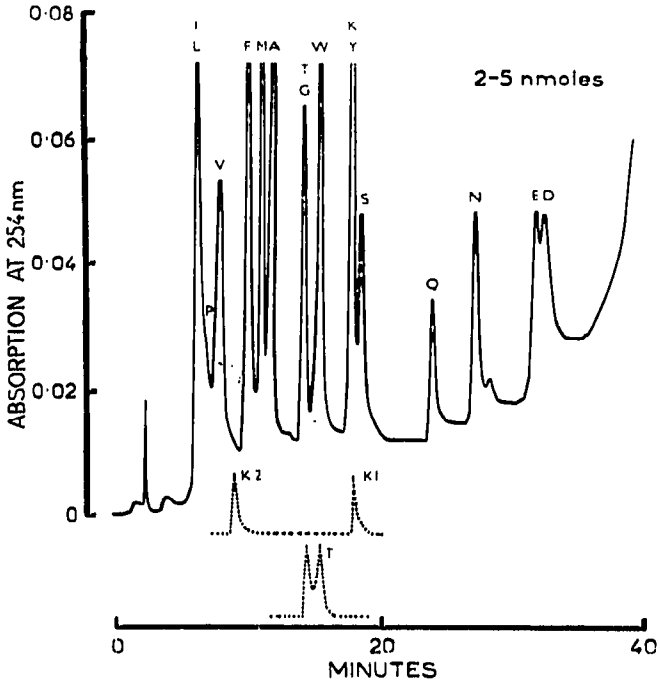

Fig. 1. Separation of PTH-amino acids by HPLC on Zorbax SIL. Elution is performed with a concave gradient solvent system from hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). Detection is by ultraviolet absorption at 254 nm. The PTH-amino acid peaks are identified by the single letter notation for the corresponding amino acids. The elution positions of the two peaks obtained both from lysine (K1 and K2) and from threonine (T) are shown below the main diagram.

lysine and threonine usually give two peaks when present in peptides sequenced by Edman's degradation. These are presumably due to the formation of N^{ε}-phenylthiocarbamoyl-N^{α}-PTH-lysine and dehydro-PTH-threonine, respectively, in addition to the expected PTH-amino acids.

In Fig. 2 we show three analyses of PTH-amino acids derived from sequenator runs which demonstrate some of the advantages of this method over GLC. The left-hand chart shows the sample obtained at cycle 2 of an automated degradation of β-1-24-adrenocorticotrophin: a sharp peak of PTH-tyrosine is seen. In contrast this compound is eluted late and as a broad peak on GLC systems both before and after silylation. The central chart shows the analysis of cycle 5 of the same degradation; without the derivatisation required in GLC the polar PTH-glutamic acid is identified directly and is quantifiable. Finally, the right-hand chart shows the analysis of PTH-asparagine derived from cycle 3 of the degradation of human calcitonin. The negligible quantity of the breakdown product PTH-aspartic acid is in direct contrast to experience with GLC systems. PTH-glycine is carry-over from cycle 2 and is a consequence of the use of a peptide-programme in the sequenator[3,4].

Some contaminating peaks are seen in the charts of Fig. 2 .They are probably due to the omission of washing steps in our peptide sequencing programme[4]. They are generally consistent within a run on the sequenator but occasionally vary between

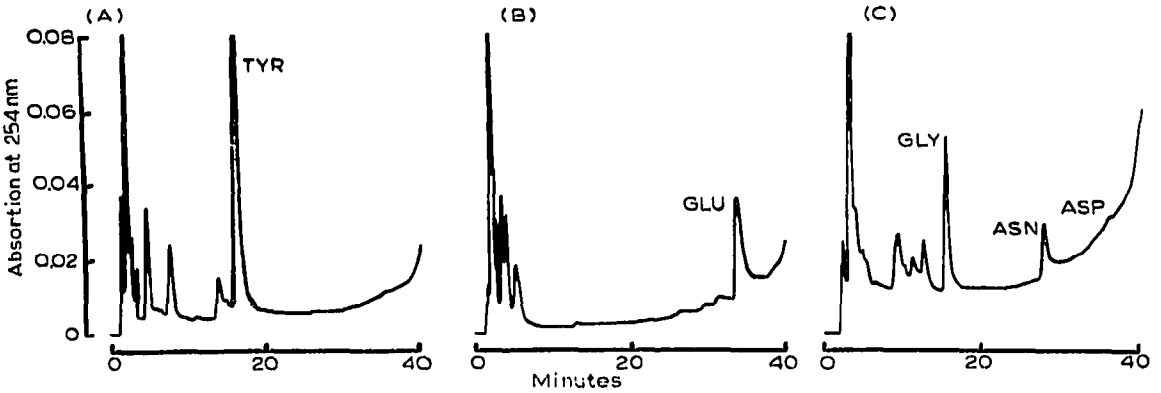

Fig. 2. Analysis of PTH-amino acids derived from sequenator runs. (A), The result from cycle 2 of the degradation of β-1-24-adrenocorticotrophin (N-terminal sequence ser–tyr–ser–met–glu–); (B), The result from cycle 5 of the same degradation; (C), The result from cycle 3 of the degradation of human calcitonin (N-terminal sequence cys–gly–asn–).

runs; this is probably due to batch differences in solvents and reagents. They rarely interfere with PTH-amino acid peaks.

PTH-arginine and PTH-histidine

These two basic PTH-amino acids which remain in the aqueous phase after ethyl acetate extraction are not eluted in the system described. When the pH of the aqueous phase is adjusted to approximately 8 with dimethylallylamine PTH-histidine may be extracted with ethyl acetate and provisional results indicate that it may be eluted on the silica column using a linear gradient from hexane–propanol (95:5) to propanol. As yet we have devised no system which successfully elutes PTH-arginine from the silica column.

DISCUSSION

Three other publications on the separation of PTH-amino acids using HPLC have recently appeared[5–7]. None of these reports the separation of all PTH-amino acids in a single run. Whilst the method described in the present paper cannot deal with PTH-histidine and PTH-arginine it does allow the identification and quantitation in a single run of all PTHs derived from all other amino acids commonly found in peptides and proteins.

The mild and non-destructive nature of this technique confers some additional advantages. For example, the column eluate may be collected in fractions for radioactivity determination, thus facilitating the identification of a labelled residue in a radioactive peptide or high-sensitivity sequencing using radioactively labelled phenylisothiocyanate.

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