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Note

Solvent system for the rapid identification of phenylthiohydantoin derivatives of amino acids by high-performance liquid chromatography

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The sequence determination of peptides and proteins using the Edman degradation remains an important technique in biochemical analysis. Various types of machines (liquid phase[1], solid phase[2] and gas–liquid–solid phase[3] are or have been commercially available to perform this technique automatically. Independent of the type of sequencer, the identification of the phenylthiohydantoin (PTH)-amino acid obtained from each cycle of degradation must be performed. Over the last 10 years, the use of reversed-phase (RP) high-performance liquid chromatography (HPLC) has become the accepted method for this analysis.

Numerous reports have appeared describing separations of PTH-amino acids, and these methods have generally consisted of RP chromatography on C_{18} column supports using isocratic[4] or gradient[5] elution with acetonitrile or methanol. An application note from Waters suggested to us that 2-propanol, a cheaper and safer solvent than acetonitrile, could be successfully used for the rapid separation of PTH-amino acids. More recently, a separation of PTH-amino acids within 35 min by isocratic elution of C_{18} columns with sodium acetate buffer–2-propanol–tetrahydrofuran was described[6]. However, PTH derivatives of Ser and Gln were not separated. Here we report that gradient elution of C_{18} columns with increasing concentrations of 2-propanol in 30 m*M* sodium acetate–acetonitrile produce a complete separation of the PTH derivatives of the commonly occurring amino acids in 13 min. The column lifetime is long, the method is applicable to sequencing at high sensitivity and the solvent system produces a good resolution of PTH-amino acids with different C_{18} columns.

EXPERIMENTAL

Acetonitrile and 2-propanol were of HPLC grade from Fluka (Buchs, Switzerland) and other chemicals were of analytical-reagent grade from either Fluka or Merck (Darmstadt, F.R.G.). Hibar LiChrocart Supersphere RP-18 columns (125 × 4 mm I.D.) were obtained from Merck, a Nova Pak C_{18} column (150 × 3.9 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.) and a Nucleosil C_{18} (5 μm particle size) column (200 × 4 mm I.D.) from Macherey, Nagel & Co. (Düren, F.R.G.).

The PTH-amino acids were purchased from either Pierce (Rockford, IL, U.S.A.) or Fluka. Methyl ester derivatives of PTH-aspartic acid, PTH-glutamic acid

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and PTH-carboxymethylcysteine were prepared by incubating the PTH derivatives at 50°C for 5 min in 1 *M* methanolic hydrochloric acid. Water used for the preparation of buffers was obtained from a Milli-Q water system (Millipore, Bedford, MA, U.S.A.).

Buffer A was prepared by adding 1.8 ml of acetic acid to 1 l of water and the solution was titrated with 4 *M* sodium hydroxide solution to the required pH. To five parts of this buffer was added 1 part of acetonitrile. Buffer B was 2-propanol–

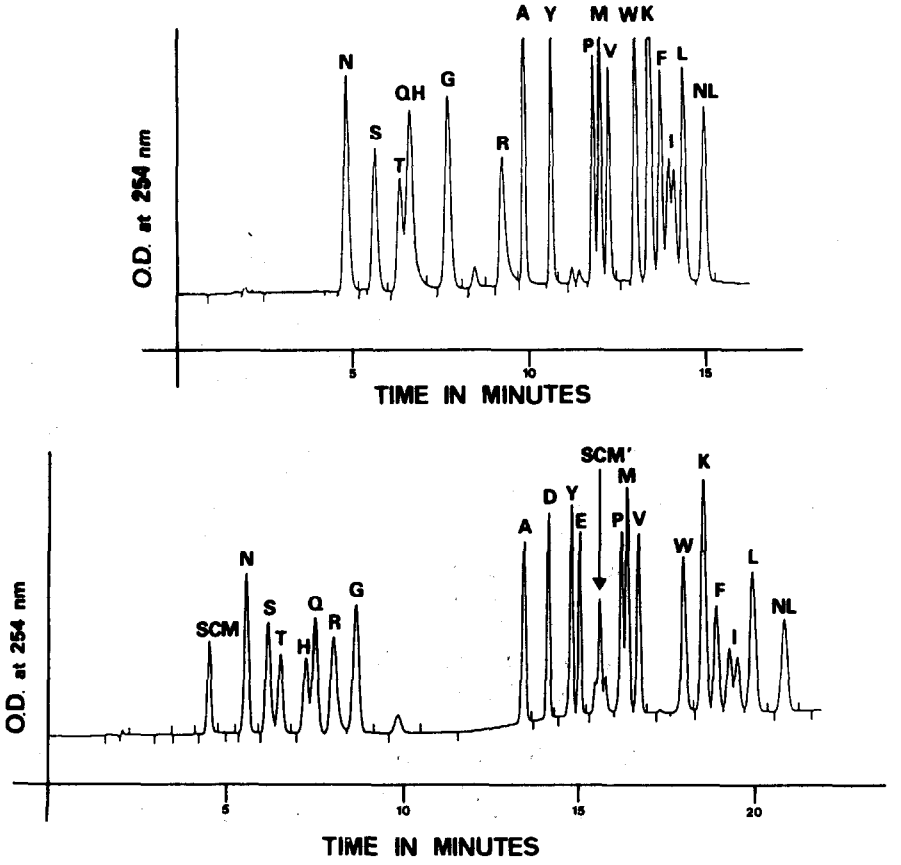

Fig. 1. Top: separation of a standard PTH-amino acid mixture on a Nova Pak C_{18} column. Buffer A was 30 m*M* sodium acetate (pH 4.8)–acetonitrile (5:1) and buffer B was 60% 2-propanol. A flow-rate of 0.7 ml/min was used and the temperature was 37°C. The programme for the elution consisted of 1.5 min of buffer A followed by an increase to 40% in B over 5 min using convex gradient No. 5. After holding these conditions for 5.5 min, the percentage of B was reduced linearly to 0% in 4 min; the cycle time was 20 min. The single-letter code for the amino acids is as recommended by the Commission on Biochemical Literature (exception: NL = norleucine). PTH-Asp and PTH-Glu are present as their methyl esters. Bottom: separation on a Nucleosil column. Buffer A was as above with the pH altered to 5.0 and equilibrating conditions were 5% in buffer B. Elution was for 6.5 min at 5% B followed by an increase to 40% B in 3.5 min using the convex gradient no. 5. Elution was continued for 8 min with 40% B and then returned to equilibrating conditions linearly over 2 min; the cycle time was 25 min. Other conditions were as described above. SCM = PTH-S-carboxymethylcysteine; SCM′ = methyl ester of PTH-carboxymethylcysteine.

water (3:2). The buffers were filtered through a 0.2 μm filter before use. Samples were injected in buffer A.

The HPLC system from Waters consisted of a WISP 710B automatic sample injector, two Model 510 pumps, a Model 680 controller and a Model 440 UV detector. Integration was performed with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390 A integrator.

Sequence analysis was performed with a Beckman 890 C instrument modified with a cold trap and conversion to the PTH derivatives was carried out automatically using a P-6 auto-converter from Sequemat (Watertown, MA, U.S.A.). Methanolic hydrochloric acid (1 *M*) was used as the conversion reagent[7].

RESULTS AND DISCUSSION

Initial experiments with a Nova Pak C_{18} column using the manufacturer's recommended procedure failed to give an adequate resolution of a standard PTH-amino acid mixture. Sodium acetate buffers (30 m*M*) of pH varying from 4.3 to 5.5 in increments of 0.1 unit were tried and a chromatogram at the optimal pH of 4.8 is shown in Fig. 1 (top). Changing the conditions, such as temperature, salt concentration and the gradient profile, failed to achieve a better separation of PTH-His from PTH-Thr (or PTH-Gln) without sacrificing the almost complete resolution of the other PTH derivatives. This failure may arise from the different chromatographic properties often observed between supposedly identical columns. Attempts with a column of Nucleosil C_{18} (5 μm) were more successful and an almost complete separation of all derivatives was obtained within 21 min, as shown in Fig. 1 (bottom).

A newly developed chromatographic support from Merck, Supersphere (4 μm particle size), became commercially available and we tested the RP-18 cartridge (125 × 4 mm I.D.) using similar conditions to those described above. As shown in Fig. 2, a nearly complete separation of all derivatives was obtained within 13 min and with a cycle time of 17 min. The reproducibility between cartridges appears to be adequate as a similar chromatogram was obtained with a second cartridge; His and Ser were resolved only on decreasing the pH by 0.2 unit; however, this did not seriously affect the resolution of the other derivatives. This procedure was adopted as an appropriate method of analysis.

The column lifetime appears to be good. Over a period of 9 months, more than 1000 samples from the Beckman sequencer have been injected on to a single column with neither a serious loss of resolution (with the exception of His–Ser) nor a marked increase in back-pressure. A slight increase in the pH (0.1 unit) of buffer A resulted in a decrease in the elution time of His relative to Ser, and such fine tuning of the chromatogram corrected the effects of column ageing.

The sensitivity of the method is adequate for sequencing at the picomole level. Fig. 2 (bottom) shows the results for a standard mixture of 10 pmol of each PTH derivative. An increase in the baseline, although aesthetically unappealing, does not hinder the identification of PTHs at this level.

No large extraneous peaks were seen from the Beckman sequencer for the degradation of peptides at the 500 pmol level. Fig. 3 shows examples from a sequence determination of a peptide derived from a tryptic digestion of the rabbit secretory component. The major contaminant peak, presumably diphenylthiourea, was eluted

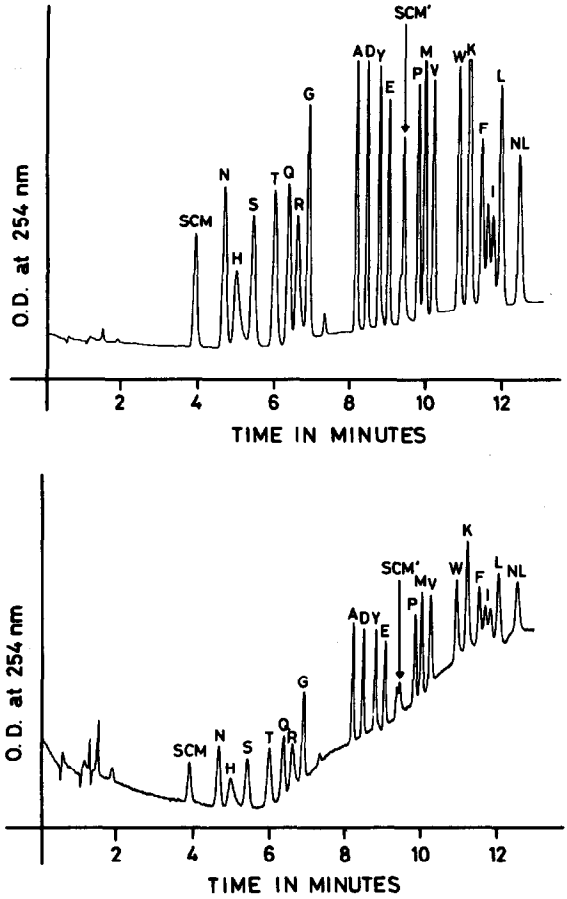

Fig. 2. Chromatography of PTH-amino acids on LiChrocart Supersphere. Amounts of 100 pmol (top) and 10 pmol (bottom) of each component were injected. Buffer A was 30 m*M* sodium acetate (pH 5.3)–acetonitrile (5:1) and buffer B was 60% 2-propanol. A flow-rate of 1 ml/min was used and the temperature was at 37°C. The programme (inject to inject time 17 min) is shown in Table I.

TABLE I

PROGRAMME FOR THE SEPARATION OF PTH-AMINO ACIDS ON LICHROCART SUPERSPHERE

Time (min)	*Flow-rate (ml/min)*	*Buffer A (%)*	*Buffer B (%)*	*Curve*
Inject	1	100	0	
1.5	1	100	0	
6.5	1	60	40	5 (convex)
11	1	60	40	6 (linear)
12	1	100	0	6

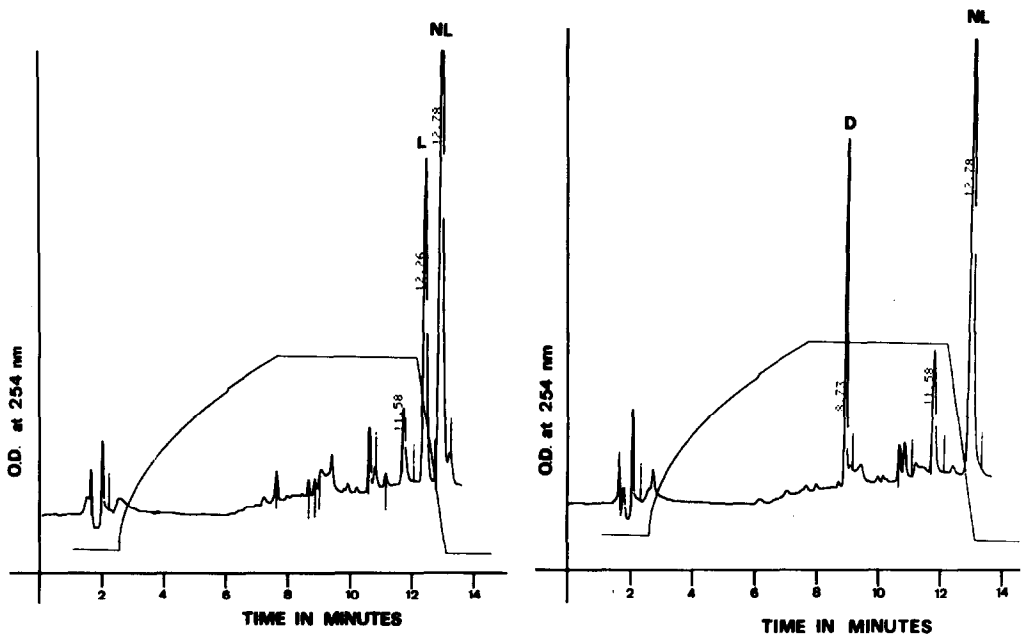

Fig. 3. Sequence analysis of a tryptic peptide derived from rabbit secretory component. The peptide (500 pmol) was degraded using a Beckman 890 C sequencer. Analyses of cycles 2 (top) and 4 (bottom) are shown; 30% of each fraction from the sequencer was injected. The gradient (see Table I) is shown superimposed.

between the Leu and Phe derivatives. When it is taken into consideration that modern gas-phase sequencers yield significantly fewer background peaks, then there should be no problem in the general application of this method.

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