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Note

Separation of phenylthiohydantoin-amino acids by high-performance liquid chromatography and some applications in dansyl Edman sequence analysis

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Reversed-phase high-performance liquid chromatography (HPLC) has, in recent years, revolutionized the techniques for both the isolation of the fragments and the analytical procedures necessary for determination of the primary structure of peptides and proteins. In particular, numerous HPLC methods have been proposed for the analysis of amino acid phenylthiohydantoin (PTH) derivatives formed during any of the various versions of the classical direct Edman degradation, including the more recent automated micro-sequencing procedures[1–6]. Most of these methods prescribe the inclusion of non-volatile buffers of low ionic strength and pH value in the range 3.5–5.5 in the formula of at least one of the solvents, and chromatography is often performed at relatively high temperatures. Thus some problems may occur, since slight alterations in these parameters will produce significant shifts in the retentions of several PTH-amino acids (PTH-AA). Moreover, adverse effects of salts and temperature on the maintenance of hydraulic components of the instrument and on lifetime of columns, respectively, must be taken into consideration. We report here an alternative procedure for the separation of PTH-AA by HPLC, which in part obviates these disadvantages. This procedure was developed as part of an attempt to overcome the major difficulty with the dansyl Edman technique of sequence analysis, namely differentiation between acids and amides, and to improve identification of histidine, tryptophan and arginine. Typical results obtained are presented.

MATERIALS AND METHODS

Analyses were performed on a chromatographic unit consisting of two Beckman Model 112 pumps, a Beckman Model 420 controler a Beckman Model 210 sample injection valve, a Beckman Model 160 UV detector, set at 254 nm, and a Perkin-Elmer Model 561 dual-pen chart recorder. The column was a Du Pont Zorbax ODS (5 μm, 250 × 4.6 mm I.D.). For the rapid separation of PTH derivatives of dicarboxylic and basic amino acids, the column was a Spheri-5 RP-18 (Brownlee, guard column, 30 × 4.6 mm I.D., 5 μm).

Standard PTH-AA (Pierce) were dissolved in methanol and stored at −20°C. Dansyl Edman degradation was performed according to Gray[7]. The ethyl acetate extracts that contain the anilino-thiazolinone derivatives usually discarded were evaporated to dryness, and then treated with 1 *N* hydrochloric acid at 80°C for 5 min.

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Most PTH-AA were extracted with ethyl acetate and the solution was dried. PTH derivatives of basic amino acids remaining in the aqueous phase were lyophilized. The dried PTH-AA were then redissolved in 0.2% methanolic trifluoroacetic acid, and sample volumes equal to or less than 25 μl were injected into the column.

Solid-phase sequencing was performed using the LKB 4020 Sequencer. Coupling of peptides was carried out with aminopropyl-glass, activated either by reaction with *p*-phenylene diisothiocyanate or through the C-terminal homoserine lactone, according to the procedures recommended in the LKB Slid-Phase Sequencing Handbook.

Acetonitrile, methanol and 2-propanol were HPLC-grade solvents, obtained from Carlo Erba; ethanol and trifluoroacetic acid were purchased from Merck. Deionized water was first glass-distilled and then passed through a Sep-pak C_{18} cartridge (Waters Assoc.).

RESULTS AND DISCUSSION

A typical elution profile of a mixture of nineteen standard PTH-AA is shown in Fig. 1; methionine was eluted between valine and proline, and lysine was eluted 1 min before tryptophan. The eluents were (A) 0.2% aqueous trifluoroacetic acid and (B) acetonitrile–2-propanol–ethanol (39.2:9.8:1, v/v/v). The column was equilibrated

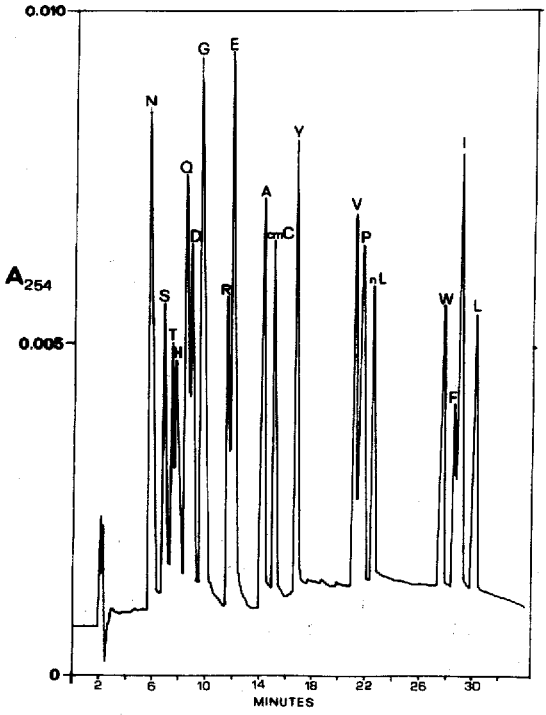

Fig. 1. Separation of standard PTH-amino acids by HPLC on a Zorbax ODS column. The chromatographic system is described in the text. Sample size: 50 pmol of each derivative. The peaks are labelled by one-letter abbreviations for the amino acids, plus nL = norleucine and cmC = carboxymethylcysteine.

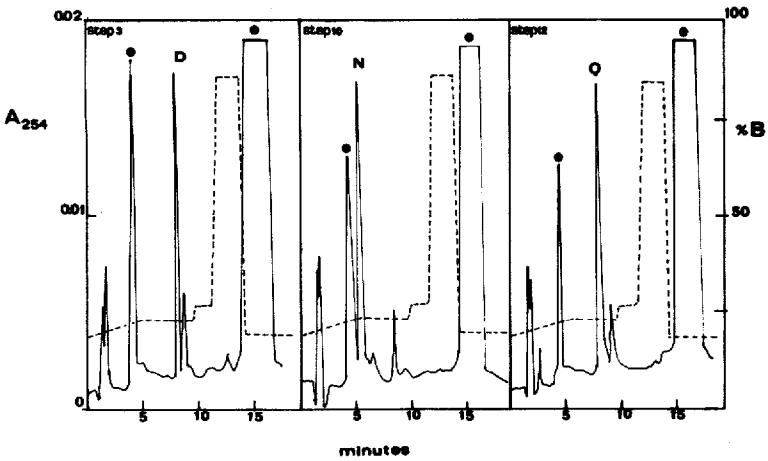

Fig. 2. Selected steps from manual dansyl Edman degradation of 25 nmol of a peptide from serine hydroxymethyltransferase. After the conversion step, an amount from 5 to 20% of the sample was injected onto the column. HPLC conditions as in the text (shorter elution program). Peaks marked with dots are the by-products of the Edman degradation, present in the samples recovered from the dansyl Edman technique. The dashed line shows the theoretical gradient.

with A–B (80:20) at a flow-rate of 1.1 ml/min, at room temperature; the separation was obtained by using the gradient programme reported in Table I; the pressure was 2500 p.s.i. It is noteworthy that immediately after elution of PTH-leucine the system is ready for a new analysis, since the column recycling is completed. Thus, the method can keep pace with the requirements of modern automated sequencers.

One advantage of this method is the simplicity of solvent preparation, which does not require distillation or delicate pH adjustment. In addition, the solvent systems and low temperature used ensure long column life and minimize instrumental

TABLE I

HPLC GRADIENT

Time (min after injection)	*Function*	*Value*	*Duration* (min)*
0.0	Flow-rate	1.1 ml/min	–
0.0	Amount B	20%	–
0.05	Amount B	23%	6.0
4.5	Flow-rate	0.7 ml/min	0.2
9.1	Flow-rate	0.8 ml/min	0.1
9.2	Amount B	27%	0.2
11.0	Amount B	33%	0.5
11.0	Flow-rate	0.8 ml/min	0.1
15.5	Flow-rate	1.1 ml/min	0.1
15.5	Amount B	36%	9.5
22.0	Flow-rate	0.7 ml/min	0.2
23.0	Flow-rate	1.1 ml/min	6.5
25.5	Amount B	20%	1.5
31.0	Start		

* Time required to reach the programmed value of the indicated function.

problems. Flow-programming during the course of chromatography was of chief importance in controlling the resolution and analysis time. In particular, a decrease in the flow-rate was essential for resolution of the polar derivatives, whereas the two subsequent increases by step from 0.7 to 1.1 ml/min allowed resolution of PTH-AA from Ala to nor-Leu in reasonable time. Finally, decreasing the flow-rate to 0.7 ml/min could split PTH-Ile from PTH-Phe. Moreover, the adverse effects of column ageing on the separation can be compensated for by decreasing the flow-rate by *ca.* 10% at steps 4, 5 and 11. When peak broadening increases with column use, a regeneration treatment, consisting of a washing with 100 ml of benzene–2-propanol (30:70, v/v), restored column performance. These precautions ensured satisfactory reproducibility of the retention times of the peaks over more than 6 months of continuous use (more than 1000 injections) of a single column with standard PTH-AA and with derivatives generated during the operation of a solid-phase sequencer; essentially similar results were obtained with a substitute Zorbax column.

The method has been applied to the solution of some problems with the dansyl Edman method of sequence analysis. This procedure is still very popular for the structural analysis of peptides at the nanomolar level because of its economy and accessibility to non-specialized laboratories, yet it has certain drawbacks. For instance, it does not allow identification of the amidation state of dicarboxylic amino acid residues, and identification of some other residues, such as those of Cm-cys, Trp, Arg and His, is often difficult. The PTH derivatives of these amino acids may be recovered from a side-fraction, usually discarded in the course of classical dansyl Edman procedure (see Materials and methods). In fact, in our laboratory they were successfully identified recently by routine application of the above-described HPLC

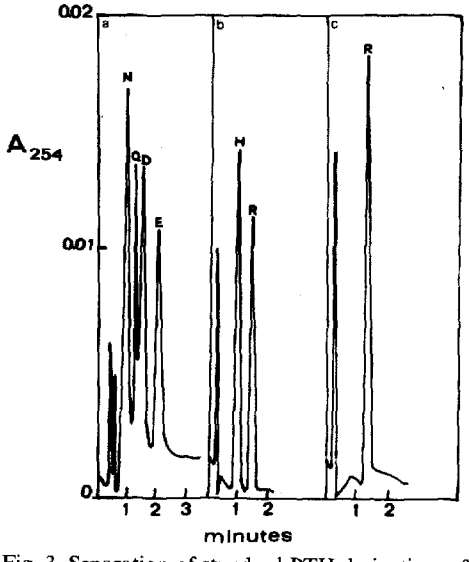

Fig. 3. Separation of standard PTH derivatives of (a) dicarboxylic amino acids and their amides and (b) basic amino acids, on a mini Spheri-5 RP-18 column. The chromatographic system is described in the text. Isocratic elution was accomplished at room temperature by 20% of solvent B at a flow-rate of 0.9 ml/min (a) or 1.1 ml/min (b). Identification of step 8 from manual dansyl Edman degradation of 25 nmol of a peptide from serine hydroxymethyltransferase is reported in (c).

method. The by-products of the Edman degradation, which in the dansyl Edman version are present in the ethyl acetate extracts, together with the anilino-thiazolinone derivatives of the amino acids, do not interfere with the analysis of polar PTH-AA. A shorter elution protocol may be chosen for their identification, with recycling starting after 11 min and analysis being completed in 18 min. The chromatograms in Fig. 2 illustrate the identification by the latter procedure of some PTH-AA recoverd in the course of the dansyl Edman degradation of the peptide GLDPQCWGVNVQPYSGSPANFAVYTALVEPH from cytosolic serine hydroxymethyltransferase, an enzyme from rabbit liver, the sequence of which is under investigation in our laboratory.

In addition, we have set up conditions for the faster and more economical solution of specific problems individually encountered in the course of dansyl Edman degradation, by following a minicolumn approach, similar to that reported by Bhown and Bennett[8]. Fig. 3a shows the separation of the PTH derivatives of Glu, Gln, Asp and Asn, obtained isocratically in 3 min on a Spheri-5 RP-18 guard column. Under similar conditions (Fig. 3b), it is possible to separate PTH-Arg and PTH-His, present in the aqueous phase after conversion of the anilino-thiazolinones. An obvious advantage of these identification procedures is the dramatic reduction of expenditure for the column (the cost of a Guard column is one-sixth that of a Zorbax ODS). An example of the application of this method in the course of sequence determination of peptide IFYRRGVRSVDPKTGKE from serine hydroxymethyltransferase is presented in Fig. 3c.

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