Journal of Chromatography, 326 (1985) 321–327
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 591

MICROSCALE ISOCRATIC SEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACID DERIVATIVES

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SUMMARY

High-performance liquid chromatography is the most common method used for the identification of phenylthiohydantoin amino acid derivatives of amino acids. Isocratic separation conditions offer advantages over gradient conditions in baseline stability and reproducibility. The isocratic separation of the common phenylthiohydantoin amino acid derivatives on two columns, suitable for routine analyses, is described. In microsequencing, identification and quantification of the phenylthiohydantoin amino acids at the femtomole level is necessary. With normal analytical columns the detection limit is about 4 pmol. Therefore, microbore columns were used. With the usual equipment, the 2 mm I.D. columns seem to be most effective for the highly sensitive and reliable identification of the phenylthiohydantoin amino acid derivatives.

INTRODUCTION

In 1949, Edman introduced the method of the sequencial degradation of polypeptides[1]. Remarkably, the chemistry of this reaction has remained practically unchanged for 35 years. Four major contributions have led to the development of modern microsequencing technology: (a) automation of the method by Edman and Begg in 1967[2]; (b) the introduction of high-performance liquid chromatography (HPLC) for the identification of the phenylthiohydantoin (PTH) amino acids[3], with a decrease in the detection limit from about 5 nmol using thin-layer chromatography to about 5 pmol, thus permitting sequencing at the low-nanomole level: (c) the introduction of polybrene[4], a carrier that prevents the washing out of small peptides from the reaction compartment; and (d) the introduction of a new instrument, the gas-phase sequencer, which was especially designed for microsequencing[5]. This instrument is capable of sequencing peptide samples of less than 100 pmol, and its full potential has probably not yet been reached.

The next step towards higher sensitivity in sequencing peptides and proteins must come in the identification. To obtain a sequence from a sample of 1–10 pmol, one must have an identification system that separates and quantitates PTH amino acid derivatives reliably at the femtomole level.

A large number of gradient HPLC systems exist that separate all the common

0021-9673/85/$03.30

amino acids within a reasonable time. However, when working at the highest sensitivity, there are always problems with baseline stability and reproducibility. Therefore, we chose to separate the PTH amino acid derivatives by isocratic elution. To achieve identification at the femtomole level, microbore columns of 2 mm I.D. were used.

EXPERIMENTAL

The HPLC equipment consisted of an LKB 2150 HPLC pump, a pulsation damping unit, an LKB 2155 oven and an LKB 2151 variable-wavelength detector. LiChrospher and LiChrospher SUPER columns were obtained from E. Merck (Darmstadt, F.R.G.). Spherisorb ODS II columns were purchased from Bishoff (Leonberg, F.R.G.). Sodium acetate (analytical-reagent grade), acetonitrile, and dichloroethane (both HPLC grade) were purchased from E. Merck. The chromatographic conditions are given in Table I. The samples were dissolved in the eluent.

RESULTS AND DISCUSSION

In 1980, we described an isocratic system for the separation of the common PTH amino acid derivatives[6]. Since then, almost all well packed reversed-phase columns have given the separation described. The results obtained with two of the columns most suitable for the separation of PTH amino acids are described here in more detail. In Fig. 1, a chromatogram obtained on a Spherisorb ODS II (3 μm) is shown. The chromatographic conditions are the same as those given in the original paper[6], and are indicated in Table I. The dependences on acetonitrile concentration, molarity of the buffer, pH and temperature are still qualitatively valid and are shown in Fig. 2. All the common PTH amino acid derivatives could be separated well; even the pair PTH-Gln and PTH-Ser was separated, which was not possible with the older 5-μm materials.

The best separations were obtained on the LiChrospher SUPER CH-8 (4 μm)

TABLE I

CONDITIONS FOR THE SEPARATION OF PTH AMINO ACID DERIVATIVES

Parameter	*Column*		
	Spherisorb ODS II (250 × 4.6 mm I.D.)	*LiChrospher CH-8 SUPER (250 × 4 mm I.D.)*	*LiChrospher CH-8 SUPER (250 × 2 mm I.D.)*
Eluent	A*	A*	A*
Flow-rate (ml/min)	1.5	2.0	0.5
Temperature (°C)	62	60	60
Detection wavelength (nm)	265	265	265
Detection limit (pmol)	3	2	0.5
Analysis time (min)	16	15.5	15.5

* A = 0.01 *M* sodium acetate (pH 5.0)–acetonitrile (LiChrosolv)–1,2-dichloroethane (Uvasol) (68.5:31.5:0.5).

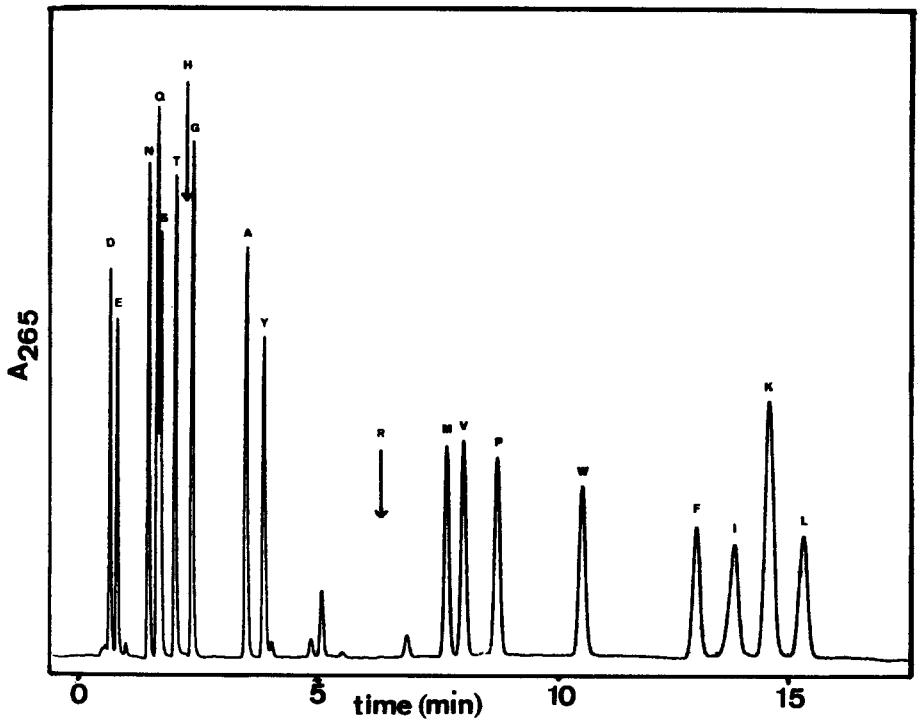

Fig. 1. Isocratic separation of PTH amino acid derivatives on Spherisorb ODS II under the conditions given in Table I. Column, Spherisorb ODS II (3 μm), 250 × 4 mm I.D.; sample, 100 pmol of each PTH amino acid dissolved in 10 μl of eluent; 0.02 absorbance units full-scale (a.u.f.s.).

column. All the common PTH amino acid derivatives are baseline-separated, as shown in Fig. 3. In this chromatogram, there is enough space to accomodate many less common PTH amino acid derivatives and the separation of some of them is shown in Fig. 4. Diphenylthiourea, usually the major artifactual peak from the sequencer, is eluted between proline and tryptophan and does not interfere with any PTH amino acid derivative. The separation conditions are shown in Table I. The only major change is the flow-rate, which is 2 ml/min, giving an analysis time of about 15 min. This high solvent consumption is the only disadvantage of this otherwise excellent column. Therefore, we routinely use the Spherisorb ODS II (3 μm) columns, despite the fact that the separation of the PTH-Gln–PTH-Ser pair is not as good. However, when the gas-phase sequencer is used with the recommended dithiothreitol-containing solvents, the normal PTH-Ser derivative will not be obtained in high yield. Instead of this derivative, we found a serine–dithiothreitol adduct, which is eluted slightly before PTH-Ala and is well separated. Again, with this column the chromatographic conditions and the dependences of the solvent composition and temperature are the same as given earlier[6].

The detection limit for PTH amino acids is 3–5 pmol with these analytical (250 × 4 mm I.D.) columns, as indicated in Fig. 5 (top). One way to lower the detection limit is to use columns of smaller inner diameter (1 or 2 mm). By using 2 mm I.D. microbore columns instead of the 4 mm I.D. analytical column the theoretical gain

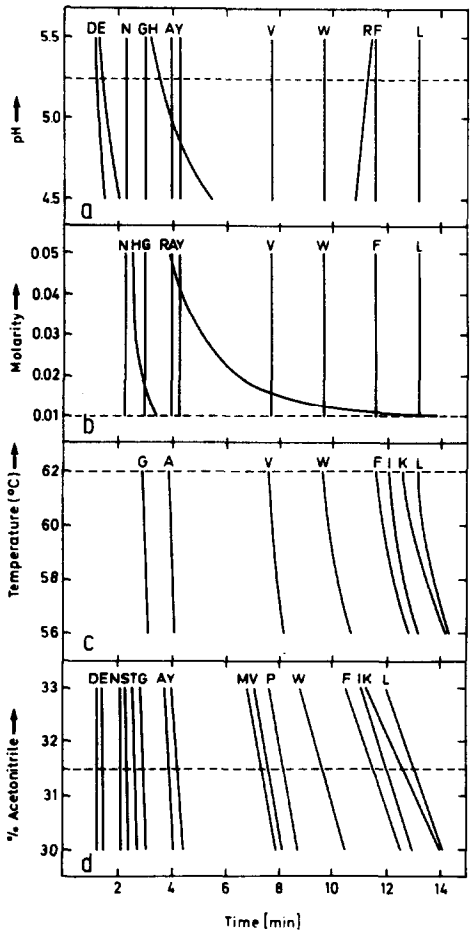

Fig. 2. Effect of (a) pH, (b) molarity of the sodium acetate buffer, (c) temperature and (d) acetonitrile concentration on the retention times of PTH amino acids. The broken lines indicate the standard conditions[6].

in sensitivity is a factor of 4. We were able to obtain 2 mm I.D. columns, packed with LiChrospher SUPER CH-8 (4 μm), giving the results shown in Fig. 5 (middle and bottom). Surprizingly, we could obtain a nearly theoretical improvement in detectability of the PTH amino acid derivatives without a major decrease in selectivity with the ordinary HPLC equipment used in the separations with the 4 mm I.D. columns. The detection limit was in the femtomole range (Fig. 5, bottom). As indicated in Fig. 6, injecting amounts as large as 10 μl did not destroy the resolution. The separation conditions are given in Table I. One very desirable side-effect was the reduction in the flow-rate to 0.5 ml/min, as this reduces costs.

Even though these columns are not yet commercially available, the results indicate that it is possible to pack 2 mm I.D. columns of the same quality as 4 mm I.D. columns. In the next few years, these columns should prove suitable for use in routine PTH amino acid analyses, mainly because they allow highly sensitive detec-

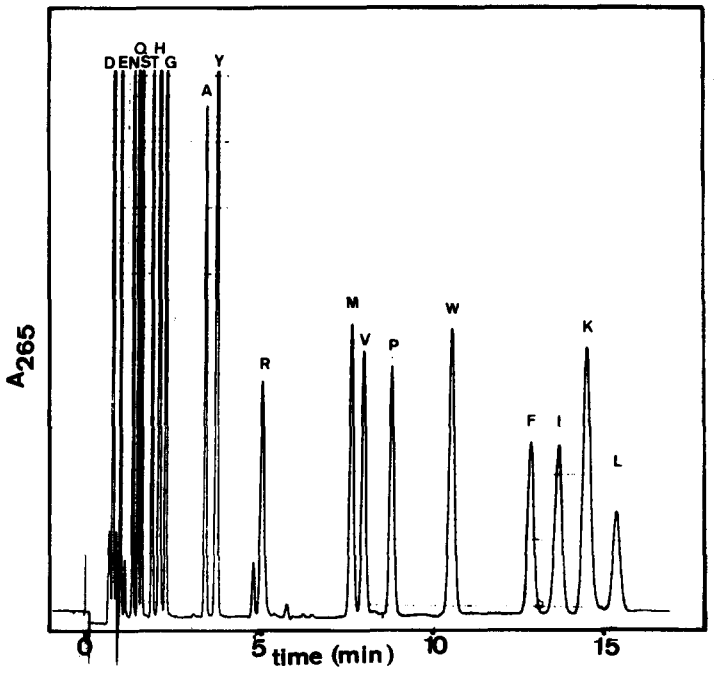

Fig. 3. Isocratic separation of PTH amino acid derivatives on LiChrospher CH-8 SUPER under the conditions given in Table I. Column, LiChrospher CH-8 SUPER, 250 × 4 mm I.D.; sample, 100 pmol of each PTH amino acid dissolved in 10 μl of eluent; 0.02 a.u.f.s.

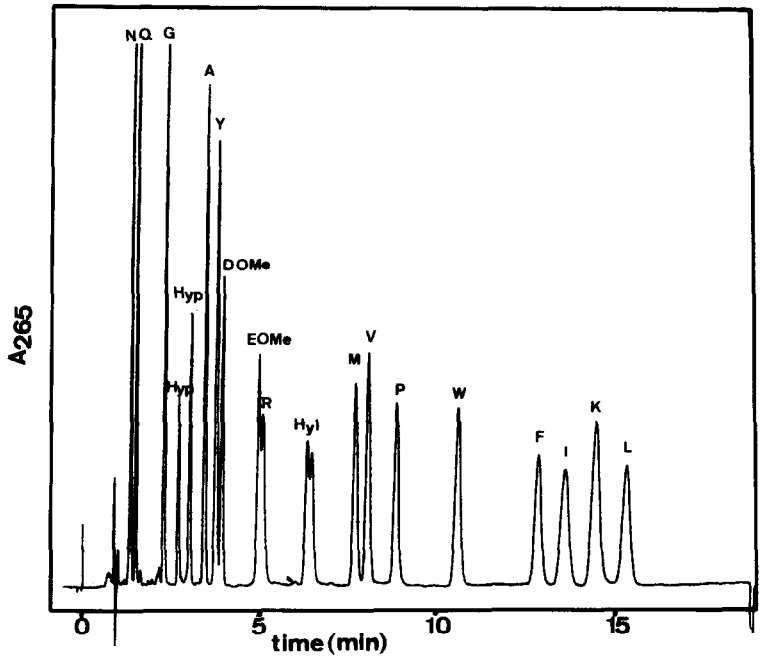

Fig. 4. Isocratic separation of PTH amino acid derivatives on LiChrospher CH-8 SUPER. Conditions as in Fig. 3. Hyp, hydroxyproline; DOMe, methyl aspartate; EOMe, methyl glutamate; Hyl, hydroxylysine.

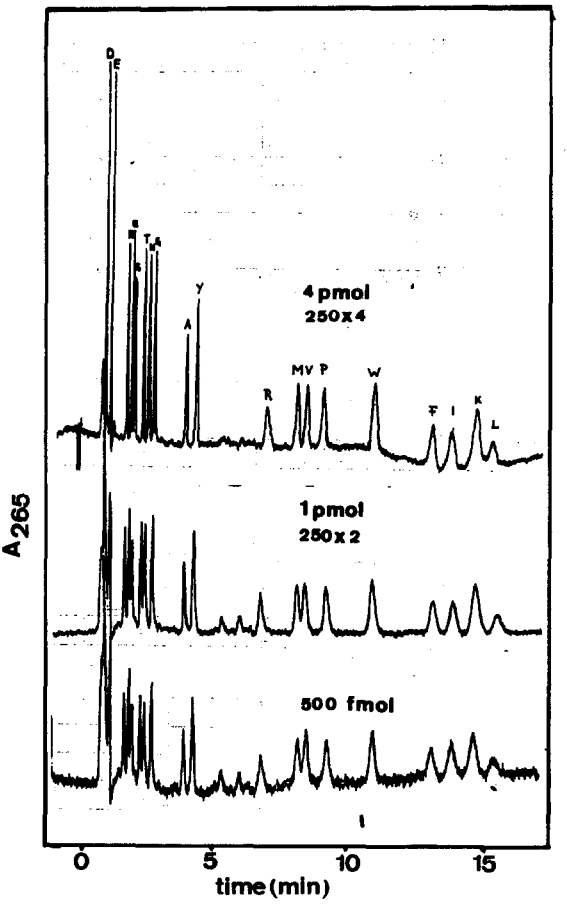

Fig. 5. Isocratic separation of PTH amino acid derivatives under the conditions given in Table I. Top: column, LiChrospher CH-8 SUPER, 250 × 4 mm I.D.; sample, 4 pmol of each PTH amino acid, dissolved in 10 μl of eluent; 0.0025 a.u.f.s. Bottom: column, LiChrospher CH-8 SUPER, 250 × 2 mm I.D.; sample, 500 fmol of each PTH amino acid, dissolved in 5 μl of eluent; 0.00125 a.u.f.s.

tion of PTH amino acids of the femtomole level, the solvent consumption is low and one can use normal high-quality HPLC equipment.

Some attempts have been made to effect the separation on different 1 mm I.D. microbore columns. However, with these columns, the external dead volumes have such a strong influence that the HPLC equipment must be highly optimized to give minimal peak broadening. Further, it seems that manufacturers are still unable to deliver 1 mm I.D. columns of the same quality and stability as 4 mm I.D. columns. This is necessary for this complex microscale separation of PTH amino acids.

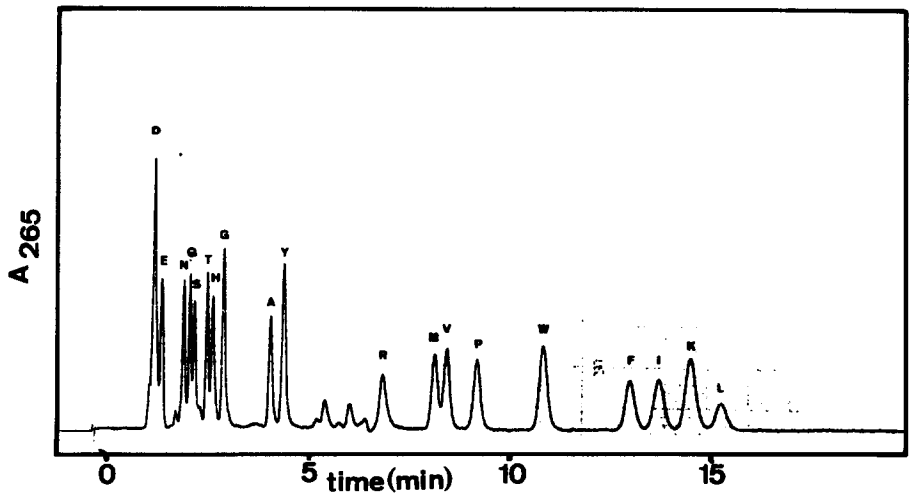

Fig. 6. Isocratic separation of PTH amino acid derivatives under the conditions given in Table I. Column, LiChrospher CH-8 SUPER, 250 × 2 mm I.D.; sample, 5 pmol of each PTH amino acid, dissolved in 10 μl of eluent; 0.01 a.u.f.s.

ACKNOWLEDGEMENTS

We are grateful to Dr. F. Eisenbeiss, Merck, Darmstadt, F.R.G., for the gift of the LiChrospher Super CH-8 columns.

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