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Note

Separation of PTH-amino acids by isocratic high-performance liquid chromatography

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Separation of amino acids as derivatives of phenylthiohydantoin (PTH-amino acids) has been performed by paper and thin-layer chromatography, gas chromatography and high-performance liquid chromatography (HPLC). HPLC, being a rapid and highly efficient technique, is advantageous because all PTH-amino acids (even non-volatile ones) can be determined as such. Up till now, separations have required several different eluent systems[1–4] or gradient elution[2,5–11]; reversed-phase systems with bonded phases have been used most frequently.

The use of isocratic elution, as proposed by Zimmermann *et al.*[12] should lead to simple and inexpensive analytical systems, especially with a one-column design. Fong and Grushka[13] used a bonded tripeptide phase, giving unique retention orders, but low-efficiency chromatograms.

In this paper we describe an efficient isocratic reversed-phase system with which 21 PTH-amino acids out of the 22 normally encountered in the Edman degradation of peptides and proteins can be separated. Increased column temperature is a prerequisite for the separation.

EXPERIMENTAL

Reagents

The eluent was a mixture of 25 parts (v/v) of acetonitrile (Merck, UVASOL, or Rathburn Chemicals, HPLC grade S) with 75 parts of 0.01 *M* sodium acetate; the pH of the mixture was adjusted to 4.6 with concentrated acetic acid.

PTH-amino acids were obtained from Pierce and from Sigma; stock solutions in methanol (Rathburn Chemicals) or a mixture of methanol and ethyl acetate (Merck) were *ca.* 20 m*M*.

Apparatus and chromatographic technique

The column consisted of two stainless-steel tubes (each 15 cm × 3.9 mm I.D.)

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connected by a low-dead-volume Swagelok fitting. The column was packed with Merck LiChrosorb RP 8 (5-μm particles) by the balanced-density slurry-packing technique. The column was water-jacketed and operated at 50° (controlled by a Haake thermostat). The sample was injected with a Waters U6K injector and consisted of 10 μl of solution containing about 1 nmole of each PTH-amino acid (prepared by diluting the stock solution with the eluent).

The chromatograph consisted of a Waters pump (Model 6000), operated at a flow-rate of 0.9 ml/min and at a pressure drop of about 3000 p.s.i. The column effluent was monitored by a Cecil variable-wavelength detector (Model 212), with a 10-μl flow-through cell. The detector was operated at 254 nm (0.1 a.u.f.s.). However, for statistical evaluation an LDC LC/SSU I chromatograph equipped with an LDC 1203 UV monitor and a Vitatron 2001 potentiometric recorder was used.

RESULTS AND DISCUSSION

Our aim was to find a simple and reliable chromatographic system capable of separating the 22 PTH-amino acids (See Table I) normally encountered in the Edman degradation of proteins.

TABLE I

CAPACITY RATIOS (k') AND RETENTION TIMES (t_R) OF PTH-AMINO ACIDS

The chromatographic system was as indicated in Fig. 1. Results were obtained from the injection of a mixture of the 22 PTH-amino acids indicated.

Compound No.	*Compound*	*t_R (min)*	*k'*	*Coefficient of variation* (%)*
1	PTH-Cysteic acid (K salt)	3.30	0.04	±3.9
2	PTH-Aspartic acid	4.60	0.45	±2.4
3	PTH-(S-Carboxymethyl)cysteine	5.20	0.64	±0.9
4	PTH-Asparagine	6.00	0.89	±1.9
5	PTH-Serine	6.80	1.14	±1.8
6	PTH-Glutamine	7.00	1.20	±1.0
7	PTH-Glutamic acid	7.30	1.30	±0.8
8	PTH-Threonine	7.60	1.39	±1.0
9	PTH-Glycine	8.60	1.71	±1.5
10	PTH-Histidine monohydrochloride	10.1	2.18	±2.2
11	PTH-Methionine sulphone	10.7	2.37	±0.4
12	PTH-Alanine	12.4	2.90	±0.3
13	PTH-Arginine	15.0	3.72	±2.5
14	PTH-Tyrosine	15.6	3.91	±0.8
15	PTH-Methionine	27.8	7.75	±2.3
16	PTH-Proline	27.8	7.75	±0.8
17	PTH-Valine	28.6	8.00	±2.3
18	PTH-Tryptophan	46.6	13.7	±3.8
19	PTH-Isoleucine	49.8	14.7	±1.8
		51.6 **	15.2 **	±2.9
20	PTH-Phenylalanine	53.6	15.9	±3.6
21	PTH-Leucine	57.6	17.1	±2.8
22	PTH-N$^{\epsilon}$-Phenylthiocarbamoyllysine	71.0	21.3	±7.2

* Calculated from the ratio t_R (PTH-amino acid) to t_R (internal standard) (see text).

** Produced two peaks in the chromatogram.

Chromatographic system

Support. Different bonded hydrophobic supports were tested, *i.e.*, C_8 and C_{18} types, with particle sizes of 5 and 10 μm. Commercial columns (Partisil ODS and μBondapak C_{18}) as well as columns slurry-packed in our laboratory (from LiChrosorb RP 8 and RP 18) were tested at room temperature, with acetonitrile–0.01 *M* sodium acetate (42:58, v/v), pH 4.6, as eluent (*cf.* Zimmermann *et al.*[12]).

We could not make a relevant comparison between the separating powers of the columns, as they affected retention differently. The best separations were obtained, however, on a column packed with LiChrosorb RP 8 (5-μm particles). More than 10,000 theoretical plates [determined with acenaphthene at a flow-rate of 0.9 ml/min and with acetonitrile–water, (60:40, v/v) as eluent] were necessary for succesful separation.

pH of the eluent. Eluents having pH values between 4.0 and 7.4 were tested; in this range, pH had no significant effect on retention times or separation.

Composition of the eluent. The influence of the composition of the eluent was studied with the PTH derivatives of methionine, proline, tryptophan, isoleucine, phenylalanine, leucine and N^{ε}-phenylthiocarbamoyllysine; these hydrophobic PTH-amino acids were especially difficult to separate. Different proportions of acetonitrile were tested, and the results are shown in Table II. As can be seen, the effects of increasing retention on the difference between the capacity ratios (k') of the PTH-amino acids were contradictory.

TABLE II

EFFECT OF COMPOSITION OF ELUENT ON CAPACITY RATIO (k')

System: LiChrosorb RP 8 (particle size 5 μm); two 15-cm columns; operated at room temperature (23°).

Amount of acetonitrile in 0.01 M sodium acetate (pH 4.6) (%, v/v)	*Value of k' for PTH-amino acid No.**						
	15	*16*	*18*	*19*	*20*	*21*	*22*
42	—	—	—	—	3.61	—	3.92
38	3.69	—	—	6.10	5.78	6.80	6.67
35	4.96	5.12	7.84	8.44** 8.54	8.68	9.66	10.1

* The figures refer to Table I.

** Produced two peaks in the chromatogram.

In general, it is preferable to use a wide k' range for a sample containing components of highly different polarities. In isocratic elution, the negative effect of peak broadening with increasing retention must, however, be borne in mind.

Effects of temperature. The use of 35% (v/v) of acetonitrile in the eluent produced fairly good results, but the need for narrower peaks was obvious. This was achieved by increasing the temperature, which also decreased the k' values considerably, and a less lipophilic eluent had to be used, *i.e.*, 25% (v/v) of acetonitrile.

A mixture of 22 PTH-amino acids (see Table I) was chromatographed at 23° (room temperature), 33°, 40°, 50°, 55° and 62°. The optimum temperature was 50°, and the k' values and retention times (t_R) obtained are shown in Table I. As can

be seen from Fig. 1, all the PTH-amino acids except PTH-methionine and PTH-proline were separated in one chromatographic run.

Changes in temperature affected the chromatographic behaviour of the individual PTH-amino acids somewhat differently, *e.g.*, the separation of PTH-arginine and PTH-tyrosine was increased at temperatures below 50°, whereas PTH-valine was better separated from PTH-methionine and PTH-proline at temperatures above 50°.

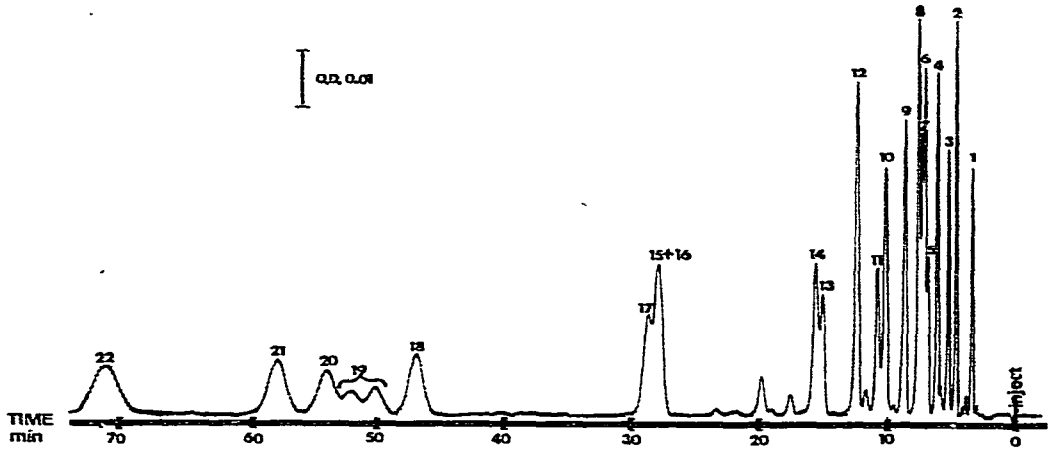

Fig. 1. Separation of a mixture of 22 PTH-amino acids (*ca.* 1 nmole of each) at 50° under isocratic conditions on LiChrosorb RP 8 (particle size 5 μm) (two 15-cm columns). The number of theoretical plates is 11.500. Eluent, acetonitrile–0.01 *M* sodium acetate of pH 4.6 (25:75, v/v;) flow-rate, 0.9 ml/min; detection wavelength, 254 nm (0.1 a.u.f.s.); chart speed, 5 mm/min.

Application to sequence analysis

To determine the reliability of the chromatographic system in sequence analysis, we have statistically evaluated both short- and long-term effects on the chromatographic behaviour of individual PTH-amino acids. The use of an internal standard and calculation of the retention-time ratios between the PTH-amino acids and the standard greatly diminished, for example, instrumental variation. In this study, PTH-hydroxyproline (Pierce kit No. 11171*5C 073), being derived from a rare natural amino acid, was chosen as standard as it yielded favourable retention times (two peaks, with k' 2.6 and 3.4) not seriously interfering with those of the PTH-amino acids of interest.

The coefficients of variation presented in Table I are the results of 20 injections divided into 7 discrete series during a period of 2 months; these values thus reflect the susceptibility of the different PTH-amino acids (in relation to PTH-hydroxyproline) to time-dependent changes in the properties of the column, which are unavoidable when the column is routinely used in sequence analysis. Notably, the hydrophobic PTH-amino acids, together with the water-soluble ones, show the greatest variation. For 20 consecutive injections, however, the coefficients of variation can be kept below ±1% for all PTH-amino acids except those of cysteic acid (±1.3), asparagine (±1.3), methionine (±1.1), valine (±1.1), tryptophan (±2.1), phenylalanine (±1.7), leucine (±1.4) and N^{ε}-phenylthiocarbamoyllysine (±2.5). This means that, if the ratios of t_R (PTH-amino acid) to t_R (internal standard) are up-

dated periodically, only the PTH-derivatives of glutamic acid and threonine, methionine, proline and valine or tryptophan, isoleucine, phenylalanine and leucine cannot be identified unambiguously in one chromatographic run, but will require additional qualitative analysis. However, introduction of a second (more hydrophobic) internal standard may lead to the direct identification of proline, tryptophan, isoleucine, phenylalanine and leucine.

It is our experience that this chromatographic system works well in sequence analysis. The use of isocratic conditions greatly reduces instrument costs, and, by recycling the eluent, consumption of solvent can be kept at a minimum.

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