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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND QUANTITATION OF PHENYLTHIOHYDANTOIN DERIVATIVES OF 25 AMINO ACIDS, INCLUDING THOSE OF CYSTEIC ACID, 4-HYDROXYPROLINE, METHIONINE SULFONE, S-CARBOXYMETHYLCYSTEINE AND S-METHYLCYSTEINE

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SUMMARY

A high-performance liquid chromatography system is presented which allows separation and quantitation (in the range 4–1000 pmol) of all common phenylthiohydantoin amino acids, including derivatives of 4-hydroxyproline, methionine sulfone and three differently modified forms of cysteine. By showing the actual solvent gradient during elution (as opposed to the programmed gradient) and by supplying information on the effects of minor changes in solvent-pH, column temperature, flow-rate, and concentration of 2-propanol in the gradient, we make guidelines available for fine-tuning the separation with new Ultrasphere-ODS (C_{18}) columns.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) of phenylthiohydantoin (PTH) amino acids in combination with automated Edman degradation has become an established method in microsequencing of proteins and peptides[1]. Various systems have been described which use either isocratic[2–4], concave[5], linear or step-gradient[6,7], or a combination of isocratic and gradient elutions[2,8–12].

Factors that contribute to an effective PTH-amino acid separation system are: (a) baseline separation of all amino acid derivatives in one run (no need for pre-column derivatization); (b) sharp peaks and low solvent background (to allow quantitation in the low picomol range); (c) reproducibility of separation; (d) short re-

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versed-phase columns (*i.e.* no "tandem columns"[4] or 25-cm columns, which are usually more expensive than 15-cm columns); (e) low flow-rates (longer lifetime of column and guard column); and (f) short analysis time (including re-equilibration of the column).

In this paper we describe a PTH-amino acid separation system that evolved from that described by Hawke *et al.*[11]. Compared with the original system we have increased the sensitivity (determined as peak height per pmol) of PTH-amino acid detection by 100%, while reducing the background absorbance by 50%. In addition we describe the separation of the PTH derivatives of cysteic acid, 4-hydroxyproline, methionine sulfone, S-carboxymethylcysteine, and S-methylcysteine and present experimental results that should facilitate the fine-tuning with other ODS HPLC columns to obtain the same separation pattern.

EXPERIMENTAL

Chemicals

PTH-amino acid standards (Pierce, Rockford, IL, U.S.A.) were prepared in acetonitrile (HPLC grade; Baker, Phillipsburg, NJ, U.S.A.) and kept at −25°C in the dark. The system was calibrated with quantitative PTH-amino acid standards (Pierce). Water, 2-propanol (both HPLC grade), glacial acetic acid and sodium hydroxide pellets were obtained from Baker, trifluoroacetic acid (Sequenal grade) from Pierce, 2-fluorobenzoic acid from Aldrich (Milwaukee, WI, U.S.A.), and α-naphthylacetic acid from Sigma (St. Louis, MO, U.S.A.).

Buffers

All buffers were directly weighed in sulfuric-acid-cleaned and dry glass bottles with polyethylene-lined screw caps. Weighing out the solvents proved to be accurate and reproducible and eliminates possible contaminations by graduated cylinders.

Buffer A. Buffer A consisted of 10% acetonitrile, 42 m*M* trifluoroacetic acid, 21 m*M* acetic acid, pH 3.90 [920 g of water, 86.43 g (110 ml) of acetonitrile, 5.28 g (3.43 ml) of trifluoroacetic acid and 1.39 g (1.32 ml) of glacial acetic acid were adjusted at room temperature to pH 3.90 with x ml of 2 N sodium hydroxide (in HPLC-grade water). The solution was made up to a final volume of 1100 ml by addition of y g of water ($y = 65 - x$)].

Buffer B. Buffer B consisted of 22% acetonitrile, 34% 2-propanol, 20% buffer A, and 24% water [43.2 g (55 ml) of acetonitrile, 66.8 g (85 ml) of 2-propanol, 49.0 g (50 ml) of buffer A, and 60.0 g of water]; pH adjustment was not necessary. The final volume was slightly less than 250 ml, owing to solvent mixture contraction.

Buffers were used without filtration and were stable for at least one month in the coldroom (4°C).

High-performance liquid chromatography

Analyses were performed on a Beckman Model 332 gradient liquid chromatograph, equipped with two Model 110A solvent pumps, a Model 210 sample injection valve with a 2000-μl sample loop, a Model 160 absorbance detector (254 nm; 10-mm flow-cell, 18.5 μl) with a microcontroller, and a Model 420 system controller programmer. Chromatograms were recorded with a Linear recorder, Model 555.

The chromatographic unit consisted of an Ultrasphere-ODS (5 μm, 15 cm × 4.6 mm I.D.); serial No. 4U E2630N) and a guard column (2.3 cm × 4.6 mm I.D.), dry-filled with Vydac A (Varian, Sunnyvale, CA, U.S.A.). Both columns were kept in an electric HPLC column heater (Rainin, Woburn, MA, U.S.A.).

Routinely, 5 μl of a PTH-amino acid mixture in the range 4–400 pmol of each derivative [N^{α}PTH/N^{ε}-phenylthiocarbamyl (N^{ε}PTC)-lysine, 2–200 pmol] were injected, together with the internal standards 2-fluorobenzoic acid (fba, 22-fold molar excess) and α-naphthylacetic acid (naa, 6-fold molar excess). Volumes exceeding 7 μl led to band broadening in the early part of the chromatogram. The standard separation was performed at 33°C with a flow-rate of 0.75 ml/min (leaving the sample loop in the system) and the following gradient (A + B = 100%): 0–0.2 min: 0% B (isocratic); 0.2–1.7 min: 0–27% B (linear); 1.7–4.2 min: 27% B (isocratic); 4.2–14.2 min: 27–95% B (linear); 14.2–17.2 min: 95% B (isocratic); 17.2–17.7 min: 95–0% B (linear); 17.7–38.0 min: 0% B (isocratic).

The sample was injected at 0.1 min (0 min in Fig. 1). At 38 min the system is ready for the next injection.

Increasing the flow-rate to 0.85 ml/min (see below) and omitting the 2000-μl sample loop reduces the overall analysis time (injection, separation, re-equilibration) to 32 min.

The actual gradient was directly measured in a blank run by monitoring the decreasing absorbance of trifluoroacetic acid and acetic acid at 214 nm. The absorbance of 0, 25, 50, 75 and 100% A (Fig. 1β) was determined by equilibrating the system with the indicated percentage of A in A + B. The background absorbance was measured in a blank run at 254 nm.

RESULTS AND DISCUSSION

Separating a standard mixture of PTH-amino acid derivatives, the same degree of resolution is obtained with 1 nmol and 4 pmol. Fig. 1 shows separations at 400, 40, and 4 pmol. Besides the PTH derivatives of the common amino acids we included those of cysteic acid and methionine sulfone (performic acid oxidation products of cysteine and methionine, respectively[13]), S-carboxymethylcysteine and S-methylcysteine (reaction products of cysteine with iodoacetic acid[14] and methyl iodide[15]) and 4-hydroxyproline (commonly found in collagen[16]). Baseline separation is achieved for all derivatives except PTH-histidine and PTH-arginine, which show tailing and partial overlapping with PTH-S-carboxymethylcysteine and PTH-alanine, respectively. The separation of the derivatives of the hydrophobic amino acids proline, methionine, and valine is easily accomplished by the indicated mixture of acetonitrile and 2-propanol in buffer B. PTH-Isoleucine and PTH-4-hydroxyproline both appear as doublets, representing the isomerized forms[17].

The superimposed actual solvent gradient (Fig. 1β) reveals that PTH-cysteic acid is eluted isocratically with buffer A, which explains why it is the only derivative that is not eluted as a sharp peak. We determined the retention times of the PTH-amino acid derivatives (single-letter code, Fig. 1) relative to 2-fluorobenzoic acid: Ca (−4.3 min), fba (0.0 min), N (0.7 min), D (1.0 min), S (1.4 min), T (1.75 min), Q (2.1 min), G (2.4 min), H (2.75 min), cmC (2.9 min), E (3.4 min), Ms (3.6 min), first hP (3.85 min), R (4.2 min), A (4.4 min), second hP (4.95 min), Y (5.5 min), mC (6.75

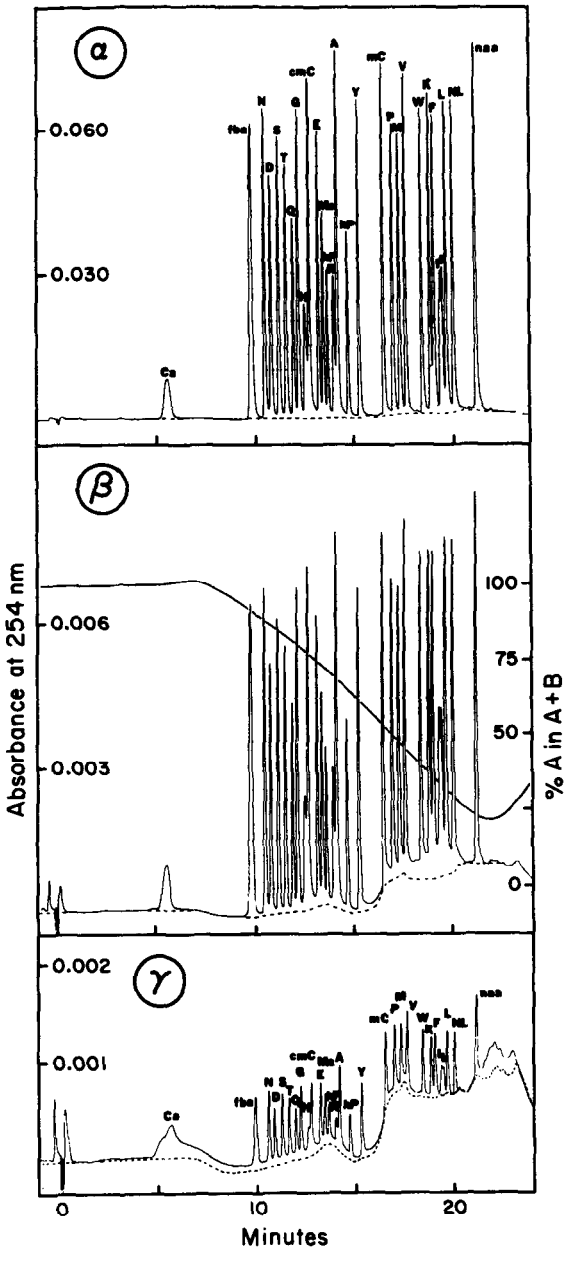

Fig. 1. Separation of a standard mixture of PTH-amino acids by reversed-phase HPLC. (α) 400 pmol, (β) 40 pmol, (γ) 4 pmol of each amino acid derivative (N^{α}PTH/N^{ϵ}PTC-lysine half the indicated amounts); accuracy of quantitation, ±7%. The one-letter code for PTH amino acids is used: Ca = cysteic acid; fba = 2-fluorobenzoic acid (internal standard); cmC = S-carboxymethylcysteine; Ms = methionine sulfone; (first and second) hP = 4-hydroxyproline; mC = S-methylcysteine; NL = norleucine; naa = α-naphthylacetic acid (internal standard). Samples were injected at 0 min; separation of derivatives and measurement of the background (dashed lines) and the actual gradient (β, solid line) were performed as described in the Experimental section.

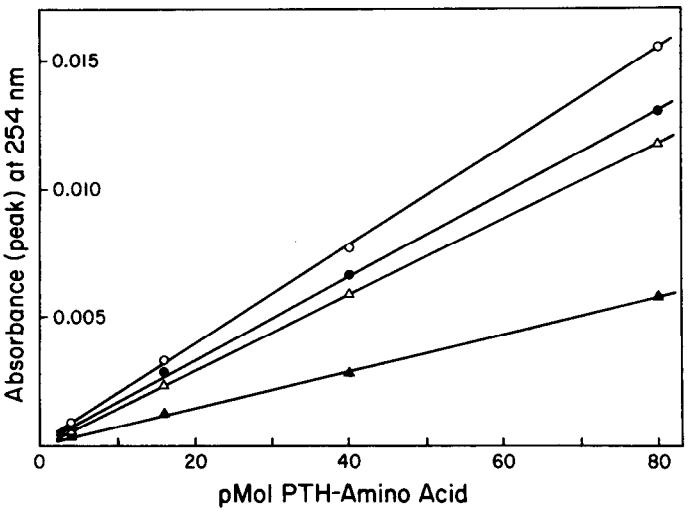

Fig. 2. Quantitation of four selected PTH-amino acids by correlating the peak height with the amount of the PTH derivative. Standard separation was performed as described in the Experimental section. Peak heights were corrected for the background contribution, shown in Fig. 1. The values for PTH-alanine were not corrected for the overlap of PTH-arginine. ○ = PTH-alanine; ● = PTH-glycine; △ = PTH-methionine; ▲ = PTH-arginine.

min), P (7.2 min), M (7.55 min), V (7.9 min), W (8.7 min), K (9.1 min), F (9.3 min), first I (9.65 min), second I (9.75 min), L (9.95 min), NL (10.35 min) and naa (11.5 min).

Owing to the sharpness of the peaks, a linear relationship is found between peak height and amount of PTH-amino acid over the range 4–1000 pmol for all derivatives except cysteic acid. Fig. 2 shows this linearity in the low picomole range for four selected PTH-amino acids. Differences in absorbance maxima (*cf.* PTH-glycine and PTH-arginine, Fig. 2) are due to differences in the baseline width (the extinction coefficients ε_{254} of PTH amino acids are very similar[6]). To achieve linear correlation, accurate background determination is required. In blank runs we found the background absorbance to be highly reproducible. During protein and peptide sequencing we consider the blank run and the immediately preceding run.

Comparing three Ultrasphere-ODS columns (serial Nos. 4UE 2630N, 4UE 2634N, and 4UE 2635N) from the same batch, we found that the front part of the chromatogram (PTH-cysteic acid to PTH-threonine) and the second half (including PTH-alanine and the amino acid derivatives thereafter) were absolutely reproducible among the three columns. However, in the region from PTH-glutamine to PTH-arginine we noticed co-elution of two or three peaks with columns 34N and 35N but not with 30N (Fig. 1). With the intention of providing guidelines for the fine-tuning of new columns, we varied selected parameters (one at a time) of the standard separation system (described above; Fig. 1). Our results can be summarized as follows:

Increasing the flow-rate from 0.75 to 0.85 ml/min reduces the *absolute* retention times (measured from injection) by *ca.* 2 min. The separation pattern remains unchanged. *Relative* retention times (compared with fba = 0 min) are reduced by *ca.* 5% (chromatogram compressed).

Lowering the column temperature from 33 to 25°C increases the absolute retention times by 0.5–1 min for all derivatives (overall pattern unchanged) except for the first PTH-4-hydroxyproline peak and PTH-histidine, which show smaller increases.

Raising the column temperature from 33 to 40°C shortens absolute retention times by 0.5–1 min for all derivatives (overall pattern unchanged); those for PTH-histidine and PTH-arginine are shortened the most.

Lowering the pH of buffer A from 3.90 to 3.75 (and also that of buffer B) leaves the absolute retention times of all non-acidic PTH-amino acids unchanged but retards fba by 1 min, PTH-aspartic acid by 0.4 min, PTH-S-carboxymethylcysteine by 0.4 min, and PTH-glutamic acid by 0.2 min.

Raising the pH of buffer A to 4.05 (and also that of buffer B) leaves the absolute retention times of all non-acidic PTH amino acids unchanged but shortens the elution times of fba by 1 min, PTH-aspartic acid by 0.4 min, PTH-S-carboxymethylcysteine by 0.4 min (which elutes now before PTH-histidine), and PTH-glutamic acid by 0.2 min.

Reducing the concentration of 2-propanol in buffer B from 34% to 32.5% increases the absolute retention times of all derivatives by *ca.* 0.5 min (chromatogram extended). Relative retention times are increased by *ca.* 5% and N^{α}PTH/N^{ε}PTC-lysine migrates closer to PTH-phenylalanine.

Increasing the concentration of 2-propanol in buffer B (at the expense of water) from 34% to 35.5% shortens the absolute retention times by 0.5 min and the relative retention times by *ca.* 5%. The chromatographic pattern is compressed.

Systematic evaluations of parameters that affect the reversed-phase HPLC separation of PTH-amino acids have been reported for an isocratic system by Lottspeich[3] (column: Hibar 250-4, LiChrosorb RP-18, 5 μm) and for a gradient system by Somack[9] (column: Ultrasphere-ODS, 250 × 4.6 mm I.D.). Owing to different standard conditions (columns, temperature, pH, and salt composition), results derived from these two systems and from ours cannot be expected to be in total agreement. As far as reproducibility of chromatographic patterns is concerned, other authors[18,19] also found small changes in retention times when comparing the same kind of HPLC columns. Nevertheless, re-adjustment is often possible, if parameters affecting the separation are well defined and can be controlled. We believe therefore that detailed information should accompany the description of new or modified HPLC separation systems; *i.e.*, the gradients, shown in most publications[2,5,9,11], never turn out to be as angular as programmed and are certainly affected by the flow-rate, the size and shape of the mixing chamber, the sample loop and the guard column.

Introducing the direct measurement of the actual solvent gradient (Fig. 1β) and supplying information on small perturbations of our standard conditions and how they affect the separation, may help establish the general usefulness of the above described separation system for PTH-amino acids.

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