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Note

High-performance liquid chromatographic support for the baseline separation of all phenylthiohydantoin amino acids

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Resolution of the phenylthiohydantoin (PTH) amino acid derivatives by high-performance liquid chromatography (HPLC) is essential for the sequence analysis of proteins by the Edman degradation. The PTH-amino acid derivatives must be analyzed in a short time, be resolved in a single run with a maximum of peak sharpness, and be quantified at low levels (< 10 pmol).

Separations of the PTH-amino acid derivatives by HPLC have been reported for several supports [1—5]. These separations generally show at least one pair of poorly resolved peaks or broad peaks. We report here the results using a support, diphenyl modified silica, which has been demonstrated to have different elution characteristics than straight-chain alkanes [6]. This support provides superior resolution of the difficult to separate PTH-amino acids.

MATERIALS AND METHODS

Reagents

Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid, sodium acetate and sodium hydroxide were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Trifluoroacetic acid and the PTH-amino acid standards were purchased from Pierce (Rockford, IL, U.S.A.). A stock PTH-amino acid mixture containing 200 nmol of each was prepared in 1 ml acetonitrile and stored at $-80°$C until use. The water used in the buffer preparations was HPLC grade prepared by a Hydro Services system (Research Triangle, NC, U.S.A.).

The buffer system used with the cyano column was prepared according to Hunkapiller and Hood [5]. The buffer system used with the C_{18} support was prepared likewise, except the pH of buffer A was varied in order to obtain

better resolution of the PTH-amino acids. The trifluoroacetate—acetate buffer system of Hawke et al. [1] was used with the Altex column, with the exception that the pH of buffer A was varied from 4.8 to 6.0 in order to obtain resolution of certain PTH-amino acids.

A modified version of the trifluoroacetate—acetate buffer system of Hawke et al. [1] was used with the diphenyl support. Buffer B was prepared as above [1], however, buffer A was prepared as follows: 66 m*M* trifluoroacetic acid and 4 m*M* acetic acid adjusted to various pH values (5.6—6.2) with 1 *M* sodium hydroxide and dilute ammonium hydroxide. Sodium hydroxide was used to take the buffer to pH 4.9 and then ammonium hydroxide to the desired pH.

HPLC equipment

A Beckman Model 421 controller, two Beckman Model 112 solvent delivery modules, a Beckman Model 340 organizer (Beckman Instruments, Berkeley, CA, U.S.A.), and a Spectroflow Model 757 absorbance detector (Kratos, Westward, NJ, U.S.A.) were used.

The columns used in the separation of the PTH-amino acids were: (a) an Altex Ultrasphere-ODS column (5 μm particle size, 250 × 4.6 mm) obtained from Rainin Instrument (Woburn, MA, U.S.A.), (b) a Cyano (5 μm particle size, 250 × 4.6 mm), (c) a Bakerbond C_{18} (5 μm particle size, 250 × 4.6 mm) and (d) a Bakerbond wide-pore diphenyl column (5 μm particle size, 250 × 4.6 mm) all obtained from J.T. Baker.

RESULTS

The twenty PTH-amino acid derivatives could not be completely resolved using a Bakerbond cyano column even with extensive variation of the gradient. The PTH-amino acids that could not be well resolved were valine/proline/methionine and tryptophan/isoleucine/phenylalanine.

The best gradient using the Altex ODS column also yielded incomplete resolution of some PTH amino acids. Valine/proline could not be resolved and complete separation of glutamine/glycine and tryptophan/isoleucine/phenylalanine could not be obtained. In addition, the resolution of tryptophan/isoleucine/phenylalanine was not reproducible from run to turn.

The best gradient using the Bakerbond C_{18} column showed no resolution of valine/methionine and incomplete resolution of glutamic acid/carboxymethyl cysteine, isoleucine/phenylalanine, and valine/proline.

Three Bakerbond wide-pore diphenyl columns from different lots were used to resolve the twenty PTH-amino acids and all gave complete separation. One support was pre-treated with a pyridine—acetate buffer prior to use. The resolution of the PTH amino acid derivative mixture using this support is shown in Fig. 1A. Incomplete separation of carboxymethyl cysteine/asparagine/serine, proline/methionine and phenylalanine/tryptophan was obtained. The separations, however, are easily sufficient to clearly identify the twenty amino acid derivatives. The same separations were obtained using samples of 10 pmol of each amino acid (data not shown).

The other two columns had no pre-treatment with pyridine—acetate buffer, and were used as they came from the manufacturer. The resolution of the PTH-

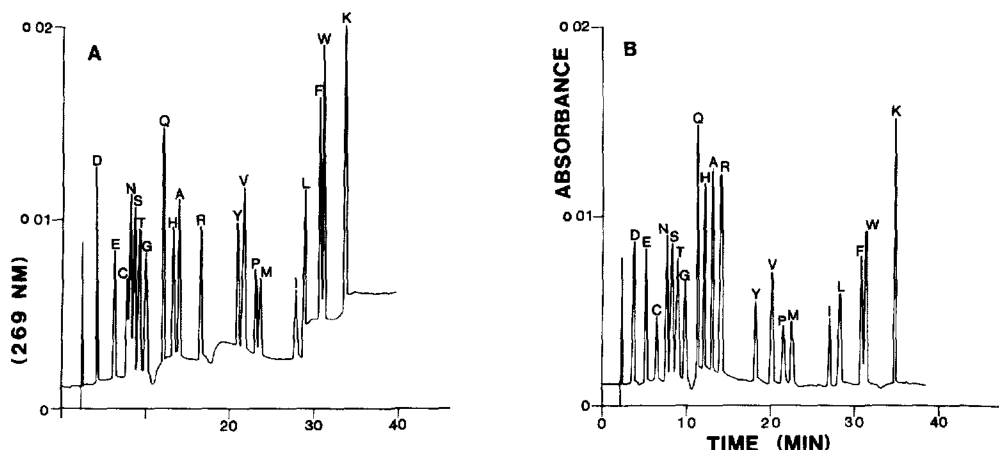

Fig. 1. Separation of PTH-amino acids on Bakerbond wide-pore diphenyl columns. Each peak represents 300 pmol (15 μl of stock solution injected) of the respective amino acid. For each chromatographic run the recorder range was set at 10 mV, the detector at 0.02 a.u.f.s. and the flow-rate was 1.5 ml/min. (A) Buffer A is 66 m*M* trifluoroactate—4 m*M* acetate, pH 5.6 and buffer B is acetonitrile—35 m*M* trifluoroacetate, pH 3.6 (75·25). The program (time in min) is as follows: (0) 5% B, (7) 18% B, (14) 28% B, (25) 45% B, (30) 50% B, (32) 100% B, (36) 5% B. The column temperature was 30°C. (B) Buffer A is 66 m*M* trifluoroacetate—4 m*M* acetate, pH 5.8 and buffer B is acetonitrile—35 m*M* trifluoroacetate, pH 3.6 (75:25). The program (time in min) is as follows: (0) 8% B, (7) 26% B, (20) 32% B, (25) 40% B, (30) 50% B, (36) 100% B, (40) 8% B. The column temperature was 27°C. Peaks: D = aspartic acid; E = glutamic acid: C = carboxymethyl cysteine; N = asparagine; S = serine, T = threonine; G = glycine; Q = glutamine; H = histidine; A = alanine; R = arginine; Y = tyrosine; V = valine; P = proline; M = methionine; I = isoleucine; L = leucine; F = phenylalanine; W = tryptophan; K = lysine.

amino acid mixture is shown in Fig. 1B. As with the first diphenyl column, the same separations were achieved with an amino acid mixture containing 10 pmol of each. A slightly different gradient than for the other diphenyl column was found to give the best resolution. Complete separation of all PTH derivatives was obtained. Although the gradient can be adjusted to provide a shorter run time we have chosen this gradient to provide a separation that prevents any possible ambiguity in the assignment of unknowns from the sequencer.

The pH of buffer A plays a major role in the elution times of arginine and histidine in this gradient. At a high pH (6.2) both elute early, histidine eluting with glutamine and arginine eluting with alanine. As the pH is lowered to 5.8 both arginine and histidine elute later, at the positions shown in Fig. 1B. Changing the pH of buffer A has essentially no effect on the elution times of the remaining eighteen PTH-amino acids.

The buffer A system used, that is adjusting the pH with both sodium hydroxide and ammonium hydroxide, was found to be necessary to give the best resolution of the PTH-amino acids at the beginning of the gradient elution. Without the presence of the ammonium ion, the resolution of asparagine/serine is not possible and the use of ammonium ion alone results in loss of resolution of other PTH amino acids.

Lower column temperatures also enhance the resolution of the PTH-amino acids. A column temperature of 27°C was found to give optimum resolution.

As the temperature was increased to 46.5°C, the resolution decreased substantially (data not shown).

DISCUSSION

In our efforts to obtain complete separation of all twenty PTH-amino acids we used a number of published methods [1—5] and modifications of these methods. In all cases one or more pairs of PTH-amino acids were poorly resolved or not resolved at all. In order to achieve a different selectivity than the cyano or C_{18} columns previously used, a diphenyl support was used. With this column we were able to achieve the complete separation of all twenty PTH-amino acids.

The reasons for the lack of success using the published methods may be due to variations in the column packings, the HPLC systems, or mobile phase components. In order to test the variation in the diphenyl column packings we tested three different columns from three separate lots and all were capable of achieving the resolution shown in Fig. 1. Although the separations shown in Fig. 1 are using conditions optimal for our work we have used other flow-rates, gradients, and pH values which provided complete separation of all peaks as well.

The key features of the separation described here are: (1) using a pH value that allows PTH-histidine and PTH-arginine to elute in a proper position, (2) using both NH_4^+ and Na^+ ions, and (3) the diphenyl packing. This separation system has been in virtually continuous use in our laboratory for over six months with little change in elution times or peak shapes. The use of this HPLC support, diphenyl modified silica, provides an easy method to achieve the complete separation of all twenty PTH-amino acids in 34 min and should be particularly useful for those laboratories that have had difficulties with other methods.

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