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USE OF A TERNARY GRADIENT FOR THE SEPARATION OF PHENYL-THIOHYDANTOIN-AMINO ACIDS, INCLUDING THE METHYL ESTERS OF ASPARTIC AND GLUTAMIC ACIDS, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The phenylthiohydantoin (PTH) derivatives of the common amino acids can be resolved in a single high-performance liquid chromatographic analysis by elution from a cyanopropylsilane column with a ternary gradient of methanol, acetonitrile, and ammonium acetate. The system is compatible with an automated sequencer–automated converter combination that produces the methyl esters of PTH-aspartic and PTH-glutamic acids.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for the analysis of phenylthiohydantoin (PTH) amino acids produced in protein sequencing has large-ly replaced the previously used techniques of gas–liquid and thin-layer chromato-graphies[1,2]. This technique has been found to provide greater sensitivity of detection and more reproducible single chromatographic separations of most or all of the commonly encountered PTH-amino acid derivatives[3–9]. However, two technological advances have diminished the usefulness of previously devised HPLC systems. The first of these is the introduction of the automated converter as a standard adjunct to the automated sequencer. Where previously the operator of the automated sequencer was faced with manually converting unstable 2-anilino-5-thiazolinone amino acid derivatives into stable PTH-amino acids by treatment with acid followed by extrac-tion into an organic solvent, the automated converter produces stable PTH deriva-tives in high yields, especially PTH-Ser and PTH-Thr, as part of the sequencer cycle. In so doing, it converts all free carboxyl groups into their corresponding methyl esters. Consequently, PTH-Asp and PTH-Glu are converted into PTH-aspartic acid methyl ester (PTH-Asp-ME) and PTH-glutamic acid methyl ester (PTH-Glu-ME), respectively. In most existing HPLC systems, the free acid PTH derivatives are well separated from other PTH-amino acids and from each other, whereas the correspon-ding methyl ester PTH-derivatives are not. They elute in a very congested portion of the elution profile and either overlap with or are inseparable from other PTH-amino

acids. The second technological advance has come in the area of HPLC instrumentation. Whereas most reported systems were devised for isocratic or binary gradients, more recently introduced instruments have ternary gradient capabilities and, moreover, some have, as standard equipment, data systems which automatically integrate peak areas and calculate component concentrations. While the ability to use three pure solvents has helped to overcome some previously encountered problems, such as solvent composition changes during successive runs caused by non-proportional evaporation from mixtures of two or more volatile solvents, others have been introduced. One such problem is the difficulty of devising ternary gradient systems. Another, encountered with instruments equipped with data systems, is to have all components of the mixture sufficiently separated from each other so that the data system recognizes and integrates each, and yet to have the program sufficiently short that it can keep pace with the automated sequencer. In general, data systems put the following restrictions upon the HPLC gradient–column system adopted: peaks should be separated by a sufficient time interval to be recognized as individual peaks, and peaks should be sufficiently broad to allow proper integration. In this report, we present a ternary gradient which not only separates all the common PTH-amino acids (with PTH-Asp and PTH-Glu as their methyl esters) but also does so in a manner compatible with both an internal data system and an automated sequencer.

EXPERIMENTAL

Reagents

Acetonitrile and methanol were distilled-in-glass grade solvents from Burdick and Jackson (Muskegon, MI, U.S.A.). The water was deionized and doubly distilled. Ammonium acetate (reagent grade) was from Fisher Scientific (Fairlawn, NJ, U.S.A.) and the PTH-amino acid standards were from Mann Labs. (New York, NY, U.S.A.) and from Pierce (Rockford, IL, U.S.A.).

HPLC equipment

The instrument used was a Spectra-Physics Model SP 8000 high-performance liquid chromatograph fitted with the following accessories: Model 4000 data system, printer/plotter, constant temperature oven ($\pm 1°C$), automatic injector, helium degassing unit, ternary gradient unit, ultraviolet detector (254 nm) and 10-μl injector loop.

A DuPont (Wilmington, DE, U.S.A.) Zorbax CN (cyanopropylsilane, 25 × 0.46 cm I.D.) column, in conjunction with a DuPont Permaphase ETH (5 × 0.46 cm I.D.) guard column, was used for the separations. A new column was prepared for use by washing it successively with the following solvents at 1 ml/min for 45 min: acetonitrile, methylene chloride, acetonitrile, acetonitrile–20 mM ammonium acetate (50:50) and 20 mM ammonium acetate. The column was then equilibrated with the starting gradient solvent mixture at 31°C.

Analytical procedures

Methanol and acetonitrile were degassed by vigorous helium sparging for 10 min. Ammonium acetate, prepared fresh daily by dilution of a 1 M stock solution (stored at 4°C), was degassed first by sonication for 15 min in a bath-type sonicator followed by sparging for at least 10 min with helium. A helium atmosphere was

maintained over each solvent. The concentrations of ammonium acetate ranged from 14 to 24 mM and depended upon the age of the column and the elution behavior of PTH-Arg and PTH-His.

Stock solutions (ca. 5 mM) of the individual PTH-amino acids were prepared in acetonitrile, with the exceptions of PTH-Arg, PTH-His and PTH-cysteic acid, which were prepared in ethanol. Exact concentrations of stock solutions were established spectrophotometrically at 269 nm from known extinction coefficients[2]. Exact amounts of stock solutions (stored at $-16°C$) were combined and diluted to give 1 nmole of each derivative per 10 μl. Concomitant with sample injection a ternary gradient of varying percentages of methanol, acetonitrile and ammonium acetate was initiated at a flow-rate of 1 ml/min. Successive injections were made at 40–45 min intervals.

Methyl esters of PTH-Glu and PTH-Asp were usually prepared with the automated converter, but occasionally they were prepared manually in the following manner. The sample to be esterified was evaporated to dryness in a small test-tube, and 200 μl of a 1.5 N HCl solution in anhydrous methanol (acetyl chloride–cold anhydrous methanol, 1:7) were added, the tube was flushed with nitrogen, and heated for 8 min at 65°C. The sample was evaporated to dryness and made up to the original volume in acetonitrile. Quantitative conversion into the esters was observed.

RESULTS AND DISCUSSION

Common PTH-amino acids

A typical elution profile using the above conditions is shown in Fig. 1. Separation of the nineteen common PTH-amino acids was obtained with the ternary gradient given in Table I. As demonstrated by the Figure, all components of the mixture were well separated. PTH-Pro and PTH-Met were the least well resolved of the PTH-amino acids but were sufficiently so that they could be accurately integrated by the data system. With a new column the separation of PTH-Pro and PTH-Met is practically baseline, but as the column ages the results shown in Fig. 1 are typical. Another pair of PTH-amino acids whose resolution is affected by column age is that of PTH-Thr and PTH-Gln. With a new column these two derivatives almost co-elute. Their resolution can be enhanced by lowering the temperature 1–2°C or by reducing the solvent flow-rate to 0.8–0.9 ml/min. As the column is used, the separation shown in Fig. 1 is typical. The introduction of the methyl esters of PTH-Glu and PTH-Asp into an area, which in other systems is already congested, poses no problems with this system; both esters are well separated from each other and from the other PTH-amino acids. Almost all separations obtained are baseline, a feature not often observed with other systems.

The point of departure in developing the ternary gradient was the system reported by Johnson *et al.*[5], who introduced the use of the cyanopropylsilane column for the HPLC of PTH-amino acids. Their basic system (0.024 M sodium acetate, pH 5.4, and methanol–acetonitrile, 17:3) was extended to a ternary one. Aqueous ammonium acetate was found to be just as effective as sodium acetate buffer, and has been used because it does not require pH adjustment. A problem encountered with this substitution is that ammonium acetate supports microbial growth better than does sodium acetate. Therefore, the ammonium acetate solutions were made fresh
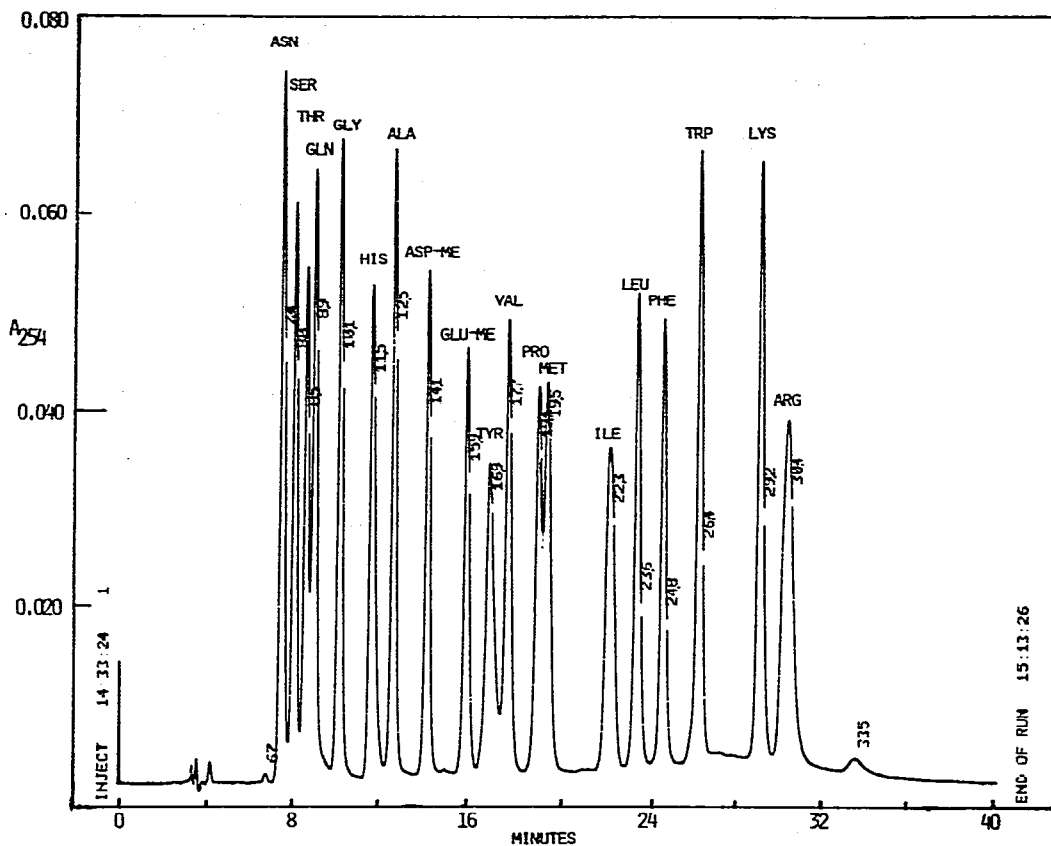
Fig. 1. Elution profile of nineteen common PTH-amino acids by HPLC using a ternary gradient and a cyanopropylsilane column. The column was equilibrated with the solvent shown at zero time in Table I. At injection of 1 nmole of each amino acid the composition of the ternary gradient changed as indicated in the table, returning to the initial conditions at 28 min. Another sample could be injected 40 min after the first. The flow-rate was 1 ml/min and the temperature was maintained at 31°C.

TABLE I

PERCENTAGE COMPOSITION OF MOBILE PHASE TERNARY GRADIENT

Time (min)	Methanol	Acetonitrile	19 mM ammonium acetate
0.0	0.0	20.0	80.0
5.0	20.0	15.0	65.0
10.0	20.0	10.0	70.0
15.0	40.0	5.0	55.0
20.0	55.0	0.0	45.0
28.0	0.0	20.0	80.0

daily from a 1 *M* stock solution, and solvent lines were washed free of it with distilled water when the instrument was left unused for more than a day.

The elution of PTH-Arg and PTH-His is dependent upon the ionic strength of

the aqueous salt component of the gradient as a result of the partial ion-exchange character exhibited by Zorbax CN columns. The elution times of these two PTH-amino acids may be controlled by adjusting the ionic strength of the ammonium acetate solution. Increasing the ionic strength shifts PTH-His towards PTH-Gly and PTH-Arg towards PTH-Lys; decreasing the ionic strength, the reverse. The compromise desired is to place PTH-His between PTH-Gly and PTH-Ala with PTH-Arg as close as possible to PTH-Lys in order to avoid prolonged analysis times. Attempts to substitute acetic or propionic acids of varying molarities for sodium or ammonium acetates resulted in the retention of both PTH-Arg and PTH-His on the column. As the column ages with use, its ion-exchange character increases; accordingly, the molarity of the ammonium acetate must be increased to maintain the desired separations. The age of the column reported in these experiments required the use of 19 mM ammonium acetate to achieve the observed elution times of PTH-His and PTH-Arg.

The elution of PTH-Arg and PTH-His is also influenced by the methanol concentration in the part of the gradient where they normally elute. Increasing or decreasing the methanol concentration slightly (1–2 %) in the 5–10-min portion of the gradient affects PTH-His and in the 15–20-min portion, PTH-Arg. However, the effect of methanol concentration on the elution of these two PTH-amino acids is not as great as is that observed with the ionic strength of the ammonium acetate.

The composition of the ternary gradient established at each step was influenced by the following observations: (1) acetonitrile affected the separation of compounds eluted in the first half of the gradient and had essentially no effect on the elution behavior of the compounds in the second half of the gradient; (2) separation of the compounds in the center of the gradient was influenced by the rate at which methanol and acetonitrile changed in relation to each other, with methanol increasing and acetonitrile decreasing; and (3) separation of compounds eluting in the second half of the gradient was largely dictated by the proportion of methanol to ammonium acetate solution.

Temperature also influenced the extent of the separations obtained. Temperatures greater than 31°C tended to sharpen the peaks of components in the first half of the gradient while broadening the peaks of components in the second half of the gradient (especially PTH-Tyr, PTH-Val and PTH-Pro, PTH-Met). Temperatures lower than 31°C tended to push components eluting in the first half of the gradient together while giving a slightly better separation of components in the second half of the gradient. Temperatures lower than 31°C also had the generally undesirable effect of broadening peaks and thus decreasing recognition by the data system.

Low pressures of operation increase the useful lifetime of the column, and this system has relatively low operating pressures. A typical experimental run begins at *ca.* 550 p.s.i. (37 atm) and reaches a maximum of *ca.* 800 p.s.i. (54 atm) after the steepest part of the gradient has been reached. It should also be noted in Table I that the gradient is returned to initial conditions by the time PTH-Arg is eluted, and there is a wash cycle and equilibration time of 12–15 min between injections. Low operating pressures and low column temperatures extend column life, and the short analysis time allows the system to keep pace with an automated protein sequencer.

Although the absolute retention times of PTH-amino acids have been observed to vary somewhat as the column ages, the relative retention times have been constant.

Other PTH-amino acids

The acidic PTH-amino acids, PTH-Glu, PTH-Asp, PTH-CM-Cys and PTH-cysteic acid, elute with the void volume of the column in an unresolved peak. When the automated converter is used, the first three will be present as their methyl esters and only PTH-cysteic acid will be in this region. Although not shown, PTH-CM-Cys-ME elutes immediately after and is not always resolved from PTH-Met. The methyl ester of this PTH-amino acid was unstable and it was generally identified from the [^{14}C]iodoacetic acid used to carboxymethylate the protein. PTH-Met sulfone elutes between PTH-Ala and PTH-Asp-ME, and is well separated from each. PTH-hydroxy-proline elutes in approximately the same position as PTH-Met sulfone, and at times merges with it. PTH-methyl cysteine elutes between PTH-Tyr and PTH-Val and is sufficiently well separated to be integrated by the data system. PTH-norleucine elutes between PTH-Leu and PTH-Phe, and is well separated from each. If desired, PTH-Nle could be used effectively as an internal standard.

Other observations

Johnson *et al.*[5] noted that one disadvantage of the Zorbax CN columns was the high baseline rise toward the end of the gradient. With this ternary gradient and the solvents used, baseline rise has not been a problem even at the higher sensitivities of the instrument, permitting the detection of as little as 10 pmoles of PTH-amino acids.

A powerful advantage of the ternary system, besides the ability to use pure solvents, is the ability to vary their combinations readily without the necessity of preparing solvent mixtures which characterize binary systems. The gradients may be linear, convex, concave, or any combination of the three. In the system developed, the column is equilibrated with 20 % acetonitrile–80 % ammonium acetate. Over the first 5-min period after injection of the sample, the ammonium acetate is decreased linearly to 65 % and the acetonitrile is decreased linearly to 15 %. At the same time, the methanol concentration is increased linearly from 0 % to 20 %. The changes at the other times are similar. It is a simple matter to hold the gradient constant for any portion of the cycle. For example, if after 10 min it were desired to hold the gradient constant for 5 min, the percentages entered at 15 min would be the same as for 10 min. Individual steps may be for any duration and can be programmed to 0.1 min.

Reversed-phase packings for HPLC columns used in the determination of PTH-amino acids have included octadecylsilane[3,4,7–9], phenylalkyl[6] and cyanopropylsilane[5]. While each has its own advantages and disadvantages, the cyanopropyl-silane column proved to give superior resolution of PTH-amino acids with the ternary gradient developed in the present study. In practice, PTH derivatives from an automated sequencer equipped with an automated converter have been identified without ambiguity. Periodically, standard PTH-amino acids are run to verify assignment of one of two closely eluting derivatives. The method shows great versatility and should prove to have general utility.

REFERENCES

1   H. D. Niall, *Methods Enzymol.*, 27 (1973) 942–1010.
2   P. Edman, in S. B. Needleman (Editor), *Protein Sequence Determination*, Springer, New York, Berlin, 1970, pp. 211–255.
3   C. L. Zimmerman, E. Apella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569–573.
4   P. W. Moser and E. E. Rickli, *J. Chromatogr.*, 176 (1979) 451–455.
5   N. D. Johnson, M. W. Hunkapillar and L. E. Hood, *Anal. Biochem.*, 100 (1979) 335–338.
6   L. E. Henderson, T. D. Copeland and S. Oroszlan, *Anal. Biochem.*, 102 (1980) 1–7.
7   J. Fohlman, L. Rask and P. A. Peterson, *Anal. Biochem.*, 106 (1980) 22–26.
8   J. U. Harris, D. Robinson and A. J. Johnson, *Anal. Biochem.*, 105 (1980) 239–245.
9   S. M. Rose and B. D. Schwartz, *Anal. Biochem.*, 107 (1980) 206–213.