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METHYLTHIOHYDANTOIN AMINO ACIDS: CHROMATOGRAPHIC SEPARATION AND COMPARISON TO PHENYLTHIOHYDANTOIN AMINO ACIDS

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SUMMARY

Most phenylthiohydantoin (PTH) amino acids and most methylthiohydantoin (MTH) amino acids may be separated from one another by thin-layer chromatography (TLC) using the same sequential development technique with the same two solvents. Similarly, a single solvent system may be used in high-performance liquid chromatography (HPLC) to separate most PTH-amino acids and most MTH-amino acids. When both TLC and HPLC separations are performed on a sample, all MTH- and PTH-amino acids can be uniquely identified. Since many solid-phase protein sequencing techniques generate both MTH- and PTH-amino acids, these analytical systems simplify identification of the amino acid derivatives. Although the chromatographic properties of MTH- and PTH-amino acids are similar, they are not identical (contrary to a previous report).

INTRODUCTION

The sequential degradation of proteins and peptides developed by Edman[1] has become the standard method for amino acid sequence determination. Automation of Edman's method, resulting in the liquid-phase sequencer[2], has greatly improved stepwise efficiency and the ability to achieve elongated sequences. Modification of the Edman technique by Laursen[3], using an insoluble solid-support for the peptide or protein, and subsequent automation of Laursen's method[4], have aided in handling the wash-out of soluble peptides and proteins in the liquid-phase sequencer, and offered a less costly alternative to the liquid-phase sequencer. The technology of both liquid- and solid-phase sequencing has advanced constantly and is the subject of recent reviews[5–7].

The amino acid removed at each sequential degradative step of the Edman method is identified as its phenylthiohydantoin (PTH) derivative. PTH-amino acids have been identified by the techniques of gas chromatography (GC)[8,9], thin-layer

chromatography (TLC)[10,11] and high-performance liquid chromatography (HPLC)[12,13]. This literature has been the subject of recent reviews[14–16].

During the course of our research, we needed to compare separations of the PTH and methylthiohydantoin (MTH) amino acid derivatives. MTH-amino acids are formed as the result of one of the solid-phase sequencing techniques. When amino-substituted resins are used for peptide or protein immobilization, excess free amino groups on the support must be blocked with methylisothiocyanate[4]. This reaction prevents the solid-support from reacting with phenylisothiocyanate used during the Edman degradation, and yields a resin with better flow characteristics. When coupling procedures are employed using peptides or proteins with unprotected amino groups[17,18], the first amino acid of the peptide or the protein becomes, after the first degradative cycle, the MTH-amino acid derivative of the first residue. Separation methods using GC[19,20] and TLC[21,22] have been reported for the MTH-amino acids. HPLC, used increasingly for the identification of the PTH-amino acids, however, has not been used for the identification of the MTH–amino acids.

Therefore we initiated studies to characterize the MTH-amino acids by HPLC, as well as to reinvestigate separations using TLC. A specific goal of the investigations was to find solvent systems which would separate both the MTH- and the PTH-amino acids, thus simplifying identification of the first amino acid derivative from the solid-phase Edman degradation.

EXPERIMENTAL

Materials

Standard PTH-amino acids were obtained from Pierce (Rockford, Ill., U.S.A.). MTH-amino acids were obtained from Pierce and Sigma (St. Louis, Mo., U.S.A.). Acetonitrile (UV grade) and methanol, used for HPLC, were purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Chloroform (0.75% ethanol as preservative) and absolute methanol, for TLC, were reagent grade (Fisher Scientific, Pittsburgh, Pa., U.S.A.). Absolute ethanol was reagent grade and was a gift from R. A. Laursen.

Methods

High-performance liquid chromatography. Solutions of the PTH-amino acids and MTH-amino acids were prepared in absolute methanol. Acetonitrile was added to promote solubility in some cases. HPLC was performed using equipment from Waters Assoc. (Milford, Mass., U.S.A.). A gradient was achieved using a Model 660 solvent programmer and two Model 6000A chromatography pumps. Samples injected with a Model U6K injector were separated on a μBondapak C_{18} column (30 × 0.4 cm I.D.) and detected at 254 nm with a Model 440 absorbance detector. Pump A delivered buffer A [0.01 *M* sodium acetate, pH 4.0–acetonitrile (9:1, v/v)], and pump B delivered buffer B [0.01 *M* sodium acetate, pH 4.0–acetonitrile (1:9, v/v] as described by Downing and Mann[23]. Chromatography was performed at room temperature at flow-rates of 2 ml/min. Identities of peaks of the amino acid derivatives were verified by single injection of individual amino acid derivatives.

Separation of the PTH-amino acids was performed using gradient elution from 5% B to 90% B over a period of 45 min, employing curve 7 of the Waters

programmer. Separation of the MTH-amino acids was performed using gradient elution from 5% B to 90% B over a period of 30 min, employing curve 9 of the Waters programmer.

Thin-layer chromatography. Solutions of the PTH- and MTH-amino acids were prepared as above. TLC was performed on 20 × 20 cm silica gel plates with fluorescent indicator (silica gel GHLF, 250 μm), obtained from Analtech (Newark, Del., U.S.A.). Thin-layer plates were developed first in solvent I [chloroform–ethanol (98:2)]. After air drying the thin-layer plate, it was developed, in the same direction, in solvent II [chloroform–ethanol–methanol (89.25:0.75:10)], using a modification of a procedure developed by Laursen[4]. Chromatography was performed in a saturated tank at room temperature. Plates were visualized under short wavelength light.

RESULTS

Most of the PTH-amino acids are resolved from one another by HPLC (Fig. 1). Three pairs of PTH-amino acids, PTH-glycine and PTH-glutamine, PTH-proline and PTH-methionine, and PTH-phenylalanine and PTH-tryptophan are not resolved using this chromatographic system. Using the same column and solvents, but employing a different gradient, HPLC provides effective resolution of most of the MTH-amino acids (Fig. 2). MTH-glycine and MTH-glutamine coelute as do the same PTH-amino acids. The MTH-derivatives of proline, valine and lysine also have nearly identical elution times.

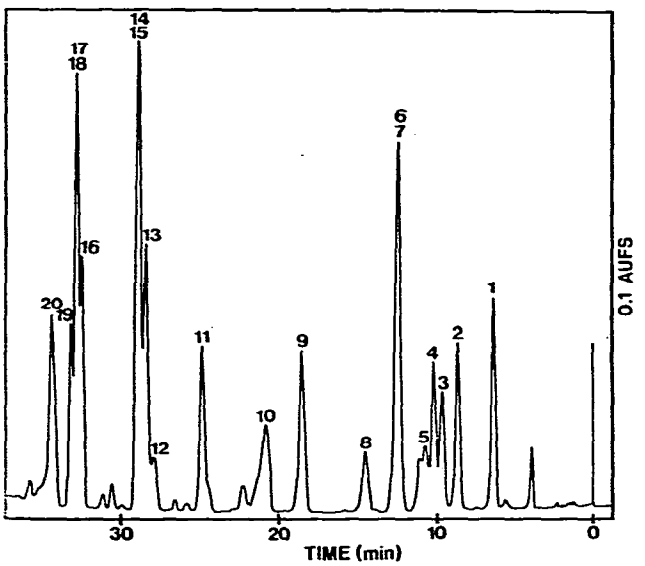


Fig. 1. HPLC separation of 20 PTH-amino acids on a 30 × 0.4 cm μBondapak C_{18} column. Conditions are described in the text. Peaks correspond to phenylthiohydantoins of the following amino acids: 1 = aspartic acid; 2 = asparagine; 3 = serine; 4 = threonine; 5 = cysteine; 6 = glycine; 7 = glutamine; 8 = glutamic acid; 9 = alanine; 10 = histidine; 11 = tyrosine; 12 = arginine; 13 = valine; 14 = proline; 15 = methionine; 16 = isoleucine; 17 = phenylalanine; 18 = tryptophan; 19 = leucine; 20 = lysine.

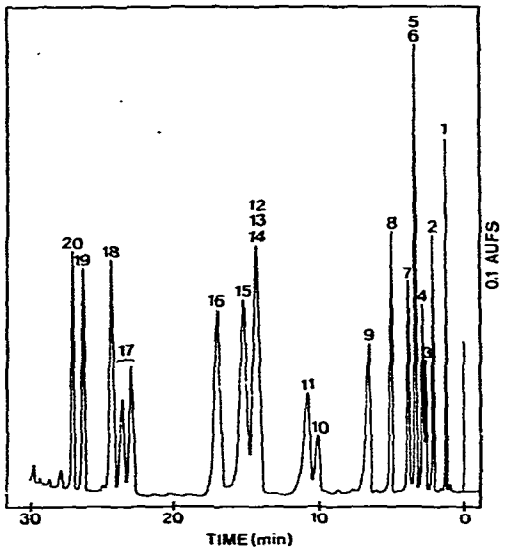
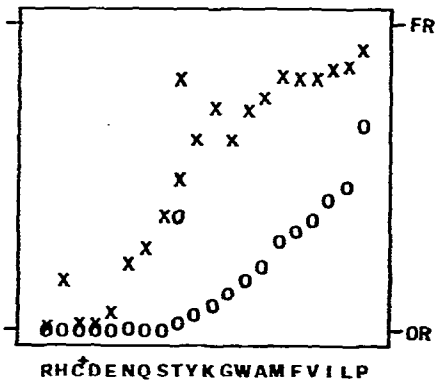


Fig. 2. HPLC separation of 20 MTH-amino acids on a 30 × 0.4 cm μBondapak C_{18} column. Conditions are described in the text. Peaks correspond to methylthiohydantoins of the following amino acids: 1 = cysteic acid; 2 = aspartic acid; 3 = asparagine; 4 = carboxymethyl cysteine; 5 = glycine; 6 = glutamine; 7 = glutamic acid; 8 = alanine; 9 = histidine; 10 = threonine; 11 = arginine; 12 = proline; 13 = valine; 14 = lysine; 15 = tyrosine; 16 = methionine; 17 = isoleucine; 18 = leucine; 19 = phenylalanine; 20 = tryptophan.

TLC of the PTH-amino acids (Fig. 3) totally resolves all but 2 of 20 common amino acid derivatives, by employing sequential development in the same direction, using two solvent systems. Residues which run at, or near, the baseline in solvent I



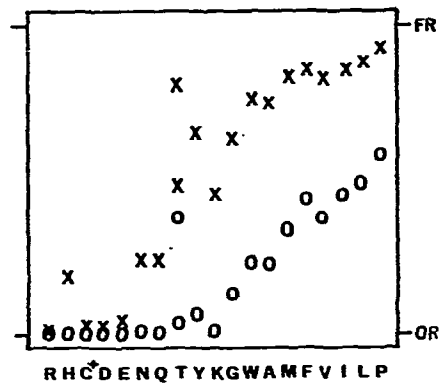


Fig. 3. TLC separation of 20 PTH-amino acids on 20 × 20 cm silica gel plates. Conditions are described in the text. Standard single letter abbreviations[24] are used to specify the amino acids, except for C^+ = cysteic acid. Other abbreviations used are: O = mobility after solvent I; X = mobility after solvent II; OR = origin; FR = solvent front. The faster migrating of the two spots in each solvent for threonine corresponds to PTH-dehydrothreonine.

Fig. 4. TLC separation of 19 MTH-amino acids on 20 × 20 cm silica gel plates. Conditions are described in the text. Abbreviations as described in Fig. 3. The faster migrating of the two spots in each solvent for threonine corresponds to MTH-dehydrothreonine.

are adequately separated in solvent II. Only PTH-aspartic acid and PTH-cysteic acid co-chromatograph.

Similar sequential development in one direction using the same two solvent systems resolves most of 19 common MTH-amino acid derivatives (Fig. 4). MTH-aspartic acid and MTH-cysteic acid co-chromatograph in both solvent systems, however, while the pairs MTH-asparagine and MTH-glutamine, and MTH-tryptophan and MTH-alanine show nearly identical migration in both solvents. The comparative R_F values for the PTH- and MTH-amino acids in solvent I and solvent II are tabulated in Table I.

TABLE I

R_F VALUES OF THE PHENYLTHIOHYDANTOIN (PTH) AND METHYLTHIOHYDANTOIN (MTH) AMINO ACIDS

Conditions described in text; standard three-letter abbreviations[24] used; Cys = cysteic acid, Δ-Thr = dehydrothreonine.

Amino Acid	*R_F after solvent I*		*R_F after solvent II*	
	PTH	*MTH*	*PTH*	*MTH*
Arg	0	0	0.01	0.01
His	0	0	0.17	0.19
Cys	0	0	0.02	0.02
Asp	0	0	0.02	0.02
Glu	0	0	0.06	0.04
Asn	0.01	0.01	0.22	0.24
Gln	0	0.01	0.27	0.24
Ser	0	*	0.38	*
Thr	0.03	0.04	0.49	0.48
Δ-Thr	0.38	0.38	0.81	0.81
Tyr	0.06	0.07	0.62	0.65
Gly	0.12	0.13	0.62	0.64
Lys	0.08	0.01	0.73	0.45
Trp	0.17	0.23	0.71	0.76
Ala	0.21	0.22	0.76	0.75
Met	0.30	0.34	0.83	0.83
Phe	0.32	0.44	0.82	0.85
Val	0.36	0.37	0.82	0.82
Ile	0.43	0.45	0.85	0.85
Leu	0.47	0.49	0.85	0.87
Pro	0.62	0.58	0.92	0.92

* MTH-serine is unstable.

It has been suggested[14] that the MTH-amino acids run in almost identical positions to the PTH-amino acids in a similar TLC system. While this may be true for some MTH-amino acids, there are several notable exceptions which can be seen clearly when chromatographed, as shown in Fig. 5.

DISCUSSION

The use of the Edman degradation for the sequence determination of peptides and proteins requires methods of identification for the PTH-derivatives generated at

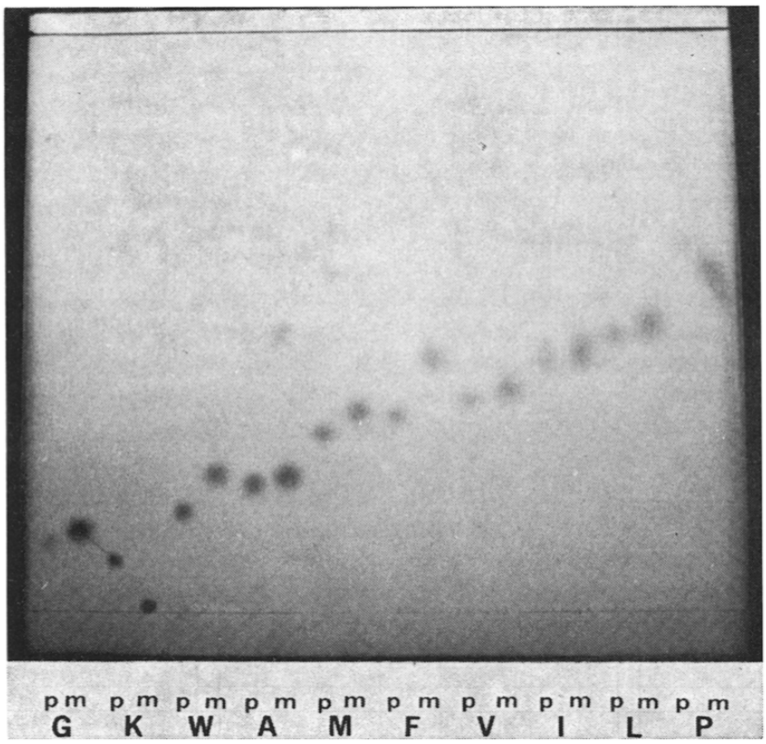


Fig. 5. Photograph of silica gel thin-layer chromatogram after development in solvent I. Conditions are described in the text. Chromatogram shows comparative mobilities of some PTH- and MTH-amino acids. Standard single letter abbreviations[24] are used to specify the amino acids. Other abbreviations used are: p = PTH-amino acid; m = MTH-amino acid. Photograph was taken under short-wavelength UV illumination.

each step of the degradation. Traditionally, TLC and GC were the methods employed for identification of the PTH-amino acids. More recently, HPLC has been used as an alternative method of analysis.

Unlike GC, TLC and HPLC are non-destructive. Thus, TLC and HPLC lend themselves more readily to use in high-sensitivity sequencing of radiolabelled samples, yielding fractions whose radioactivity may be easily quantitated.

The generation of MTH-amino acid derivatives during the first step of some solid-phase Edman degradation procedures requires identification methods similar to those used for PTH-amino acids. Preferably, systems should be employed which can identify both PTH- and MTH-amino acids. In this paper we report on modifications of published procedures for the separations of PTH-amino acids by HPLC and TLC. Using the same solvent systems, we are also able to characterize the corresponding MTH-amino acid derivatives.

Employing the same solvents and gradient as Downing and Mann[23], but using a single 30 cm μBondapak C_{18} column, instead of the 2 × 30 cm columns used by those authors, we obtained improved resolution of the PTH-amino acids (Fig. 1). The three pairs of PTH-amino acids which coelute in this system are easily distinguished by TLC (Fig. 3). The Model 660 solvent programmer on the Waters high-

performance liquid chromatograph, allows easy and rapid changes in elution parameters such as gradient profile, program time, and initial and final solvent conditions. Using the same solvents, as well as the same initial and final solvent conditions, but changing the gradient profile and shortening the program time, we are able to resolve the MTH-derivatives of the same amino acids by HPLC (Fig. 2). The one pair and one triplet of MTH-amino acids not resolved totally by HPLC are definitively resolved by TLC (Fig. 4). The order of elution of the PTH- and MTH-amino acids in the HPLC system employed is similar but not identical.

The TLC system presented allows a rapid and inexpensive method of identifying all but one pair of the PTH-amino acids (Fig. 3), as well as most of the MTH derivatives (Fig. 4). For investigators relying mainly on TLC, the members of the three coeluting pairs of MTH-amino acids may easily be distinguished. MTH-glutamine and MTH-asparagine each give secondary spots corresponding to MTH-glutamic acid and MTH-aspartic acid, respectively, arising from deamidation during the Edman procedure. MTH-aspartic acid and MTH-cysteic acid may be differentiated by treatment of the MTH-derivative with a solution of methanol–hydrochloric acid[25]. The MTH-cysteic acid derivative remains unchanged, while the MTH-aspartic acid is esterified, giving the MTH-aspartic acid methyl ester, which migrates near, but is resolved from, MTH-alanine. (PTH-cysteic acid and PTH-aspartic acid may be differentiated in a similar manner). MTH-alanine and MTH-tryptophan may be resolved by chemical staining. The thin-layer plate is heated to 100° for 5 min and, after spraying with a solution of ninhydrin (0.3%, w/v), collidine (3%, v/v), and acetic acid (10%, v/v) in absolute ethanol, the plate is reheated to 100° for an additional 5 min. MTH-alanine gives a red-orange spot, while MTH-tryptophan gives a bright yellow spot.

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