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UNIDIMENSIONAL, SEQUENTIAL SEPARATION OF PTH-AMINO ACIDS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

SHEILA A. SCHUETTE and COLIN F. POOLE*
Department of Chemistry, Wayne State University, Detroit, MI 48202 (U.S.A.)

SUMMARY

Eighteen of the twenty common protein PTH-amino acids derivatives are separated by continuous multiple development high-performance thin-layer chromatography. The separation is performed on silica gel plates using five development steps with four changes in mobile phase. The derivatives are identified by scanning densitometry, and the total analysis requires less than 1 h. The unseparated derivatives of alanine and tryptophan are baseline resolved in an alternative solvent system. Thus, all twenty of the common PTH-amino acid derivatives may be identified with the proposed method. By using the unidimensional method of development, the high sample capacity of the high-performance thin-layer chromatographic plate is preserved; samples and standards can be run simultaneously to improve the accuracy of identification. The detection limit for the PTH-amino acid derivatives determined by *in situ* reflectance scanning densitometry at 270 nm was found to be *ca.* 0.5 ng per spot.

INTRODUCTION

A major advance in analytical biochemistry was the development of automated sequential analyzers for the determination of the amino acid sequence of peptides and proteins. Although various reaction schemes may be used, one of the most popular methods is the Edman degradation reaction. In this process, the N-terminal amino acid, as its phenylthiohydantoin (PTH) derivative, is cleaved from the peptide chain at the conclusion of each cycle of the sequenator. The determination of the structure of a mammalian peptide or protein requires a support analytical method to identify which amino acid is produced at the end of each cycle of the sequenator. For this purpose gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) or mass spectrometry are used. However, no single analytical technique has established itself as the method of choice for the identification of the PTH-amino acids. The literature describing new separation schemes or modifications to existing procedures has remained buoyant over the last decade and has been reviewed comprehensively[1–4].

Recent advances in the practice of TLC have led to its wider acceptance as a powerful analytical tool for the quantitative analysis of complex mixtures. This

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changed role and accompanying expectations have spawned a new expression "high-performance thin-layer chromatography" (HPTLC). The performance breakthrough in TLC was not a result of any specific advance in instrumentation or materials, but was rather a culmination of improvements in practically all of the operations of which TLC is comprised. For the separation of the PTH-amino acids, it was expected that HPTLC would provide a significant increase in separation efficiency and detection sensitivity approaching one order of magnitude, shorter analysis times, and an increase in the number of samples and standards which can be analyzed per plate compared to conventional TLC[5,6].

That HPTLC could be used with advantage for the separation of the PTH-amino acids was recognized by Bucher[7] and Yang[8]. However, neither author was able to demonstrate an adequate separation of all 20 common protein PTH-amino acids and some sample overlaps remained in all solvent systems investigated. The position of the PTH-amino acids in the chromatogram was made by eye or photographic means which is not the most accurate or appropriate method for this purpose. In this paper we have used the method of continuous multiple development to separate the PTH-amino acids a few components at a time and precision scanning densitometry for the accurate measurement of spot location at each step in the development sequence. In this way, a complete separation of the mixture at any one point in time becomes unnecessary and more selective solvents can be used to maximize the resolution of those components separated in each development sequence. Changes in the developing solvent can be made at the conclusion of any development sequence to create a step-wise mobile phase gradient. Also, each time the solvent front transverses the plate a natural spot reconcentration phenomenon occurs whereby the bottom half of the spot is pushed into the top and the axis of the spot becomes compressed in the direction of migration[9]. This spot reconcentration mechanism works in opposition to the normal spot broadening process occurring during migration and results in an improvement in sample resolution.

EXPERIMENTAL

The PTH derivatives of the common protein amino acids were obtained in kit form from Sigma (St. Louis, MO, U.S.A.). Standard solutions were prepared by dissolving 4.5–5.0 mg of the PTH-amino acid derivatives in 2.0 ml of ethyl acetate or methanol. Standard mixtures at the same concentration as the above were prepared for all twenty PTH-amino acid derivatives, for fifteen non-polar and moderately polar derivatives (ALA, ASP, GLU, GLY, ISL, LEU, LYS, MET, PHE, PRO, SER, THR, TRP, TYR and VAL), for seven moderately polar and polar derivatives (ARG, ASN, ASP, CM-CYS, GLN, GLU, and HIS) and for the pair ALA/TRP. The standard mixtures were used for identification purposes and in the optimization studies. When not in use the standard solutions are stored under refrigeration.

Chromatography was performed on 10 cm × 10 cm HPTLC plates coated with silica gel 60 (E. Merck, Darmstadt, G.F.R.). The plates were precleaned by a single development with acetonitrile. All solvents were distilled in glass, chromatography grade from Burdick & Jackson (Muskegon, MI, U.S.A.).

Standard solutions were applied to the plate using a 200-nl Pt–Ir micropipette (Antech, Bad Durkheim, G.F.R.) attached to an EVA-Chrom applicator (W & W

Electronic Instrument, Basel, Switzerland). The spots were applied in a line 0.5 cm apart and 0.5 cm from the lower edge. The plates were developed in a short-bed continuous development chamber (Regis, Morton Grove, IL, U.S.A.). Position 2, plate length 3.5 cm or position 4, plate length 7.5 cm were used as indicated in the text.

In situ scanning of the HPTLC plates was performed with a Shimadzu CS-910 scanning densitometer (Shimadzu, Columbia, MA, U.S.A.). All measurements were made in the reflectance mode, single beam operation, at a wavelength of 270 nm. The scanning speed and recorder speed were 48 mm min^{-1}. The slit width was 4.6 mm and slit height 0.46 mm.

RESULTS AND DISCUSSION

The method used is summarized in Table I. It provides a separation of eighteen of the twenty PTH-amino acid derivatives in well under 1 h. ALA and TRP are not separated in this scheme, but a second solvent system is available for their resolution.

The first development is made with methylene chloride for 5 min at position 2 in the short-bed continuous development chamber. The function of this development step is to provide an initial ordering of the derivatives in the region of the origin, thereby improving the resolution of the mixture in subsequent development steps (Fig. 1). The least polar of the amino acid derivatives, PTH-proline, can be identified at this stage. In fact, its migration properties are so different from the other PTH-amino acid derivatives that it remains baseline resolved in each of the first four development steps.

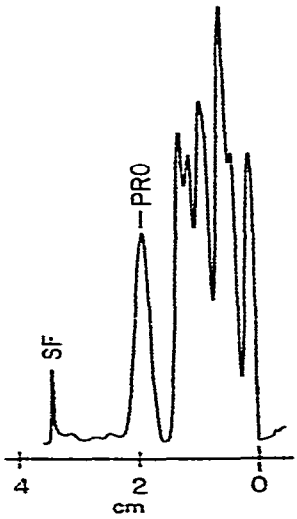
After the methylene chloride has been evaporated from the plate, it is re-developed for 10 min in methylene chloride–isopropanol (99:1) in position 4 of the short-bed chamber. Position 4 corresponds to a plate length of 7.5 cm. At this development stage any of the residues PRO, LEU, ILE, VAL and PHE can be identified (Fig. 2).

The third development sequence is a repeat of step 2. It provides a better separation of the peaks resolved in step 2 as well as enabling MET, ALA/TRP, GLY,

TABLE I

OPTIMUM EXPERIMENTAL CONDITIONS FOR THE SEPARATION OF THE PTH-AMINO ACIDS BY CONTINUOUS MULTIPLE DEVELOPMENT HPTLC

Development step	*Mobile phase composition*	*Plate length (cm)*	*Time (min)*	*PTH-amino acid derivative identified*
1	CH_2Cl_2	3.5	5	PRO
2	CH_2Cl_2–$(CH_3)_2CHOH$ (99:1)	7.5	10	PRO, LEU, ILE, VAL, PHE
3	CH_2Cl_2–$(CH_3)_2CHOH$ (99:1)	7.5	10	PRO, LEU, ILE, VAL, PHE MET, ALA/TRP, GLY, LYS, TYR, THR
4	CH_2Cl_2–$(CH_3)_2CHOH$ (97:3)	7.5	10	PRO, MET, LYS, TYR, THR, SER, GLU
5	$C_2H_5OOCCH_3$–CH_3CN–CH_3COOH (74.3:25:0.7)	7.5	10	ASN, GLU/GLN, ASP, CM-CYS, HIS, ARG



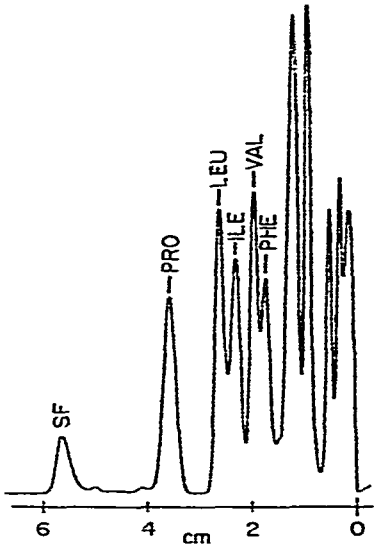


Fig. 1. First continuous multiple development. Methylene chloride, position 2, 5 min.

Fig. 2. Second continuous multiple development. Methylene chloride–isopropanol (99:1), position 4, 10 min.

LYS, TYR, THR residues to be identified (Fig. 3). TRP often appears as a shoulder on the side of the ALA peak, but it is not resolved adequately for identification purposes. ALA and TRP are separated almost to baseline by a 10-min development in hexane–tetrahydrofuran (9:1) in position 2 of the short-bed continuous development chamber (Fig. 4).

The mobile phase is changed to methylene chloride–isopropanol (97:3) for the fourth development step of 10 min in position 4. The separation between LYS, TYR and THR is improved and SER and GLU can be identified in addition (Fig. 5). The small satellite peaks accompanying the labelled peaks in this chromatogram are impurities in the original standards and not artifacts of the development process. Fig. 5 also illustrates the advantage of being able to make measurements with the densitometer at any stage in the sequence. The separation of LYS, TYR, THR, SER and GLU is to baseline at this stage and their identification is easily achieved. However, LEU/ILE, VAL/PHE and TRP/ALA/GLY have started to merge together and could not be determined individually, although well separated in earlier steps.

The very polar PTH-amino acid derivatives remain essentially unresolved in the region of the origin. A much more polar mobile phase is selected for their resolution in the fifth and final development step. A 10-min development in position 4 with ethyl acetate–acetonitrile–glacial acetic acid (74.3:25:0.7) separates all the remaining PTH-amino acid derivatives except GLU/GLN (Fig. 6). However, GLU is separated from GLN in the fourth development step so that the two are easily differentiated from each other.

The improved separating power and shorter analysis times provided by HPTLC technology in combination with the continuous multiple development mode

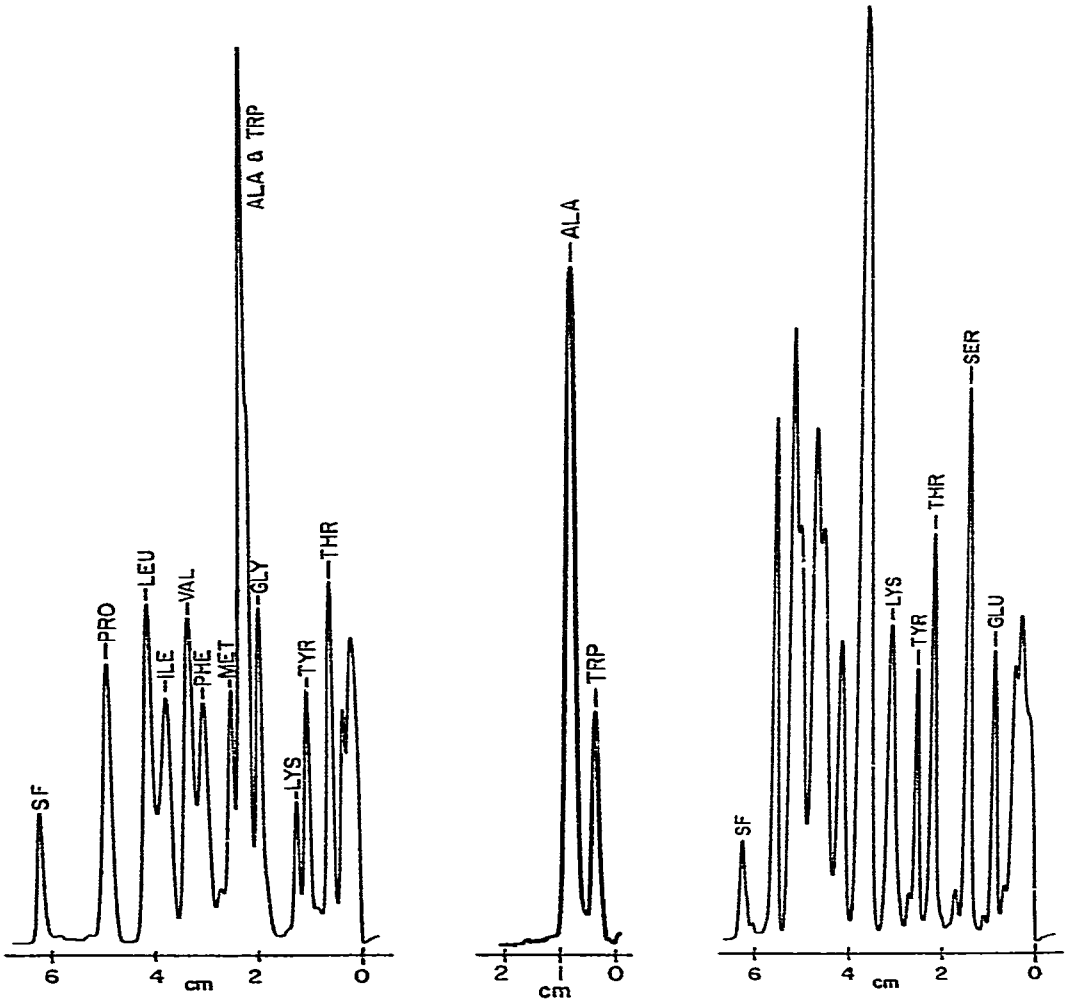


Fig. 3. Third continuous multiple development. Methylene chloride–isopropanol (99:1), position 4, 10 min.

Fig. 4. Separation of ALA and TRP by continuous development in hexane–tetrahydrofuran (9:1), position 2, 10 min.

Fig. 5. Fourth continuous multiple development. Methylene chloride–isopropanol (97:3), position 4, 10 min.

of separation have met our original goal of a fast and efficient method for the identification of the PTH-amino acid derivatives. An important advantage of this HPTLC method is that eighteen samples and standards can be separated simultaneously. Compared to closed-bed techniques such as HPLC, internal calibration can be used for improved identification. Also, on a per sample basis, the analysis time is reduced to the time required for one separation divided by the number of samples on the plate. Some additional time is required to scan each track but this is short compared to the time for development. Consequently, for multiple samples HPTLC is much faster

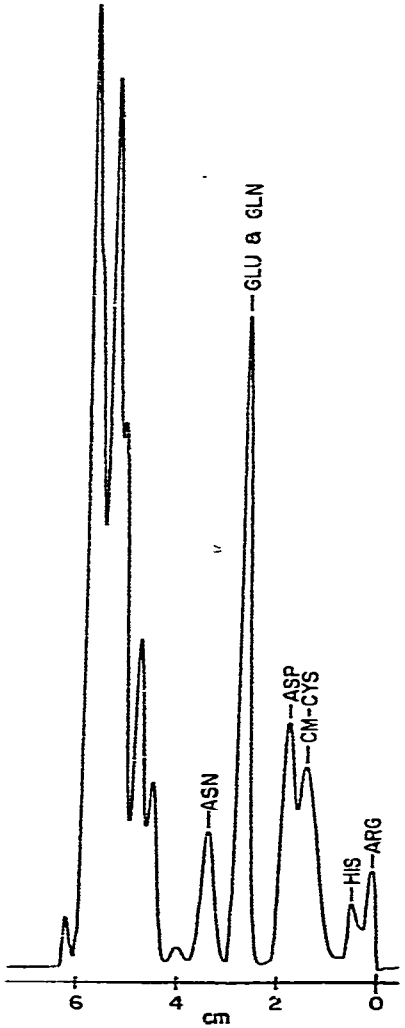


Fig. 6. Fifth continuous multiple development. Ethyl acetate–acetonitrile–glacial acetic acid (74.3:25:0.7), position 4, 10 min.

than HPLC, which relies on elution chromatography and can separate only one sample at a time.

CONCLUSIONS

The method described for the identification of the PTH-amino acids represents a considerable improvement over the results found using conventional TLC practices. Sample resolution is better and the analysis time reduced. Two-dimensional development is not necessary to enhance resolution so that the method preserves all the advantages expected of unidimensional development; namely, high sample capacity (eighteen samples on a 10 × 10 cm HPTLC plate) and the ability to separate samples and standards simultaneously to improve the certainty of derivative identification.

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