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Note

Methanol solvent system for rapid analysis of phenylthiohydantoin amino acids by high-pressure liquid chromatography

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Elucidation of the primary structure of proteins involves two fundamental steps: the sequential degradation of proteins by which the N-terminal amino acids are converted into phenylthiohydantoin (PTH) derivatives and the identification of the derivatized amino acids after each cycle of degradation. In order to maximize the efficiency of the operation, both procedures should parallel each other in terms of sensitivity, reliability, and reproducibility. The introduction of the automatic sequencer by Edman and Begg[1] greatly enhanced the speed and predictability of the former task. Developments in the second category have not been as rewarding. Regrettably, no single method for identification and quantitation of the PTH amino acids is available. This mandates the utilization of several different procedures before unequivocal identification of all the PTH amino acids can be made.

The most commonly used techniques for the identification of PTH amino acids are gas–liquid chromatography (GLC)[2], thin-layer chromatography (TLC)[3] and acid hydrolysis followed by identification of parent amino acid[4]. A more promising addition to the methodology of PTH amino acid identification and quantitation is high-pressure liquid chromatography (HPLC). Efforts are being directed by protein sequencing laboratories towards the development of HPLC primarily due to the high resolution obtained on small, rigid, spherical particles of uniform size, and the speed of the system. HPLC is more sensitive than GLC and is applicable to the analysis of the PTH amino acids without additional modification of the derivatives such as silylation of the polar PTH amino acids to increase their volatility and level of detectability. These advances have significantly improved the potential for sensitive detection and identification capabilities of the PTH amino acids[5–8]. Zimmerman *et al.*[9] have recently reported the separation of all the PTH amino acids within 20 min at 62° utilizing a sodium acetate–acetonitrile gradient. Hunkapiller and Hood[10] and Bridgen[11] have developed an acetic acid methanol program. In this communication we present a method for the separation and quantitation of the PTH amino acids in 20 min at room temperature.

MATERIALS AND METHODS

Methanol was obtained from Burdick & Jackson Labs. (Muskegon, Mich.,

U.S.A.). Ultrapure deionized water[15] was filtered through a 0.45-μm filter (Millipore, Bedford, Mass., U.S.A.) before use. Sequential grade glacial acetic acid and standard PTH amino acids were purchased from Pierce (Rockford, Ill., U.S.A.). Stock solution of each PTH amino acids (2–4 nmole/μl) were prepared in methanol or ethyl acetate. A mixture of standard PTH amino acids (600–900 pmole/μl) was prepared in methanol, stored at 20° and was changed every other day to avoid the effects of degradation.

Analyses were performed with a Waters high-pressure liquid chromatograph (Model ALC/GPC-204) equipped with a Waters Model 440 absorbance detector, Model 660 solvent programmer and a μBondapak C_{18} column (30 cm × 4 mm I.D.; part No. 27324). Solvent A and B were constituted as follows: solvent A: 900 ml water, 100 ml methanol, 2.5 ml glacial acetic acid, and 50 μl acetone. The pH was adjusted to 4.1 with NaOH and the solvent was filtered through a 0.45-μm filter (Millipore) before use. Solvent B: 100 ml water, 900 ml methanol, 0.25 ml glacial acetic acid; it was filtered as above.

The column was developed with a 15-min linear gradient of 95% A+5% to 55% A+45% B at a flow-rate of 2.5 ml/min.

Sperm whale apomyoglobin (Beckman part No. 339182), was sequenced in the Beckman 890C sequencer using either the 0.5 *M* quadrol[12] or a 0.1 *M* THEED[N,N,N′,N′-tetrakis(2-hydroxyethyl)ethylenediamine](ICN, Plainview, N.Y., U.S.A.)[13,14] program.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the separation for the PTH amino acids within 20 min. It is especially important to note that most of the PTH amino acids have baseline separation, which makes the whole system practical when quantitation and fraction collection is desired. Selected steps from an automated sequencer run of 20 nmole of sperm whale apomyoglobin are shown in Fig. 2. The low background made it possible to pick up pmole quantities, making HPLC a most sensitive and practical method for PTH amino acid identification and quantitation. Matthews *et al.*[8] have reported partial separation of all PTH amino acids in a single run of 40 min by HPLC. In their system the PTH amino acid pairs of glycine and threonine, and lysine and tyrosine co-eluted and the remaining PTH amino acids did not have baseline resolution. Rodkey and Bennett[7] reported a 25-min program but without achieving efficient separation. Other programs have been published[5–7] but none seem to give a complete separation. Zimmerman *et al.*[9] have recently achieved complete resolution of all the 20 PTH amino acids using an acetonitrile and sodium acetate gradient at elevated temperature (62°). The methanol program reported in the present communication offers an alternative method which has good reproducibility, long column life and can be carried out at room temperature. The separation (Fig. 1) is achieved within 20 min. PTH-Val and PTH-Met co-elute, whereas PTH derivatives of Leu, Phe and Ile poorly separate in the mixture. Nevertheless, (PTHs Leu, Ile, Phe) when injected individually the difference between their elution times is 10 sec; thus making their identification feasible. The elution profile of these and the acid-soluble PTH amino acids (PTH-His and PTH-Arg) is shown in Fig. 1 with broken lines. Each residue elution time is explicitly defined. Because of the extreme reproducibility and adequate elution time difference, we have encountered no problem in the identification

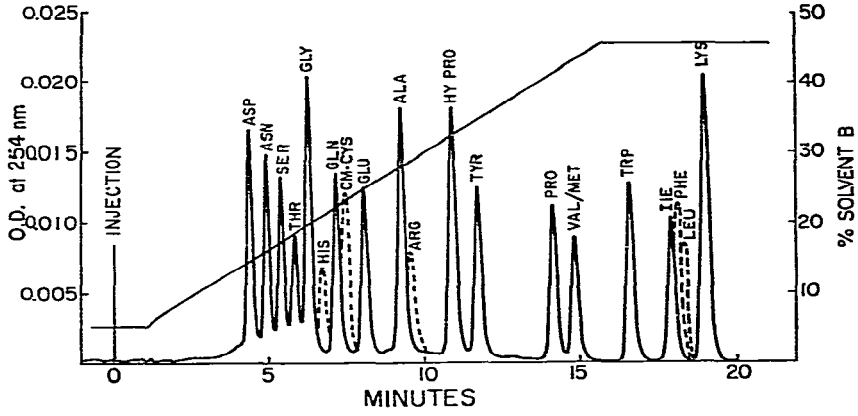

Fig. 1. Separation of PTH amino acids on a μBondapak C_{18} column (30 cm × 4 mm I.D.) with a linear gradient from 95% A + 5% B to 55% A + 45% B. A flow-rate of 2.5 ml/min was employed. Sample size was 600–900 pmoles of each PTH amino acid. Acid-soluble PTH amino acids and those which co-elute when in mixture are shown with broken lines (for details see text).

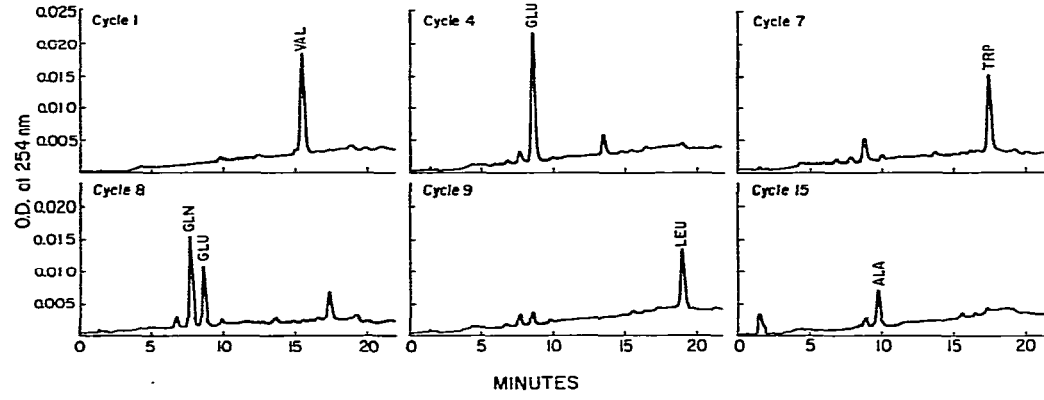

Fig. 2. Selected steps from the automated Edman degradation of sperm whale apomyoglobin (20 nmoles). A 0.1 *M* THEED program was used and 10% of the sample was injected for identification.

of PTH-Phe, PTH-Ile and PTH-Leu. Furthermore, the present column has been in continous use for a period of 5–6 months without observing any adverse effects on the separation efficiency or elution times. We have observed however that an overnight wash with solvent B alone at 0.2 ml/min helps increase the column life and separation reproducibility. Since baseline separation could be achieved for most of the PTH amino acids, it is feasible to cut the peaks utilizing an optical density-level sensor as they elute from the column when ^{35}S-PITC (phenylisothiocyanate) is used. Work in this direction is in progress.

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