THE USE OF METHANOL IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENYLTHIOHYDANTOIN–AMINO ACIDS

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1. Introduction

The extensive and continuous use of the automated Edman degradation to determine the amino acid sequence of proteins had led to the development of three main methods of identification of phenylthiohydantoin (PTH) amino acids obtained at each cycle. Thin-layer chromatography (TLC) [1], the oldest method, has the main drawback of being only qualitative. Gas–liquid chromatography (GLC) [2] is quantitative but not for all amino acid derivatives. Certain residues need additional derivatization and others cannot be identified at all. Amino acid analysis after acid hydrolysis [3] requires time and the use of an analyser. It does not permit the distinction between Ala/Ser/Cys, Asp/Asn or Glu/Gln nor the identification of tryptophan which is destroyed by hydrolysis.

Recently high-performance liquid chromatography (HPLC) was introduced to separate the PTHs and appears to be quickly replacing the previously used methods. In this report we would like to present a number of improvements over the methods [4–10]. These improvements are:

(i) Speed of analysis – 16 min for separation of 17 PTHs.
(ii) Low cost of operation and safety – the use of methanol instead of the ten-times more expensive and toxic acetonitrile.
(iii) Simplicity – a single program for the organic phase soluble derivatives.

2. Materials and methods

A Waters high-performance liquid chromatograph, model ALC/GPC-204 equipped with two 6000 A pumps, model 660 solvent programmer for gradient elution, model U6K injector, model 440 absorbance detector and a Microbondapak C_{18} column (3.9 mm × 30 cm). Three buffers were used:

(i) Buffer A, composed 23% methanol in 0.005 M sodium acetate, pH 5.3/20 μl acetone (to raise absorption of A to the level of B).
(ii) Solvent B, composed 44% methanol.
(iii) Solvent C, composed 22% methanol/5% acetic acid.

Water was deionized and glass distilled. Methanol (Pro Analyse) was from Merck, Darmstadt. All solvents were passed through a 0.47 μm Millipore filter before use. The gradient in program 1 was generated using curve 10 of the Model 660 programmer in 3 min followed by isocratic elution for 13 min. Program 2 used for the separation of PTH–Arg, –His and –Cys was an isocratic elution with solvent C. A constant flow rate 2.5 ml/min was maintained. The absorption was recorded at 254 nm. Temperature was set constant at 26°C. PTH–amino acid derivatives were dissolved in methanol before injection.

Automated sequence analysis was performed on 0.1–0.3 μmol protein in a Beckman 890C sequencer using 1 M quadrol and a single cleavage.

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3. Results and discussion

Methanol is used here for performing a separation of phenylthiohydantoin amino acids by high-performance liquid chromatography, in much the same conditions as those described using acetonitrile. Methanol, however, is a considerably less expensive and more innocuous solvent.

Two chromatograms of standard PTHs are presented in fig.1A,B. The top separation is made in 80 min; the lower analysis takes 16 min. Identification is obtained for all organic phase-soluble PTH residues except for the Val/Met pair, which are easily distinguished by GLC or TLC. Although PTH–Ile and PTH–Phe are not well resolved, they may be distinguished by their different retention times. PTH–Lys may be completely resolved from PTH–Leu by lowering the temperature or by decreasing the final methanol concentration from 44–42%. Tryptophan is completely separated from the other apolar PTHs when methanol is used, which is not the case with acetonitrile [4,5,9].

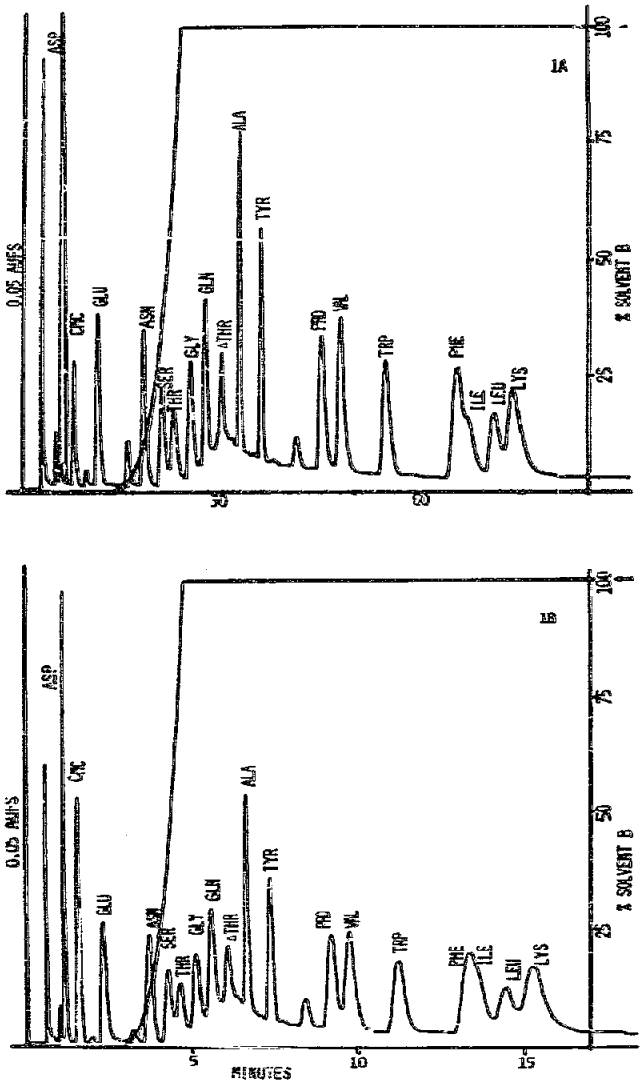


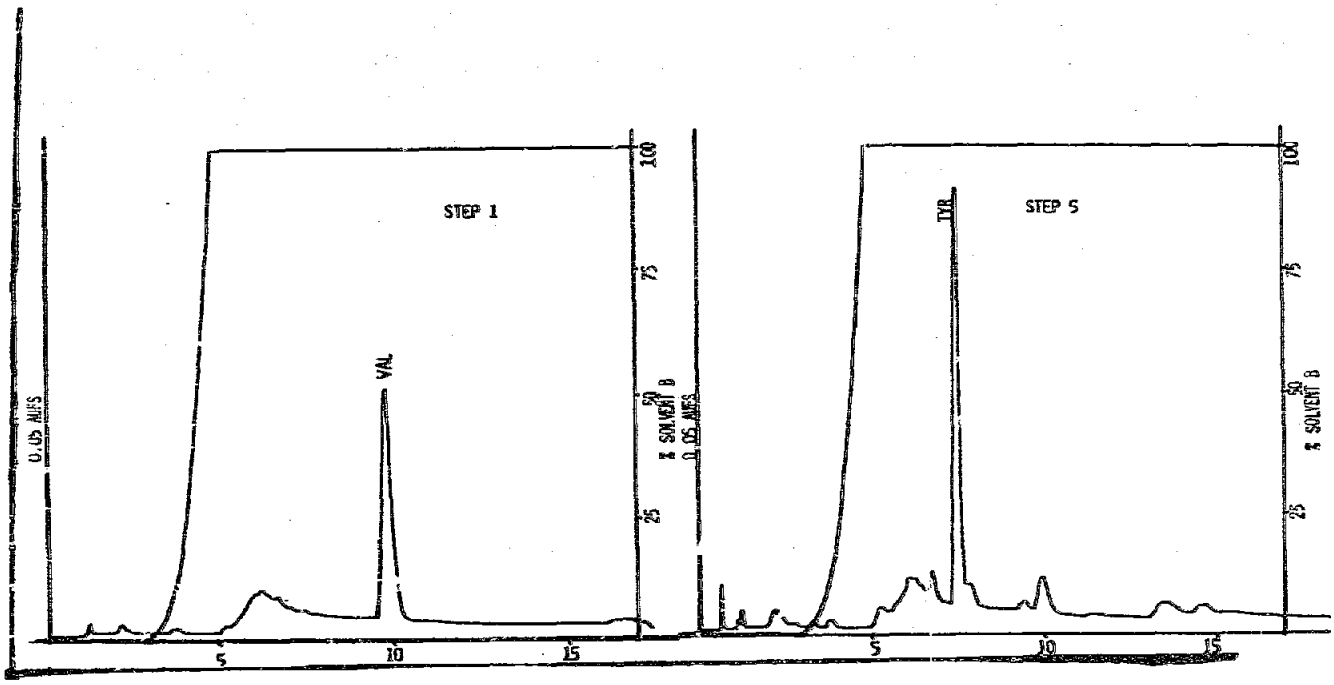
Fig.1B. Gradient elution of 0.5 nM PTH–amino acids from a Microbondapak column using program 1. The concave line running throughout the chromatogram illustrates the increasing concentration of solvent B. Flow rate = 2.5 ml/min. Fig.1A. The same conditions as in B except that the flow rate has been reduced from 2.5–0.5 ml/min.

The usefulness of the HPLC was demonstrated by the analysis of samples obtained from the automated degradation of *Lotus tetragonolobus* lectin. Only 1/25th of each sample was injected in the chromatograph, thus illustrating the sensitivity of the method. Steps 1, 5, 7, 8, 11, 12 are presented in fig.2.

The aqueous phases are examined using program 2, an isocratic elution with 22% methanol/5% acetic acid. The PTH derivatives of Cys, His and Arg are resolved within 3.5 min. The isocratic elution allows 17 injections/h. Most of the ultraviolet-absorbing contaminants from the automatic Edman degradation remain in the aqueous phase, are eluted from the column in the void volume and do not interfere with the identification of the PTHs. The detection of PTH–Arg at step 19 of the *Lotus* degradation is shown in fig.3 together with the preceding and following steps.

When comparing our results with the methanol-use report [7], the method has been improved both by separation quality and by speed: 16 min instead of 30 min. Time is an important factor when peace has to be kept with the automatic sequenator, for which degradation times are steadily reduced [10].

We are attempting to further improve the resolution of the separation by using a Zorbax ODS column with a particle size of 5 μm as suggested [8] instead of the 10 μm material used here.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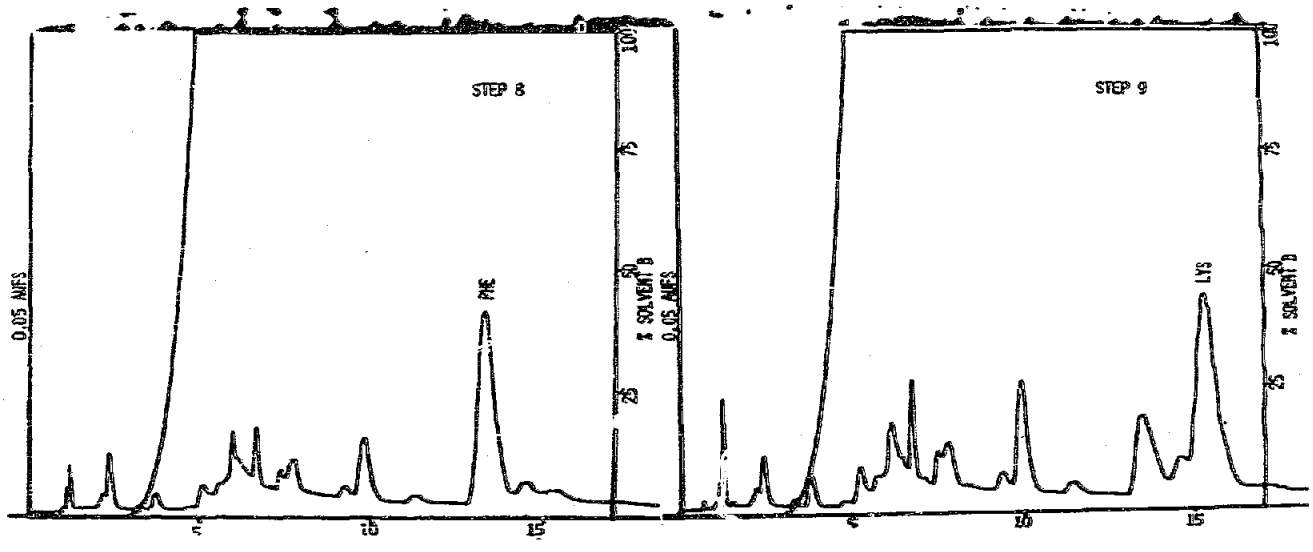
VAL
STEP 5
TYR
0.05 AUFS
% SOLVENT B
100
75
50
25
5
10
15


STEP 8
PHE
STEP 9
LYS
0.05 AUFS
% SOLVENT B
100
75
50
25
5
10
15

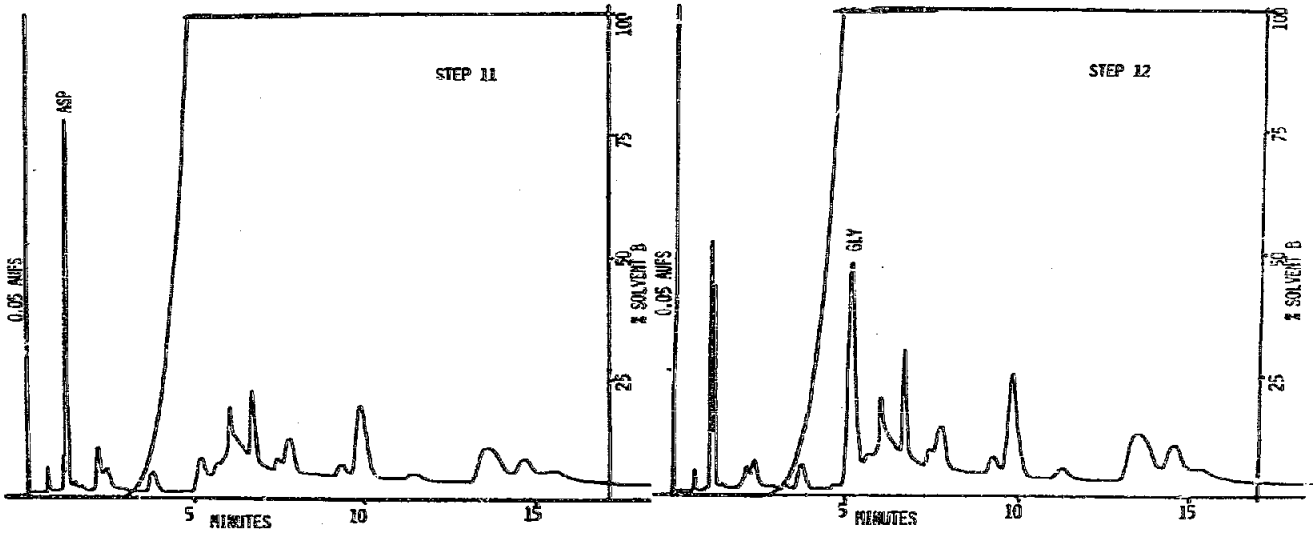


Fig.2. Selected steps from the automated Edman degradation of *Lotus tetragonolobus* lectin. After conversion, extraction, and drying with nitrogen the samples were dissolved in methanol and 4% was injected (and analyzed using program 1).

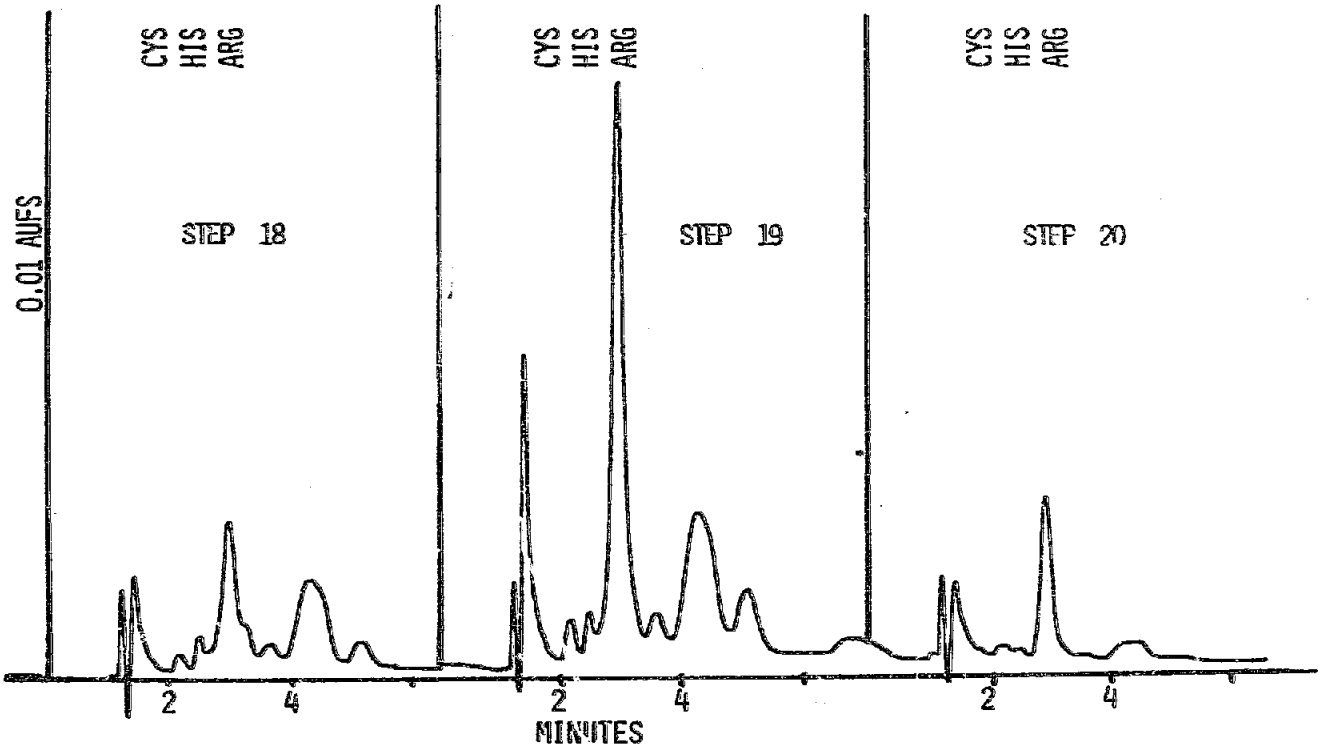


Fig.3. Aqueous phases from steps 18–20 from automated Edman degradation of *Lotus tetragonolobus* lectin, analyzed using program 2.

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