Isocratic Separation of PTH-Amino Acids at Picomole Level by Reverse-Phase HPLC in the Presence of Sodium Dodecylsulfate

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The phenylthiohydantoin (PTH) derivatives of protein amino acids have been separated by reverse-phase high performance liquid chromatography (HPLC) on a fully end-capped C18 column using an isocratic solvent system. The developing solvent was 0.01 M sodium acetate buffer (pH 4.5) containing 39.5% acetonitrile and 0.02% sodium dodecylsulfate (SDS). With an automated liquid chromatography equipped with a dual-channel detector, operating at 254 and 313 nm, the present isocratic separation system was quite useful for routine microanalysis of PTH-amino acids released with a “gas-phase” sequencer. The time for one run was ~23 min and the limit of analysis ~2.5 pmol of a PTH-amino acid.

It has been well recognized that high performance liquid chromatography on reverse-phase columns is a rapid and reliable method for the identification of PTH-amino acids obtained on Edman degradation of proteins and peptides. A variety of chromatographic conditions with gradient and isocratic elutions has been reported (1), but none are satisfactory for microanalysis with respect to sensitivity, resolution and reproducibility. To facilitate microsequencing with a gas-liquid-solid phase (“gas-phase”) sequenator (2), a new isocratic elution procedure for PTH-amino acids was established with a C18 reverse-phase column at picomole level. The procedure is characterized by the use of sodium dodecylsulfate as a component of the developing solvent. This paper describes the separation of nineteen standard PTH-amino acids on the C18 column and the results of a typical protein sequencing experiment in which the present chromatographic method was successfully used with the “gas-phase” sequencer.

All analyses of PTH-amino acids were performed with a Waters liquid chromatograph (QA-1 Analyzer) equipped with an automatic sampler (WISP, Model 710B, Waters Associates), a dual-channel detector operating at 254 and 313 nm, and a Hewlett-Packard 3390A integrator. Acetonitrile was HPLC-grade (Wako Pure Chemicals) and the other chemicals were reagent-grade. The stock solution of standard PTH-amino acids was prepared by dissolving each amino acid derivative
(Seikagaku Kogyo) in methanol to the concentration of 10 nmol per ml and it was stored in the dark at -80°C. For standard runs for daily analysis, the stock solution was diluted with the chromatographic developing solvent to the concentration of 250 pmol per 100 µl of each PTH-amino acid. The C18 reverse-phase column was reactivated by flushing with methanol for 30 min at the flow rate of 1.0 ml per min at 37°C, when necessary.

Typical chromatograms obtained for synthetic mixtures composed of 10 and 2.5 pmol of each PTH-amino acid are shown in Fig. 1. The elution was carried out with an isocratic solvent composed of 39.5% acetonitrile, 0.02% SDS and 0.01 M sodium acetate buffer, pH 4.5. The elu-

![Chromatogram](image-url)

Fig. 1. Isocratic separation of 19 PTH-amino acids on a C18 reverse-phase column. Chromatography was performed with a Toyo Soda ODS-120T column (4.6 × 250 mm) at a flow rate of 1 ml per min and 37 ± 0.5°C. The details of the elution solvent are given in the text. After 8 min, the detection sensitivity was increased 2-fold by programming the attenuation setting from 4 to 2 mV (0.0005 AUFS) at 10 pmol-level analysis (A) and from 1 to 0.5 mV (0.000125 AUFS) at 2.5 pmol-level (B). At the same time, the chart speed was decreased from 1 to 0.5 cm per min. The chromatogram of the above 19 PTH-amino acids and the dehydrated derivatives of PTH-Ser and PTH-Thr separately recorded at 313 nm is also presented as a composite illustration at 10 pmol-level. All protein amino acids are denoted by one-letter symbols. Other abbreviations are: J(T)(S), JThr(Ser); X, impurity.

*J. Biochem.*
tion positions of DPTU and DMPTU, the major by-products detected on protein/peptide sequencing with the "gas-phase" sequencer (Model 470A, Applied Biosystems), and five other PTH-derivatives, PTH-Nle, PTH-Cya, PTH-Cmc, PTH-\(\alpha\)Ser, and PTH-\(\beta\)Thr, are also given. PTH-Asp and PTH-Cmc were eluted at the same position under the present conditions. However, on analysis of samples obtained in our experiment, these two amino acids could be reasonably distinguished from one another by carefully watching for an additional peak of PTH-\(\alpha\)Ser, derived on partial degradation of PTH-Cmc. PTH-Cmc could be also separated from PTH-Asp when the concentration of acetonitrile in the elution solvent was reduced to 20\% (data not shown).

Monitoring of the eluate at 313 nm was effective for definite identification of PTH-Ser and

Fig. 2. Effect of SDS on the retention times of PTH-amino acids. Each PTH-amino acid is denoted by a one-letter symbol.

Fig. 3. Chromatograms of PTH-amino acids obtained on direct sequencing of a protein from snake toxin. Seven chromatograms obtained at cycles 1, 11, 21, 22, 33, 34, and 35 are presented. The amounts used for analysis of PTH-amino acids were 100 (cycle 1), 200 (cycle 11), and 400 (cycle 21-35) pmol on the basis of the amount of protein (2 nmol) loaded onto the sequencer. The PTH-amino acids identified at the indicated and the previous cycles are denoted by one-letter symbols and those in parentheses, respectively.
PTH-Thr in addition to detection of their dehydrated derivatives. The identification was made on the observation that the absorbance recorded at 313 nm for the dehydrated derivative was greater than that at 254 nm for the remaining intact PTH-amino acid under the present conditions. The limit of detection at 313 nm was 2.5 pmol for PTH-ĞSer and PTH-ĞThr.

Except for the addition of SDS, the solvent system for isocratic elution is essentially the same as that reported by Zimmerman et al. (4). The effect of SDS in the developing solvent is distinct, as shown in Fig. 2. The two cationic PTH-amino acids, PTH-Arg and PTH-His, are so sensitive to the SDS concentration that their retention times increase in parallel with an increase in the concentration of this anionic detergent. With 0.02% SDS, the peaks of both PTH derivatives were shifted from the crowded region of peaks to a vacant region on the chromatogram. This shift is probably due to the electrostatic interaction between the positive charges of cationic PTH-derivatives and the negative charge of SDS adsorbed on the C18 hydrocarbon moiety bonded to the porous silica.

The retention times of all PTH-amino acids were stable (< ±0.1 min) and the developing solvent was recycled until the resolution of PTH-Phe, PTH-Lys (PTC), and PTH-Ile became incomplete. If this happened, the resolution was readily restored by adding a few drops of acetonitrile to the solvent to maintain the critical concentration of the organic solvent (39.5±0.5%). With this care, the developing solvent of a given lot could be continuously used for at least one month. Moreover, the C18 column was completely reactivated by flushing with methanol when PTH-amino acids were poorly resolved. However, good resolution was usually maintained for more than 1,000 samples owing to the cleaning effect of SDS for the reverse-phase column and tubing during chromatography.

Figure 3 shows seven chromatograms of PTH-amino acids obtained on sequencing with a 470A sequencer of a protein (Mr, 9,000), a minor component of crude β-bungarotoxin (3), from the venom of Bungarus multicinctus. In this experiment, the solvent of the acetonitrile solution of PTH-amino acids released on sequencing was completely evaporated carefully in a vacuum concentrator before injection onto the C18 column. This process of evaporation (40°C, 2 h) was critical for good chromatographic analysis, since dithiothreitol in acetonitrile which was used to transfer PTH-amino acids in the flask to the fraction-collector tubes in the sequencer is eluted as a large, broad peak near PTH-Glu. By analyzing the chromatographic data, the amino-terminal thirty-five residues of the protein were eventually determined to be as follows. Glu·Met·(Cys)·Asn·Met·(Cys)·Val·Arg·Pro·Tyr·Pro·Phe·Met·Ser·Ser·(Cys)·Pro·Glul·Gly·Glul·Asp·Arg·(Cys)·Tyr·Lys·Ser·Tyr·Trp·Val·Asn·Glu·Asn·Gly·Lys. The amount of PTH-Lys (PTC) at cycle 35 in Fig. 3 is 18 pmol. Half-cystine residues in parentheses were not determined by the present direct sequencing but by a conventional sequence determination procedure after reduction and alkylation (data not shown).

It is concluded that the present isocratic elution on a C18 column is suitable for microdetermination of PTH-amino acids obtained by automated sequencing at picomole level.

REFERENCES

J. Biochem.