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Note

Identification of phenylthiohydantoinaminoethylcysteine by high-performance liquid chromatography

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The identification of cysteine (as its PTH derivative)* after Edman degradation[1] in the sequencing of proteins has largely been unsuccessful. This amino acid is generally identified as its oxidized product, cysteic acid, or as the alkylated derivative, S-carboxymethylated cysteine[2,3]. The quantitative determination of PTH-cysteic acid and PTH-S-carboxymethylcysteine also presents special problems[4]. Recently, the identification of cysteine as its pyridylethylated derivative was reported[5]. This paper provides information on the determination of PTH-AE-Cys by high-performance liquid chromatography (HPLC), which is now regarded as the method of choice for identifying and quantifying PTH-amino acids generated as a result of automated sequencing procedures[6,7].

We have devised simple methods for identifying PTH-AE-Cys by HPLC in which we use either acetonitrile or methanol solvent systems. The methanol buffers allow the separation under complete isocratic elution conditions of all the PTH-amino acids except Val/Met, although Phe/Ile are not well resolved. The AE-Cys at position 93 in the aminoethylated β-chain of human hemoglobin was unambiguously identified with this procedure.

EXPERIMENTAL**

All PTH-amino acids, including PTH-AE-Cys, were purchased from Pierce (Rockford, Ill., U.S.A.). Methanol and acetonitrile (glass-distilled) were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.), sequencer chemicals from Beckman (Palo Alto, Calif., U.S.A.) and Polybrene from Aldrich (Milwaukee, Wisc., U.S.A.). All other reagents were of analytical grade. Enzymes were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

* Abbreviations: PTH = phenylthiohydantoin; AE = aminoethyl; DMAA = dimethylallylamine; Polybrene = hexadimethrine bromide; TCPK = L-(1-tosylamido-2-phenyl) ethylchloromethyl ketone; DMF = dimethylformamide.

** The use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

The β-chain of Hemoglobin Detroit (β95 Lys $\rightarrow$ Asn) was reduced, aminoethylated and digested with TPCK-trypsin as previously described[8,9]; the peptide Tp X-XI was isolated by ion-exchange chromatography[10]. This peptide was sequenced in a Beckman 890C sequencer according to the DMAA program (102974) recommended by the manufacturer. Approximately 2 mg of Polybrene were added to the sequencer cup, and one cycle of the peptide program was run before the sample was added. This procedure improved the retention of the peptide in the spinning cup[11,12].

The PTH-amino acids were detected on a Waters Model ALC/GPC-204 chromatograph equipped with a Model 660 solvent programmer and a Model 440 absorbance detector. In the acetonitrile methodology two μBondapak C_{18} columns (30 cm $\times$ 3.9 mm I.D.) in series were used, but in the methanol procedure only one column was used. The first procedure has been described previously[13] and was followed without change. The second procedure was essentially that described by Zeeuws and Strosberg[7] but with the following modifications. Buffer A was composed of 20% methanol in 5 m*M* sodium acetate; the pH was adjusted to 5.6 with acetic acid for better resolution of Glu and Asn. Buffer B was composed of 38% methanol in water. The mixing gradient was generated by using curve 11 on the programmer. Under these isocratic conditions, buffer A was pumped for 5 min, and then buffer B was pumped for 40 min longer. The flow-rate was 2.5 ml/min and absorption was recorded at 254 nm. These analyses were performed at room temperature (23°).

RESULTS AND DISCUSSION

Fig. 1 shows the results when the acetonitrile system was used for separating a standard mixture of PTH-amino acids, including PTH-AE-Cys. The PTH-AE-Cys, dissolved in DMF, eluted as a sharp peak after lysine. The critical disadvantage of this system is that the PTH derivatives Val/Pro/Met and Ile/Trp/Phe cannot be resolved with it. In addition, acetonitrile is a toxic and expensive solvent.

The PTH-AE-Cys derived from the β-chain of Hemoglobin Detroit can be seen in Fig. 2. When the recovery of this residue (92.5 nmole) was compared with that of PTH-Leu at position 96 (120.9 nmole), we concluded that the PTH-AE-Cys derivative was stable and was probably not susceptible to side reactions as is the case with PTH-S-carboxymethyl-Cys, which is subjected to a β-elimination reaction resulting in low yields and partial recovery as dehydroserine.

The methanol solvent system provided several advantages over the acetonitrile procedure: (1) only one column is used with the methanol system, and as prepacked columns are very expensive, this would constitute a substantial saving over a period of time; (2) methanol is less expensive, in addition to being a more innocuous solvent; and (3) the time of analysis is shortened from the 60 min required for the acetonitrile system to 45 min for the methanol system.

A decrease in total time may be achieved by increasing the percentage of methanol in buffer B. However, there was some loss in resolution of PTH-Lys and PTH-AE-Cys. The addition of acetic acid to buffer B seemed to have no effect on the separation. In addition, attempts to separate the PTH-AE-Cys in a standard mixture by the aqueous methanol method of Zeeuws and Strosberg[7] were unsuccessful.

Fig. 3 shows the resolution of a mixture of PTH-amino acids, including PTH-AE-Cys, with the modified methanol system. PTH-AE-Cys was well resolved

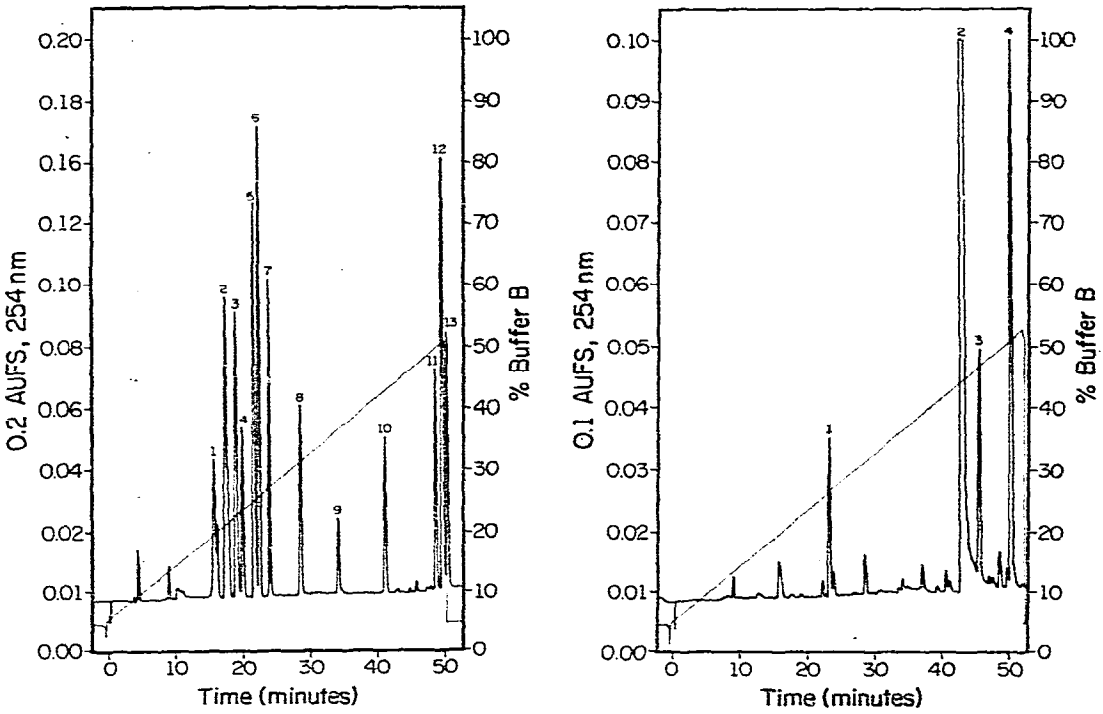

Fig. 1. Separation of a standard mixture of PTH-amino acids using the acetonitrile solvent system. The details of this procedure have been described previously[13]. The concentration of the PTH-derivatives was 5 nmoles except if otherwise indicated. PTH-Lys, Asn, Gln and AE–Cys were dissolved in DMF, the others in ethyl acetate. The recorder speed was 6 in./h. 1 = Aspartic acid; 2 = asparagine (10 nmoles); 3 = serine (10 nmoles); 4 = threonine; 5 = glutamine (10 nmoles); 6 = glycine (10 nmoles); 7 = glutamic acid; 8 = alanine; 9 = tyrosine; 10 = proline; 11 = leucine; 12 = lysine (10 nmoles); 13 = AE-cysteine (10 nmoles).

Fig. 2. Separation and identification of PTH–AE–Cys derived from the β-chain of hemoglobin Detroit (peptide X–XI). The same conditions of analysis were maintained[13]. 1 = Glutamic acid; 2 = Polybrene; 3 = DMAA; 4 = AE-cysteine.

although its retention on the column appeared to be longer than with the acetonitrile system, as evidenced by the broader peak. It should be noted that all PTH-amino acids were resolved except for the Val/Met pair. Although PTH-Phe/Ile were not well resolved, these derivatives can be identified by their different retention times. Complete resolution of PTH-Lys and PTH-Leu was obtained with this methanol system, in contrast to the Zeeuws and Strosberg method[7] in which lowering the temperature of analysis or decreasing the final concentration of methanol was required for the resolution of this pair. PTH-Val/Met can easily be distinguished by gas–liquid chromatography, thin-layer chromatography or by back-hydrolysis with hydrochloric acid–tin(II) chloride[14] followed by amino acid analysis.

A further advantage of our modification of the methanol solvent system is that the isocratic elutions, first with buffer A and then with buffer B, can be accomplished

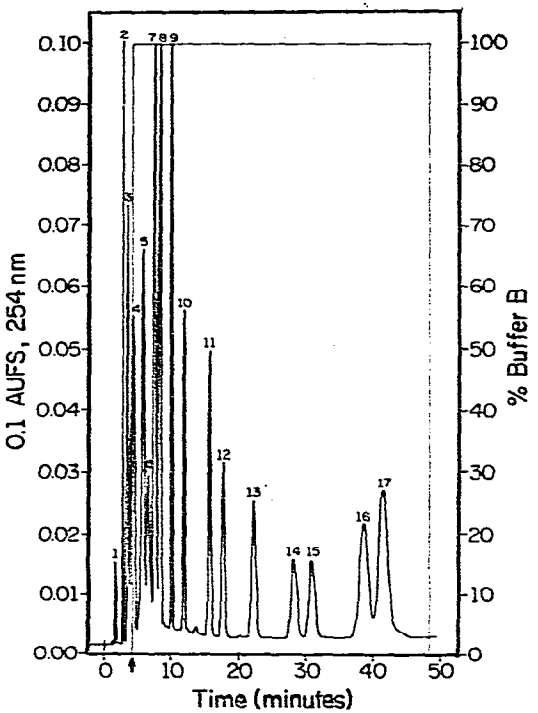

Fig. 3. Separation of a standard mixture of PTH-amino acids using the modified methanol solvent system. The isocratic conditions are indicated by the thin line representing the gradient profile. The arrow denotes the end of the first isocratic elution (5 min). This event appears on the figure at less than 5 min and is due to the recorder pen offset. 1 = Solvent; 2 = aspartic acid; 3 = glutamic acid; 4 = asparagine (10 nmoles); 5 = serine (10 nmoles); 6 = threonine; 7 = glycine (10 nmoles); 8 = glutamine (10 nmoles); 9 = alanine; 10 = tyrosine; 11 = proline; 12 = valine; 13 = tryptophan; 14 = phenylalanine; 15 = leucine; 16 = lysine; 17 = AE-cystine (10 nmoles).

with only one pump and a timing mechanism (instead of a gradient programmer) to permit the switch from buffer A to buffer B at the designated time. Such a system would result in considerable savings on equipment expenditure.

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