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Note

Improved high-pressure liquid chromatographic separation of amino acid phenylthiohydantoins

JACQUES ELION
Institut de Pathologie Moleculaire, 24, rue du Fb. Saint Jacques, 75014 Paris (France)
MICHAEL DOWNING
Cardiovascular Research Unit, Mayo Clinic, Rochester, Minn. (U.S.A.)
and
KENNETH MANN*
Hematology Research Unit, Mayo Clinic, Rochester, Minn. (U.S.A.)
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The determination of NH_2-terminal sequences of peptides or proteins by the Edman degradation involves the identification of the residue removed from the peptide at each cycle (for a recent review, see ref. 1). The phenylthiohydantoin (PTH) derivatives of the amino acids are commonly identified by thin-layer chromatography (TLC) or by gas–liquid chromatography (GLC) both before and after silylation. High-pressure liquid chromatography (HPLC) has been more recently used for this purpose and presents several advantages over other procedures, including identification and quantitation of all the residues, including histidine and arginine, without derivatization. Several HPLC procedures have been described[2–6] and we have reported the separation of most of the PTH amino acid derivatives in 40 min on a μBondapak C_{18} column utilizing an acetonitrile–acetate solvent system[7]. We describe here a new procedure utilizing the same column and a methanol–acetate solvent system. This procedure results in a shorter time of analysis and better resolution. All of the PTH derivatives are resolved in 32 min with the exception of the pair PTH-valine–PTH-methionine.

MATERIAL AND METHODS

Standard PTH derivatives were obtained from Pierce (Rockford, Ill., U.S.A.). Methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and used without further purification. Water used in the preparation of the buffers was purified on a Milli-Q four place Millipore system (Millipore, Bedford, Mass., U.S.A.). Prior to use on the high pressure liquid chromatograph, the buffers were filtered through a 0.45-μm Millipore filter.

Analysis of the PTH derivatives was carried out on a Waters Assoc. ALC/GPC 202 high-pressure chromatograph equipped with a second Model 6000

* To whom correspondence should be addressed.

pump and a Model 660 programmer. Samples (1 to 20 μl) were injected without solvent interruption through a Waters Assoc. U6K injector. Separations were carried out at room temperature on a μBondapak C_{18} column (30 cm $\times$ 4 mm). Amino acid PTHs were detected by absorption at 254 nm utilizing the UV detector of the liquid chromatograph.

Solvents were pumped at a total flow-rate of 3 ml/min, resulting in a pressure of 4000 p.s.i. Solvent A was 0.01 *M* sodium acetate pH 4.0–methanol (9:1) and solvent B 0.01 *M* sodium acetate pH 4.0–methanol (1:9). The column was equilibrated with solvent B contributing 5% to the total volume of the eluent. PTH derivatives were eluted from the column by a concave gradient (curve 7 on the programmer) from initial conditions (5% solvent B) to 40% of solvent B over 22 min, followed by an isocratic elution with 40% solvent B for 10 min. Both solvents A and B were stirred continuously while being supplied to the solvent delivery pump.

RESULTS AND DISCUSSION

HPLC is the technique of choice for the quantitative identification of all the PTH amino acids without derivatization. The limitations of this technique up to now have been the time of analysis, incomplete resolution of PTH derivatives, cost of the instrumentation and of solvents.

Fig. 1 shows the elution profile of a standard mixture of PTH derivatives of ten amino acids. The elution positions of the twenty usual PTH amino acid derivatives

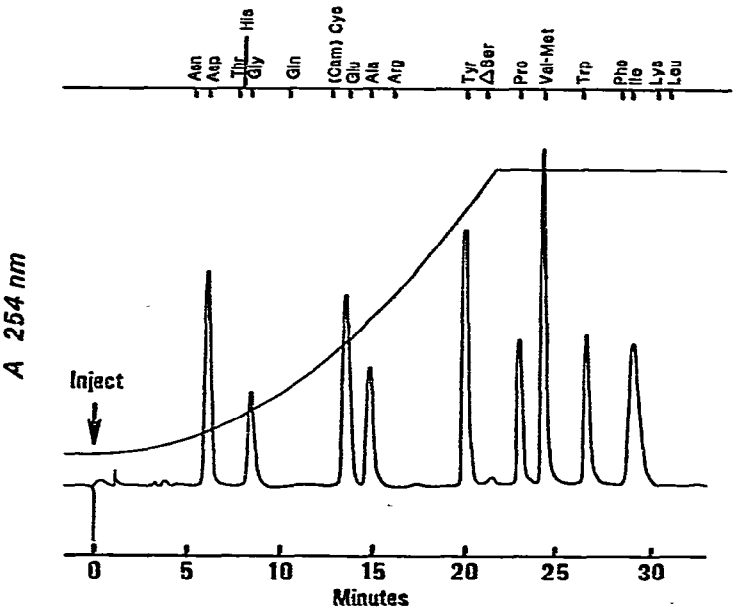

Fig. 1. Elution profile of a mixture of 10 PTH derivatives of amino acids by HPLC. Elution conditions are described in the text. The profile of the elution gradient is shown by the concave line running through the chromatogram, and followed by an isocratic elution for 10 min. The full-scale deflection represents 0.32 absorbance units for 3–7 nmoles of each PTH amino acid derivative.

are illustrated on the upper part of the figure. The PTH amino acids are resolved one from the other in 32 min, with the exception of PTH-valine and PTH-methionine which are eluted as a single peak. The PTH derivatives of phenylalanine, tryptophan and isoleucine which were eluted in a single peak in the acetonitrile–acetate system[7] are all resolved in the present system. Similarly proline is clearly separated from the valine–methionine peak.

Several hundred residues obtained from an automatic liquid-phase sequencer have been positively identified utilizing this HPLC program, with very high reproducibility. Reproducibility is enhanced if the column is washed with the second solvent (solvent B) for 10 min after every 5 identifications. Standard PTH serine is eluted between PTH-aspartic acid and PTH-threonine. However, the PTH serine derivatives produced by the sequencer are always found as the derivative dehydroserine (PTH Δ Ser). This derivative gives a characteristic double peak eluting after PTH-tyrosine. Since PTH-histidine and PTH-arginine are resolved from the other PTH amino acids, the extraction of the organosoluble PTH derivatives from the aqueous conversion medium is theoretically no longer necessary. Good results are obtained when N,N,N′,N′,-tetrakis (2-hydroxypropyl)ethylenediamine (Quadrol) is used as the coupling buffer in the sequencer. However, when the N,N′-dimethylallyamine (DMAA) buffer is used, a DMAA peak is present in the void volume which may interfere with the identification of PTH-asparagine, and extraction is therefore preferable. Utilization of a DMAA sequencer program often results in an additional peak eluting near the valine–methionine peak which occasionally interferes with the identification of these two residues.

In addition to improved resolution and shorter analysis time, which allows identification to keep pace with the automatic sequencer, the use of methanol instead of acetonitrile represents a substantial decrease in the running cost of the liquid chromatograph.

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