PTH-AMINO ACID ANALYSIS BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (MHPLC)

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1. Introduction

High-performance liquid chromatography has in recent years become an extremely valuable method for the analysis of PTH-amino acids. It offers the advantage over the routinely used gas chromatographic methods that it also allows one to identify easily the thermolabile or poorly volatile PTHs of asparagine, aspartic acid, glutamine, glutamic acid, tryptophan, threonine, serine and lysine. Several elution programmes for liquid chromatography of PTH-amino acids have been described [1–7]. All these methods use stainless steel columns of sizes varying between 2 X 250 mm and 4.6 X 500 nm and apply pressures, depending on the elution rates wanted (100–150 ml/h), of 1000–3000 psi.

We here describe the determination of PTHs using a micro-high pressure liquid chromatograph (MHPLC), an apparatus very recently introduced [8]. With this method the separations are carried out on a column made of an ordinary piece of PTFE tubing of 1.5 mm o.d. The advantages of the method are discussed.

2. Materials and methods

The analyses were carried out on a FAMILIC-100 microhigh performance liquid chromatograph (Japan Spectroscopic Co., Tokyo). The column was a piece of PTFE tubing, 0.5 mm i.d., 0.5 mm thick and 9 cm long, filled with a 5 μm ODS-18 packing for reversed phase chromatography (SC-01-05150, Jasco, Japan). The sample volume was injected in the column with a microfeeder, a push-button accentuated system that allows one to draw off the set volume (usually 0.2 μl) from the sample solution. A constant flow rate of 8 μl/min was delivered by a 250 μl airtight syringe driven by the microfeeder as in [8].

The column eluate was monitored at 254 nm in a UVIDEC-100 variable wavelength spectrophotometer using a special quartz flow cell of 0.3 mm light pathlength.

At 4 mM stock solution in methanol containing the PTH-derivatives of all the amino acids except histidine, arginine and cysteine, was used to work out a satisfactory methodology for separation. All solvents used were purchased from Merck (p.a. grade).

3. Results and discussion

Figure 1 shows the separation of the polar PTH-amino acids from the reference mixture, using 23% methanol in 5 mM sodium acetate, pH 5.3, as eluant (solvent A, also used in [6]). The retention times are 4.6 min for PTH-Asp, 6.7 min for PTH-Glu, 9.3 min for PTH-Asn, 11.0 min for PTH-Ser, 12.1 min for PTH-Thr, 13.7 min for PTH-Gly and 15.3 min for PTH-Gln. The analysis can be continued by manually refilling the syringe with the microfeeder, a manipulation which takes some 30 s. In the next 20 min only two other PTHs are eluted, PTH-Ala after 28.4 min and PTH-Tyr after 51.5 min. The other PTHs from the reference mixture remain adsorbed on the column. We have repeatedly established that none of these components interfere with a subsequent analysis of a new sample, probably

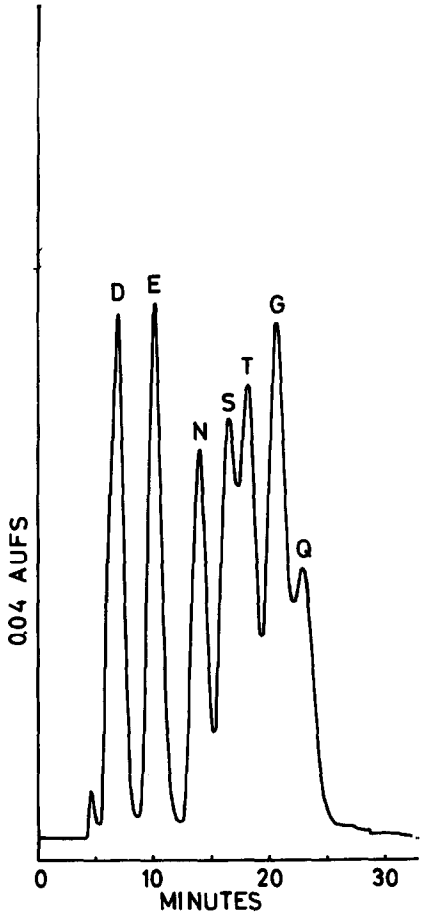

Fig.1. Separation of polar PTH-amino acids with solvent A on a microcolumn of ODS-18. Amount of each component, 1.25 nmol. Injection vol. 0.3 μl.

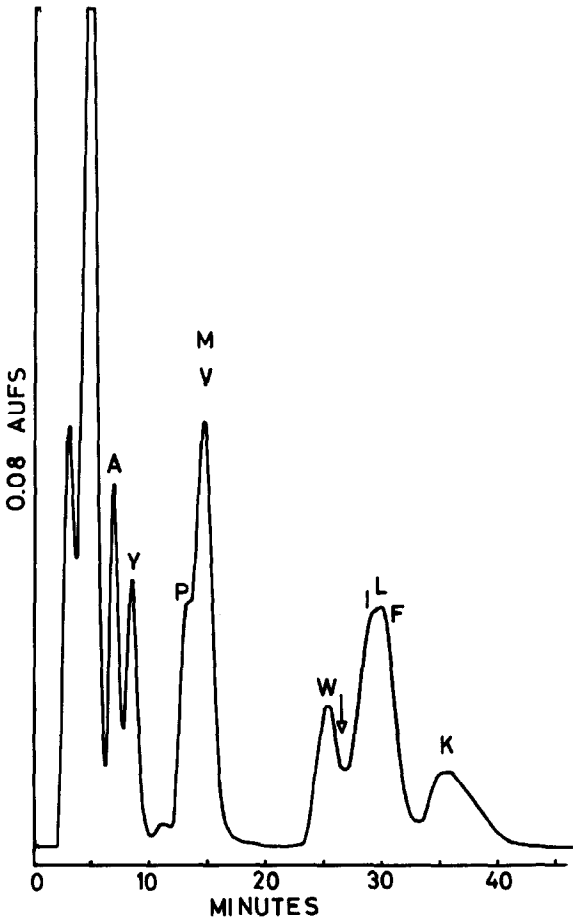

Fig.2. Separation of non-polar PTH-amino acids and PTH-Lys with solvent B on the microcolumn. The arrow indicates the start of the second delivery of solvent. Amounts and injection volume as in fig.1.

because when eluted, they do so as a broad peak undistinguishable from the baseline.

For the separation of the non-polar PTH-amino acids we used the mobile phase water/acetonitrile/isopropanol, as proposed [9], but in the proportion 7.5/1.5/1 (solvent B). In order to use the same components as in solvent A we have tested the effects of increasing concentrations of methanol in water, and different pH values. None of these mobile phases, however, showed such a good separation as did solvent B. A separation using solvent B is shown in fig.2. The PTHs of Ala and Tyr are eluted as the first components after the total peak of the polar amino acids. The retention times are 6.9 min and 8.4 min, respectively. They are followed by PTH-Pro at 13.4 min, and by the coeluted PTHs of Met and Val at 14 min. After refilling the syringe manually, the analysis yields PTH-Trp after 25.5 min, PTH-Ile after 29.1 min, PTH-Leu and PTH-Phe both after 30 min and PTH-Lys after 46 min.

Although the MHPLC can be equipped with a gradient system [8], we did not apply a continuous gradient as suggested for the identification of the polar and non-polar PTHs in a single run by ordinary high performance liquid chromatography (HPLC)

[3–7]. There are two main reasons why we limited ourselves to isocratic elutions:

(1) The approximately equal distribution of the PTHs over both chromatograms (fig.1,2) do not suggest that all the PTHs can be satisfactory separated within the 25 min needed to empty the syringe.

(2) The injected sample volume is so small (0.1–0.01 μl) that it is not justifiable to spend time making the gradient, a procedure which in the MHPLC must be carried out prior to an analysis.

One may, instead, perform two analyses with a separate sample volume, one to detect the polar, the other to detect the non-polar amino acids. Should one want a complete analysis with only one sample injection, very satisfactory and reproducible results can be obtained using a stepwise gradient. It is only necessary to refill the syringe with solvent B after the initial charge of solvent A has been delivered, and to continue the analysis. An example is given in fig.3.

Figure 3 also illustrates the sensitivity of the method. The experiment was carried out on 40 pmol each of the PTHs with a photometric setting of 0.04 a.u.f.s. The lowest sensitivity of the UVIDEC-100 spectrophotometer used in the FAMILIC-100 HPLC is 0.005 a.u.f.s.

In comparison with analyses of amino acid phenylthiohydantoins on ordinary liquid chromatographs, we conclude from our work that the micromethods on the MHPLC do not result in a shortened analysis time, nor in an improved sensitivity of detection. HPLC and MHPLC seem to be competitive in these respects. The main advantage of MHPLC is the great ease with which one can work out quickly the best conditions for separation, including the search for the best packing material. A column can easily be packed using the microfeeder [8]. Moreover, it is self-evident that the miniaturization of the column to about 1% vol. of an ordinary HPLC column implies the use of such small amounts of packing and of eluant that the working costs are impressively reduced. The need to apply small sample volumes is advantageous when other identification methods for the PTHs are required as is usually the case in automatic protein sequencing. Since the routinely used methods such as thin-layer chromatography and gas chromatography (GC) are less sensitive than MHPLC, the availability of more material for analysis with these methods is certainly welcome.

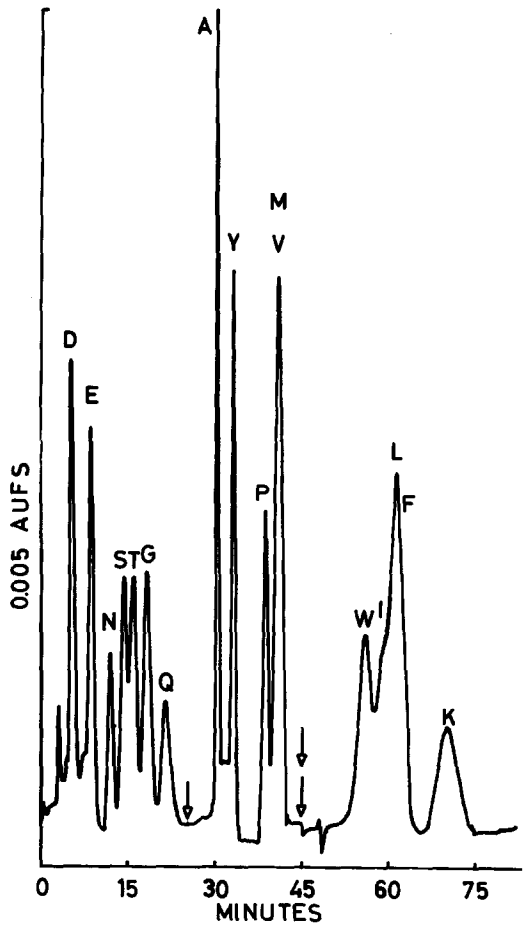

Fig.3. Stepwise elution of polar and apolar TPH-amino acids with solvents A and B after a single injection of 0.01 μl sample containing 40 pmol each component. The single arrow is the start of solvent B, the double arrow is the start of the second delivery of 0.25 ml solvent B.

We have applied the MHPLC methodology to confirm some residues of the N-terminal region of *Agrobacterium tumefaciens* II Chrys cytochrome *c*-556 (J.v.B. et al., unpublished). Tyrosine-31 had been particularly difficult to determine by GC but was obvious from the analysis by MHPLC.

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