A new isocratic HPLC separation for Pth-amino acids, based on 2-propanol

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The described isocratic separation for phenylthiohydantoin (Pth) amino acid derivatives offers advantages over gradient systems: the baseline is more stable at high sensitivity, the resolution and reproducibility is excellent and the cost and consumption of solvent is very low. The separation of low picomolar quantities, on 2 different types of reverse phase columns, are presented. The method is used 'off line', i.e. as a separate analytical unit, or in an 'on line' mode, where it is directly connected to a protein sequencer for automatic identification of the released amino acid derivatives.

Phenylthiohydantoin amino acid *2-Propanol* *Tetrahydrofuran* *Isocratic separation* *HPLC*

1. INTRODUCTION

The introduction of the method of sequential degradation of polypeptides by Edman [1], marked the beginning of a new period in the study of protein chemistry. The basic technique has remained remarkably unaltered since its appearance. A number of developments have led from this beginning to the presently employed microsequencing technology. These are: (1) the automation of the degradation by Edman and Begg [2]; (2) the availability of high performance liquid chromatography (HPLC) for the identification of phenylthiohydantoin (Pth) amino acids [3] whereby the sensitivity has been increased; (3) the improvement of the Beckman machine, e.g. by changing the vacuum system and the delivery valves [4], and the incorporation of an autoconverter [5] and 'on-line HPLC' [6]; (4) the advent of the gas phase sequencer [7], which can be operated with as little as 100 pmol of starting material of unknown sequence (peptide or protein).

The use of a gradient system for the identification of the Pth-amino acids, particularly at low picomolar concentrations often causes problems of baseline stability, due to changes in the eluent composition, and with retention time reproducibility, because of difficulties with gradient mixing. Isocratic separations suffer to a far less extent from these difficulties and have the additional advantage of being of low cost. The equipment required is simpler and the solvent consumption is low, especially as it can be recycled in a closed system. Up to now the published isocratic systems, which resolved most of the Pth-amino acid derivatives, have been based on an aqueous mixture with acetonitrile [8,9]. Here we offer an alternative to this solvent by using a system with 2-propanol. The separation that can be obtained with this solvent is as good as that with acetonitrile. 2-Propanol also has the advantage that it is cheaper and a less poisonous and aggressive substance. Therefore, the HPLC equipment is less likely to fail. In addition, we have substituted dichloroethane with tetrahydrofuran, which is more readily soluble in an aqueous mixture.

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2. MATERIALS AND METHODS

The standard Pth mixture was purchased from Pierce, dissolved in 100% methanol and diluted with 20% 2-propanol in water shortly before the injection. 2-Propanol was 'pro analysis' grade from Merck, and tetrahydrofuran 'HPLC grade' from Rathburn Chemicals. The water used was of HPLC quality from a Millipore Milli-Q purification system.

2.1. *Isocratic separation of Pth-amino acids*

The HPLC system was equipped with a Knauer HPLC pump, model 64, with either a Knauer filter UV photometer (fig.1a) or a Jasco variable wavelength photometer, model Uvidec II (fig.1b), a Rheodyne injection valve and a Kipp and Zonen BD41 chart recorder. The columns employed were, Spherisorb C8 3μ from Phase Separations Ltd. (250 × 4 mm, filled by Knauer or self-packed with a Shandon filling apparatus) as shown in fig.1b, or Lichrokart HPLC cartridge Supersphere RP8 4μ from Merck (250 × 4 mm) as in fig.1a. The conditions for the isocratic separation were as given in table 1. The eluent was mixed as follows: first, 195 ml 2-propanol were made up to 1 l with water, i.e. 19.5% 2-propanol in water, and degassed; second, to this mixture were then added 10 ml tetrahydrofuran, 10 μl sodium azide (4 mg/ml) and 5 or 15 ml 1 M sodium acetate, pH 5.3, to give a final concentration of about 5 or 15 mM, respectively and sonicated. The 1 M sodium acetate was prepared by titrating 1 M acetic acid with sodium hydroxide.

3. RESULTS AND DISCUSSION

The separation of 10 and 50 pmol of the reference Pth-amino acid derivatives by isocratic HPLC on reverse phase C8-bonded silica support is shown in figs 1a and 1b, respectively. In fig.1a the support was Lichrokart Supersphere RP8, 4μ particles from Merck and in fig.1b, Spherisorb C8, 3μ particles from Phase Separations Ltd. The HPLC chromatograms show the excellent resolution of virtually all the derivatives with simple HPLC equipment. Since only picomolar quantities of Pth derivatives are released in microsequence analysis, the eluent mixture can be recycled: a litre of eluent is sufficient for a week of chromatography. The solvent mixture was continuously purged with a gentle stream of nitrogen, and no additional degassing was required during the week.

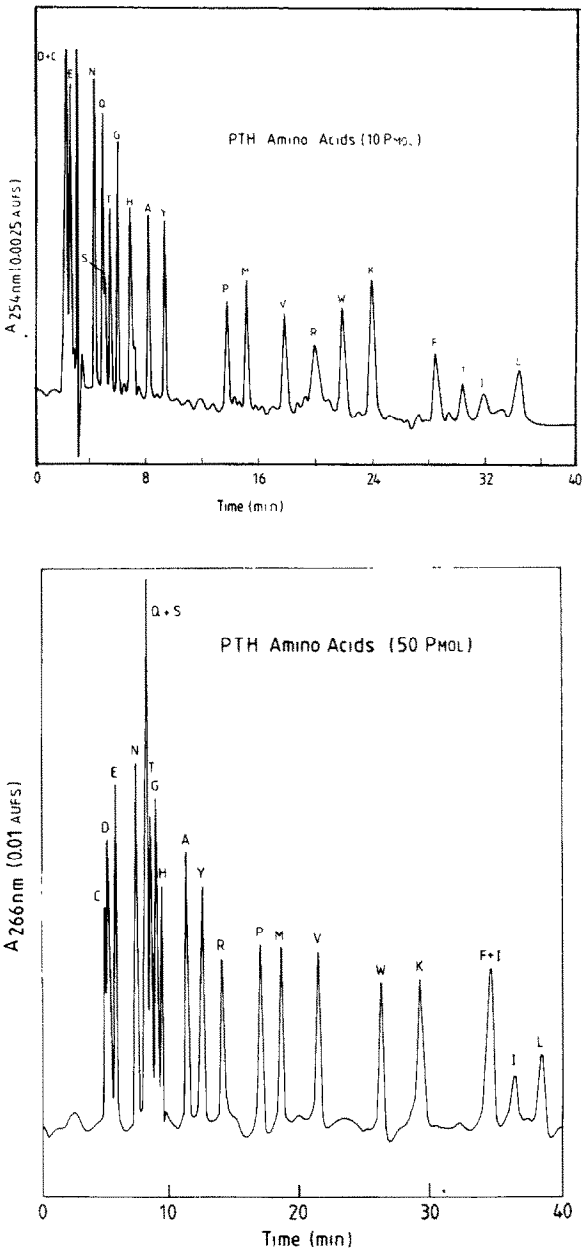

Fig.1. Isocratic separation of Pth amino acid derivatives. (A) On Lichrokart Supersphere RP8. (B) On Spherisorb C8. For details see table 1.

Depending on the sensitivity of the detector, the

Table 1

Chromatography conditions for the isocratic separation of Pth-amino acids

	Lichrokart Supersphere RP8 (column fig.1a)	Spherisorb (column fig.1b)
Temperature	58°C	58°C
Flow rate	0.4 ml/min	0.4 ml/min
Column pressure	~80 bar	~100 bar
Detection	Knauer UV photometer 254 nm 0.0025 AUFS	Jasco UVIDEC II 266 nm 0.01 AUFS
Recorder speed	5 mm/min	5 mm/min
Column dimensions	250 × 4 mm	250 × 4 mm
Support	Lichrokart Supersphere RP8 4μ	Spherisorb C8 3μ
Eluent	2-Propanol:water: tetrahydrofuran: 1 M sodium acetate pH 5.3	2-Propanol:water: tetrahydrofuran: 1 M sodium acetate pH 5.3
Mixture (ratio)	220:780:10:5	195:805:10:15

detection limit for the Pth-amino acid derivatives was 1–5 pmol at 0.0025 AUFS (Knauer detector). Especially at such high sensitivities, the isocratic separation shows a stable baseline as well as constant retention times. These two facts are of the greatest importance for the automatic on-line identification of the Pth derivatives in sequencers, because the system must operate reliably for long periods of time. The most difficult derivatives to separate were Pth-serine and Pth-threonine. Here the Lichrokart Supersphere RP8 column was slightly better (see fig.1a). However, both serine and threonine produce dehydrated derivatives that can be detected at 313 nm, and at this wavelength the other Pth amino acid derivatives show little or no absorption.

As previously described [8] the elution of Pth-histidine and Pth-arginine is highly dependent on the ionic strength of the buffer (sodium acetate concentration). This fact is clearly illustrated in figs 1a and b. The lower ionic strength of the eluent in fig.1a causes the Pth-histidine and particularly the Pth-arginine to elute later. The 2 isomers of Pth-isoleucine separated well with the system, although in fig.1b the one isomer coelutes with Pth-phenylalanine. Both Pth-proline and Pth-lysine elute earlier than with acetonitrile [8,9]. The separation was remarkably independent of sample volume, with injection volumes between 10–100 μl, provided the injection was made on 20% 2-propanol/water and the flow rate was kept low (about 0.4 ml/min). However, as one would expect, a smaller volume will improve the separation slightly, especially where two peaks are close together. The isocratic on-line detection system has been installed in the Berlin sequencers [4,6], and the 2-propanol system described here is in use in three different machines; sample quantities of 20, 50 and 100 μl are automatically injected.

In conclusion, it should be stressed that the method described here offers a cheap, simple isocratic HPLC system for the separation and quantitative determination of the Pth amino acid derivatives generated during automatic Edman degradation of peptides and proteins. It is comparable in sensitivity with the gradient systems employed so far. Further improvements in columns, for example the introduction of microbore (2 or 1 mm internal diameter) columns and advanced detectors will help to decrease the detection limit further. Since lower flow rates (which generate lower back pressures in the columns) can be used in the isocratic mode than those that have to be applied to any gradient system, this facilitates the use of microbore columns and guarantees the columns longer life.

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REFERENCES

[1] Edman, P. (1949) Acta Chem. Scand. 4, 281.
[2] Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91.
[3] Zimmermann, C.L., Appella, E. and Pisano, J. (1977) Anal. Biochem. 77, 569–573.
[4] Wittmann-Liebold, B. (1983) in: Modern Methods in Protein Chemistry (Tschesche, H. ed.) pp.229–266, De Gruyter, Berlin.
[5] Wittmann-Liebold, B., Graffunder, H. and Kohls, H. (1976) Anal. Biochem. 75, 621–633.
[6] Wittmann-Liebold, B. and Ashman, K. (1985) in: Modern Methods in Protein Chemistry (Tschesche, H. ed.) pp. 303–327, De Gruyter, Berlin.
[7] Hewick, R.M., Hunkapiller, M.W., Hood, L.E. and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990–8005.
[8] Lottspeich, F. (1980) Biol. Chem. Hoppe-Seyler 361, 1829–1834.
[9] Lottspeich, F. (1985) J. Chromatogr. 326, 321–327.