Identification of Subnanomole Amounts of PTH-Amino Acids by High Performance Thin-Layer Chromatography

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Summary
High performance thin-layer chromatography (HPTLC) of PTH-amino acids on 5 x 5 cm silica gel plates pre-coated with a fluorescence marker gives 10–20 fold increase in sensitivity compared to ordinary silica gel plates. Separation of PTH-Leu from PTH-Ile is easily achieved in contrast to chromatography on polyamide sheets. Only two solvent systems are required and as many as 12 samples can be chromatographed on each plate. However, if the sample is contaminated with N-phenylthiourea a third solvent system is necessary.

Introduction
One of the major problems in Edman degradation is the identification of the phenylthiohydantoin (PTH) amino acids. Among the methods of choice are gas chromatography [1], high performance liquid chromatography [2], amino acid analysis [3, 4], thin-layer chromatography on silica gel plates [5, 6] or on polyamide sheets [7–9]. None of the methods are, however, without difficulties [10] and several problems arise when automated micro sequence methods are employed [11, 12].

A common practice is to use a combination of at least two methods in order to achieve more reliable identification. Here thin-layer chromatography offers the advantage of being rapid and inexpensive. The use of polyamide sheets [7–9] has increased the sensitivity to 0.05–0.2 nm making it a valuable tool in sequencing nanomolar amounts of proteins.

High performance thin-layer chromatography (HPTLC) utilizes silica gel with a small particle size and a narrow particle size distribution. It is 10 to 20 times more sensitive than ordinary silica gel plates containing an internal fluorescent marker [5, 6] and offers the same sensitivity as polyamide sheets.

This paper describes solvent systems for HPTLC where all the PTH-amino acids usually found in the ethyl acetate phase after conversion are separated.

Materials and Methods
HPTLC silica gel plates 60 F254, acetic acid (pro analysis), and 1,2-dichloroethane (reinst) were obtained from Merck. Chloroform and ethyl acetate (both pro analysis) were from May and Baker. PTH-standards were from Sigma or Pierce.

Solvent 1 is prepared by mixing 9 parts of chloroform containing 1.5 % ethanol with 1 part of ethyl acetate by volume resulting in the ratio: chloroform:ethanol:ethyl acetate 88.65 : 1.35 : 10. Solvent 2 is made from 90 parts of chloroform containing 1.5 % ethanol, 10 parts of ethanol and 2 parts of glacial acetic acid resulting in the ratio: chloroform:ethanol:glacial acetic acid 88.65:11.35 : 2 v/v/v.

Application of the samples was made with a 10 mm 3 syringe operated with a micrometer gear. A marker mixture containing PTH-Pro, Leu, Ile, Val, Phe, Ala, Trp, Gly, Tyr, Glu, Asp and Gln is spotted near each edge and at the centre of the plate. The presence of several marker samples is necessary since U-shaped solvent fronts are occasionally seen. Twelve samples can be applied on each plate.

250 cm 3 cylindrical beakers with glass lids as described by Kulbe [8] were used for the chromatography. The ratio between the volume of the solvent and that of the chamber being about 1:40. After chromatography in the first solvent the plate is dried in a stream of air and viewed under UV-light at 254 nm. The plate is then developed in the next solvent system and in the same direction. Chromatography in each system takes about ten minutes. A permanent record can be made by photographing through a yellow filter with a Polaroid camera.

Results and Discussion
The first solvent resolves the following PTH-amino acids: Pro, Leu, Ile, Phe, Met, Val, Ala, Trp, Gly, Tyr, Lys and Thr (Fig. 1 A). The other PTH-amino acids remain at the origin except PTH-Ser which moves slightly above the origin. The second system resolves PTH-Asn, Glu, CMCys, Asp, Glu, Ser and MetSO 2 (Fig. 1 B). Irregular solvent fronts may sometimes result in insufficient resolution of PTH-Lys from PTH-Tyr in system 1. However, in system 2 they are well separated and PTH-Lys is located between PTH-Ala and PTH-Trp.

If the extractions following coupling in the Edman procedure are insufficient the sample may contain N-phenyl-
Thin-layer chromatography of PTH-amino acids. Chromatography was performed as described in text.
A: System 1
B: System 1 + 2 in same direction.
C: System 1 + 2 + 3 in same direction.
Abbreviations:
dPTU: N,N'-diphenylthiourea,
PTU: N-phenylthiourea,
CMC: carboxymethylcysteine,
Msf: methionine sulfoxide.

High contrast pictures of plates illuminated by UV-light at 254 nm were obtained with 35 mm Agfaortho 25 film exposed through 3× yellow filter. The film was developed in Agfa Rodinal 1 + 9 for 5 minutes at 20 °C.

The higher load capacity of HPTLC plates makes application faster than on polyamide sheets without increasing spot size.

One of the greatest advantages of HPTLC is the easy separation of PTH-Leu from PTH-Ile. They are poorly separated on polyamide sheets and a separate run is necessary [8].

Detection of PTH-amino acids by staining on thin-layer plates suffers from the disadvantage that a new sample