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Note

Separation of phenylthiohydantoin-amino acids by overpressured-layer chromatography

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The most widely used approach to determine amino acid sequences of proteins and peptides is the Edman procedure[1]. Phenylthiohydantoin (PTH)-amino acids have routinely been identified by gas chromatography (GC)[2], high-performance liquid chromatography (HPLC) on bonded packings[3,4] thin-layer chromatography (TLC) on silica gel chromatoplates[5,6] and polyamide sheets[7]. Bucher[8] elaborated a separation method on a fine-particle silica gel chromatoplate and identified sub-nanomol amounts of PTH-amino acids using two or three solvent systems successively on the same plate. More recently, Schuette and Poole[9] used a continuous multiple development on a silica gel chromatoplate. However, these methods have some drawbacks.

GC requires further derivatization of some phenylthiohydantoin derivatives before injection. HPLC is a direct and sensitive method but the reproducibility of the retention time using gradient elution is not good. TLC is not sufficiently sensitive, and high-performance TLC employs only a relatively short migration distance, *e.g.*, 5–10 cm, which is not sufficient for the separation of complex mixtures containing large numbers of components such as PTH-amino acids. The multiple development has other disadvantages such as the need for a subsequent development and detection.

In this paper we demonstrate the known advantages of overpressured-layer chromatography (OPLC)[10–12] for the separation and the quantitation of PTH-amino acids.

EXPERIMENTAL

Eluent 1 was prepared by mixing chloroform, ethanol (95%) and acetic acid in the ratio of 90:10:2 (v/v), and eluent 2 was prepared by mixing dichloromethane and ethyl acetate in the ratio of 90:10 (v/v). All solvents were of reagent grade used without further purification.

A standard mixture was prepared by dissolving PTH-amino acids (each 1 mg/ml) in acetone–methanol (1:1, v/v). The sample volume was 0.4 μl.

The sorbent layer was HPTLC silica gel F_{254} (Merck, Darmstadt, F.R.G.) with impregnated edges on three sides. The sample was spotted by use of a 1-μl Hamilton syringe. For the chromatographic separation a CHROMPRES 10 chamber was used (Labor MIM, Budapest, Hungary). The chromatoplate was dried between

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the two developments. The quantitative evaluation was accomplished by a CS-920 High Speed TLC/HPTLC Scanner (Shimadzu, Japan) at 275 nm.

RESULTS AND DISCUSSION

More than 50 solvent systems were investigated to find a suitable eluent pair for the linear OPLC separation, and the migration distance was optimized. The following systems were found to give the best results:

(1) chloroform–ethanol (95%)–acetic acid (90:10:2, v/v)

(2) dichloromethane–ethyl acetate (90:10, v/v)

The first solvent resolved the polar PTH-amino acids, cysteic acid (K salt) ($CySO_3K$), His, Asn, Glu, S-methyl-cysteine (CySCM) and Asp, and the others were on the β-front (the secondary demixing front). The second solvent system separated the less polar and non-polar PTH-amino acids, Ser, Glu, Thr, Lys, Tyr, Gly, Trp, Ala, S-carboxymethyl-cysteine (CySM), Met, Phe, Nle, Ile, Leu and Pro (Fig. 1). The optimal migration distance in the first development was 16 cm and that in the second was 20 cm, therefore continuous development was used for the separation. The total migration time was 1 h.

Fig. 2 shows the one-dimensional separation of PTH-amino acids using rechromatography with the two optimal solvent systems and continuous development.

Fig. 3 illustrates the densitogram of PTH-amino acids at 275 nm. The peak areas obtained using the linearization routine of the scanner give linear calibration

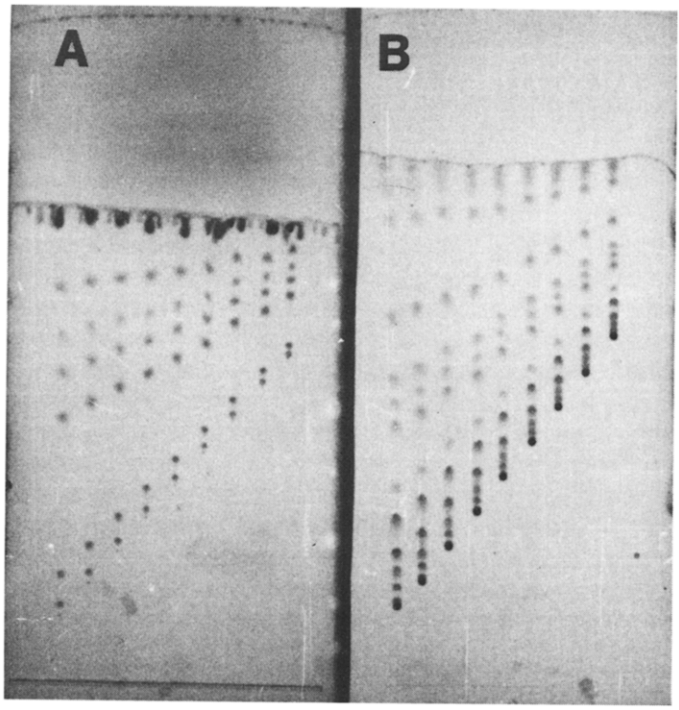

Fig. 1. Separation of PTH-amino acids by OPLC using solvent system 1 (A) and solvent system 2 (B).

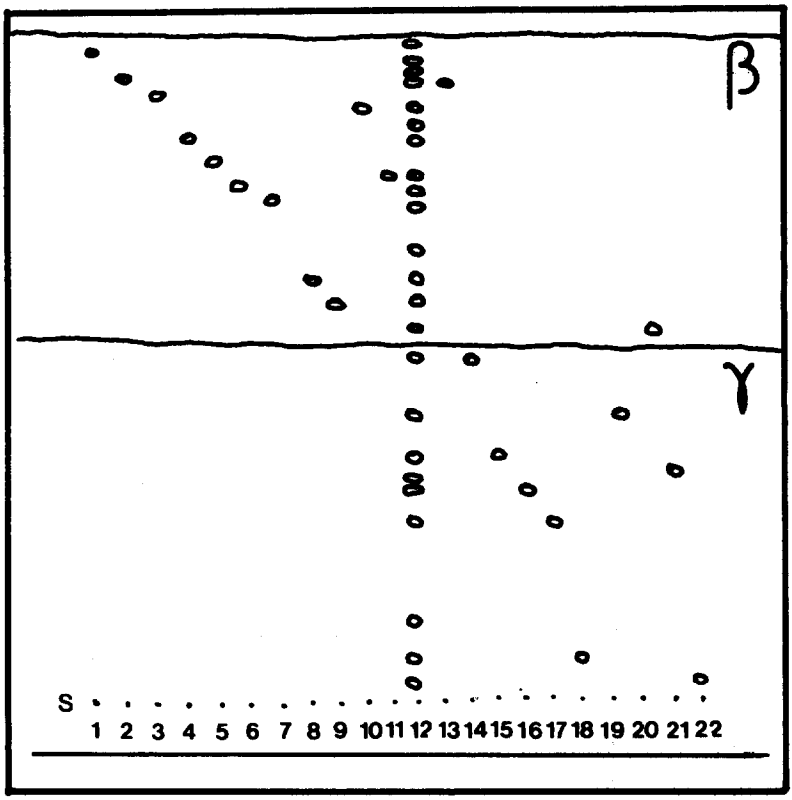

Fig. 2. Separation of PTH-amino acids by OPLC. Flow-rate: 0.30 ml/min. Migration times: first development, 20 min; second development, 34 min. Migration distances: first development, 16 cm; second development, continuous. External pressure on membrane: 1.0 MPa. Amino acids: 1 = Pro; 2 = Leu; 3 = Ile; 4 = Phe; 5 = Met; 6 = Ala; 7 = Trp; 8 = Tyr; 9 = Lys; 10 = Val; 11 = CySM; 12 = standard mixture; 13 = Nle; 14 = Glu; 15 = Asg; 16 = Gln; 17 = Asn; 18 = His; 19 = Ser; 20 = Thr; 21 = CySCM; 22 = $CySO_3K$. S = Start; β,γ = solvent fronts.

TABLE I

REPRODUCIBILITY OF QUANTITATION OF PTH-AMINO ACIDS

PTH-amino acid	*Peak area*	*S.D.*	*C.V. (%)*
$CySO_3K$	322.0	15.8	4.9
His	282.0	9.7	3.4
Asn	306.2	3.0	0.99
Thr	220.4	9.5	4.3
Gly	268.2	12.7	4.7
Trp	382.6	19.0	5.0
CySM	152.2	12.1	7.9
Nle	1264.4	66.7	5.3
Phe	1073.2	32.1	3.0
Pro	443.8	33.2	7.5

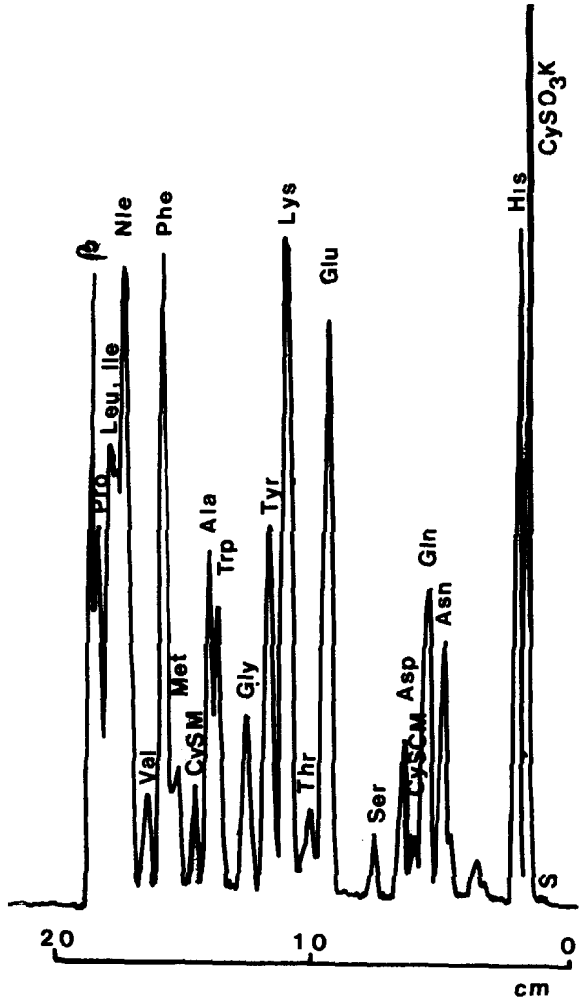

Fig. 3. Densitogram of PTH-amino acids obtained with a Shimadzu CS-920 Scanner at 275 nm.

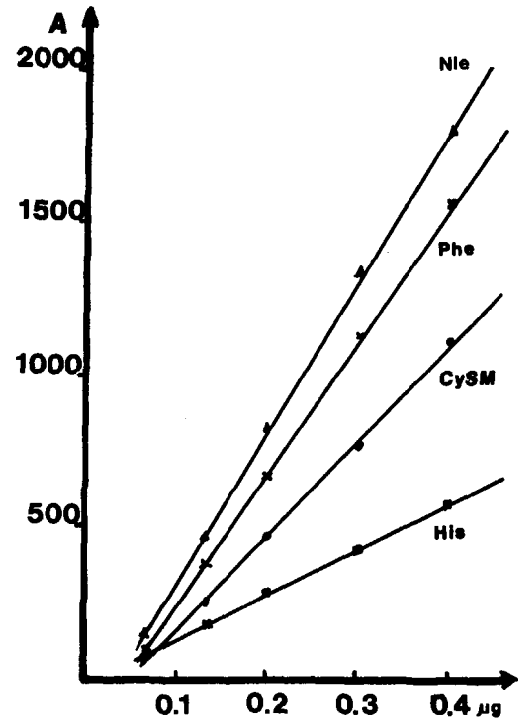

Fig. 4. Calibration curves for some PTH-amino acids.

curves for PTH-amino acids (Fig. 4) and the detection limit is 0.05 μg for every PTH-amino acid. Nine parallel measurements were used (0.2 μg of each PTH-amino acid) for the reproducibility test. The average coefficient of variation of the integrated peak area was about 5% (see Table I).

These results show that linear OPLC with rechromatography is suitable for the efficient separation and quantitation of PTH-amino acids in structural investigations of proteins and peptides.

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