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# THIN-LAYER CHROMATOGRAPHY OF AMINO ACID HYDANTOINS*

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## SUMMARY

Amino acid hydantoins were detected directly in amounts of 0.2–0.5 μg with *tert.*-butyl hypochlorite. The amino acid hydantoins were separated by thin-layer chromatography using the following solvent systems: (I) chloroform–ethanol–formic acid (2:1:1); (II) *n*-butyl acetate–ethanol–formic acid (12:2:1); (III) dichloroethane–ethanol–formic acid (80:20:1); and (IV) *n*-butanol–acetic acid–water (15:2:5). The terminal amino groups of several proteins were detected successfully.

## INTRODUCTION

Potassium cyanate reacts with the amino groups of peptides or proteins in slightly alkaline solution, and the corresponding amino acid hydantoin is formed by subsequent acidification. The hydantoin derivatives are more stable than thiohydantoins, especially against air oxidation, because the 2-position of the hydantoin ring is substituted with a carbonyl group instead of the thiocarbonyl group in thiohydantoins.

Stark[1] reported the use of amino acid hydantoins in the determination of N-terminal amino acid groups of peptides or proteins. He identified the liberated amino acid hydantoins by hydrolyzing them to the corresponding amino acids, which were detected with ninhydrin. However, he did not propose the direct identification of amino acid hydantoins on chromatograms, presumably because there was no suitable detecting agent. Ultraviolet absorption was used by Hagel and Gerding[2] for the direct identification of hydantoins in column chromatography.

We previously reported[3] the optical rotatory dispersion and circular dichroism of L-amino acid hydantoins and suggested their application in the determination of the D,L-configuration of the N-terminal amino acid groups of peptides.

We found that *tert.*-butyl hypochlorite was suitable for the detection on chromatograms of amino acid hydantoins, which were sensitively revealed by this "peptide bond detecting agent" as blue spots. Two imino protons of hydantoin react with the reagent, and imino groups at the 3-position, which has two adjacent carbonyl groups, may react more sensitively.

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The thin-layer chromatography (TLC) of amino acid hydantoins and their detection with *tert.*-butyl hypochlorite is reported in this paper.

## EXPERIMENTAL

### *Preparation of materials*

*tert.*-Butyl hypochlorite was prepared in the conventional manner[4].

All the materials used were prepared as in the previous work[3]. A representative procedure, for the preparation of 5-methylhydantoin (hydantoin derived from alanine) was as follows.

L-Alanine (4.5 g; 0.05 mole) and 4.9 g of potassium cyanate were dissolved in 30 ml of water and were allowed to react for 30 min at 70° with stirring. The reaction mixture was refluxed with 30 ml of 6 *N* hydrochloric acid and, after filtration, the solution was allowed to stand overnight in a refrigerator. Recrystallization from hot water gave 4.5 g of the hydantoin in a yield of 79.0%.

According to the above procedure, 13 hydantoins were prepared, including the derivatives of glycine, alanine, phenylalanine, aspartic acid, glutamic acid, lysine, arginine, histidine, methionine, cysteine, proline, serine and threonine. Histidine hydantoin was obtained by adjusting the pH to 5 with 2 *N* sodium hydroxide solution after cyclization. Arginine hydantoin was prepared according to the method of Boon and Robson[5]. Lysine, which has an $\varepsilon$-amino group, reacted with twice the molar amount of potassium cyanate in a similar manner to phenyl isothiocyanate. The reactions of other functional groups were similar to those of phenylthiohydantoin with the exception of serine and threonine. Hydantoins derived from serine and threonine were prepared without the need for dehydration, although the synthesis of the thiohydantoins proceeded simultaneously with dehydration.

With valine, leucine, isoleucine, tyrosine and tryptophan, pyridine (at the carbamylation stage) and acetic acid (at the cyclization stage) were added in order to dissolve the amino acids.

### *Thin-layer chromatography*

All solvents except formic acid were redistilled. The formic acid used was of commercial analytical-reagent grade and was used without further purification.

TLC of the amino acid hydantoins was carried out in a jar (24 × 21 × 5 cm) on a 20 × 20 cm plate of silica gel (Kieselgel G, Merck, 0.25 mm thick) which had been activated for 40 min at 110°.

All of the chromatograms were equilibrated with the vapour of the solvent system in a jar before the runs. The solvent systems used are described later.

The compounds in the spots were detected by spraying with 1% of *tert.*-butyl hypochlorite in dichloroethane containing 10% of acetic acid, followed by spraying an equal volume of 0.05 *N* KI and a saturated solution of *o*-tolidine in 2% acetic acid. Free amino acids were also detected with this reagent on thin-layer chromatograms, but were distinguished from hydantoins with ninhydrin as the hydantoins were not revealed.

### *Terminal amino acid group determination of beef insulin and several proteins*

The Stark procedure for the determination of N-terminal amino acids was

followed until the liberation of terminal amino acid hydantoin[1]. The determination of insulin as an example was carried out as follows. The scale was 1.0 $\mu$mole.

Insulin (6.0 mg) was dissolved and denatured in a mixture of 1.3 ml of 8 *M* urea and 1.3 ml of morpholine–acetate buffer (pH 8.0) and carbamylated overnight with 125 mg of potassium cyanate at 50°. After dialysis and lyophilization, the carbamylated protein was dissolved in acetic acid and hydrolyzed in a sealed tube with 5 ml of 6 *N* HCl at 110° for 30 min.

After evaporation to dryness, the residue was placed on a column of ion-exchange resin (Amberlite IR-120). The column was eluted with water, the first 30 ml of effluent was evaporated to dryness and the residue was dissolved in ethanol. An aliquot of the solution was chromatographed as described before.

Ribonuclease-A, lysozyme and trypsin were determined by the same procedure. Glutathione was carbamylated without denaturation.

## RESULTS AND DISCUSSION

The solvent systems used were as follows:

(I) chloroform–ethanol–formic acid (2:1:1)
(II) *n*-butyl acetate–ethanol–formic acid (12:2:1)
(III) dichloroethane–ethanol–formic acid (80:20:1)
(IV) *n*-butanol–acetic acid–water (15:2:5).

Amino acid hydantoins derived from 18 amino acids (Table I) were separated successfully; $R_F$ values are shown in Table II and Figs. 1–4. Asparagine and glutamine

TABLE I

ELEMENTARY ANALYSIS AND MELTING POINTS OF HYDANTOINS

| *Compound* | *Lit. m.p. (°C)* | *Calculated (%)* | | | *Found m.p. (°C)* | *Found (%)* | | |
|---|---|---|---|---|---|---|---|---|
| | | *C* | *H* | *N* | | *C* | *H* | *N* |
| Gly | 223–225 | 36.00 | 4.03 | 27.99 | 222 | 35.71 | 3.91 | 28.07 |
| Ala | 174–177 | 42.10 | 5.30 | 24.55 | 177 | 41.72 | 5.31 | 24.47 |
| Val | 146–148 | 50.69 | 7.07 | 19.71 | 145 | 50.45 | 7.02 | 19.43 |
| Leu | 212–214 | 53.83 | 7.74 | 17.94 | 214 | 53.63 | 7.77 | 18.24 |
| Ile | 150–151 | 53.83 | 7.74 | 17.94 | 148 | 53.83 | 7.67 | 18.04 |
| Asp | 210–213 | 37.98 | 3.83 | 17.72 | 218 | 37.67 | 3.64 | 18.01 |
| Glu | 175–176 | 41.86 | 4.68 | 16.28 | 174 | 41.70 | 4.58 | 16.17 |
| Phe | 181–183 | 63.15 | 5.30 | 14.73 | 182 | 63.35 | 5.13 | 15.00 |
| Tyr | 261–264 | 58.25 | 4.89 | 13.58 | 262 | 58.10 | 4.82 | 13.50 |
| Try | 248–251 | 62.87 | 4.84 | 18.33 | 244 | 62.64 | 4.69 | 18.41 |
| Met | 103–105 | 41.36 | 5.79 | 16.08 | 104 | 41.23 | 5.65 | 16.40 |
| $CySO_3H$ | — | 20.83 | 3.06 | 12.15 | 273 (decomp.) | 20.54 | 2.11 | 12.24 |
| Ser | 187–188 | 36.93 | 4.65 | 21.53 | 188 | 36.61 | 4.49 | 21.54 |
| Thr | 193–196 | 41.66 | 5.59 | 19.44 | 200 | 41.87 | 5.34 | 19.33 |
| Lys | 195–197 | 44.85 | 6.59 | 26.16 | 193 | 44.85 | 6.64 | 26.15 |
| Arg | 100 | 34.36 | 6.18 | 28.62 | 100 | 34.30 | 6.04 | 28.45 |
| His | 235 | 28.81 | 4.19 | 25.86 | <280 | 28.99 | 4.14 | 25.78 |
| Pro | 163–165 | 51.42 | 5.75 | 19.99 | 165 | 49.22 | 5.61 | 19.81 |

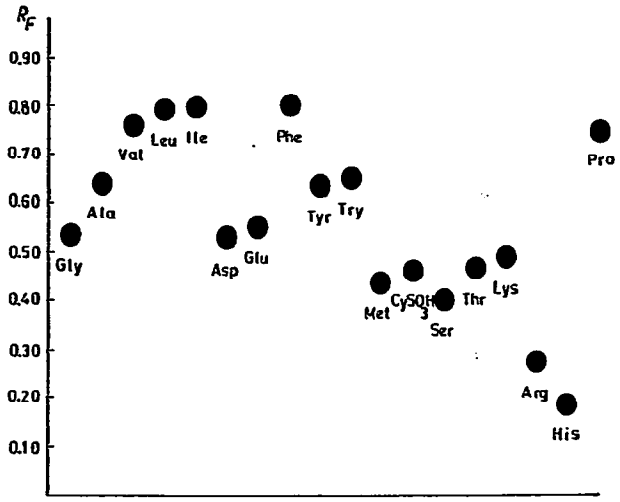


Fig. 1. TLC of hydantoins in solvent I: chloroform–ethanol–formic acid (2:1:1).

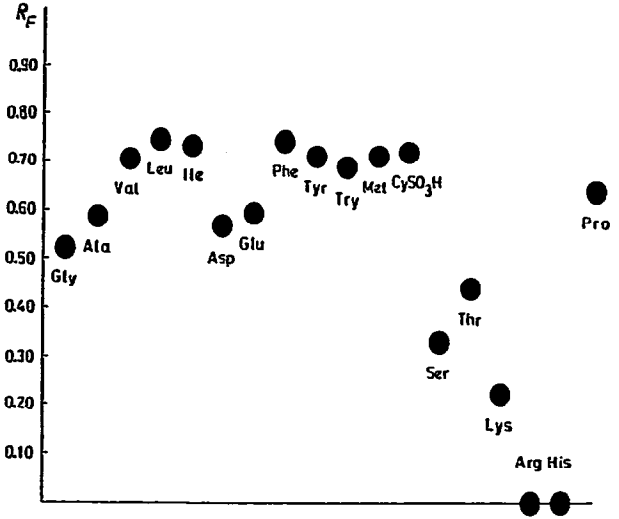


Fig. 2. TLC of hydantoins in solvent II: *n*-butyl acetate–ethanol–formic acid (12:2:1).

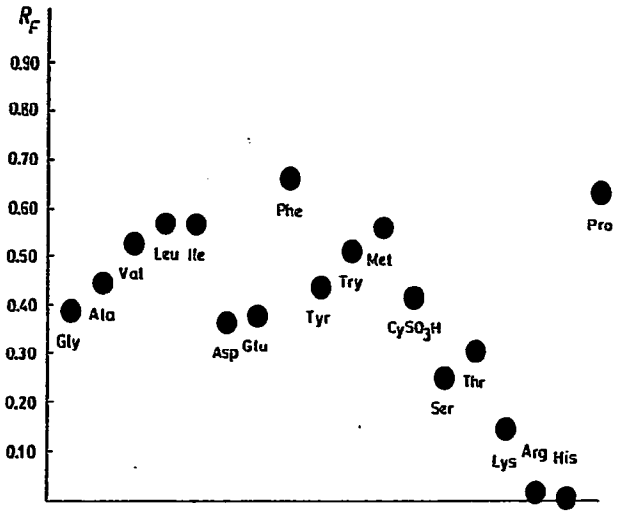


Fig. 3. TLC of hydantoins in solvent III: dichloroethane–ethanol–formic acid (80:20:1).

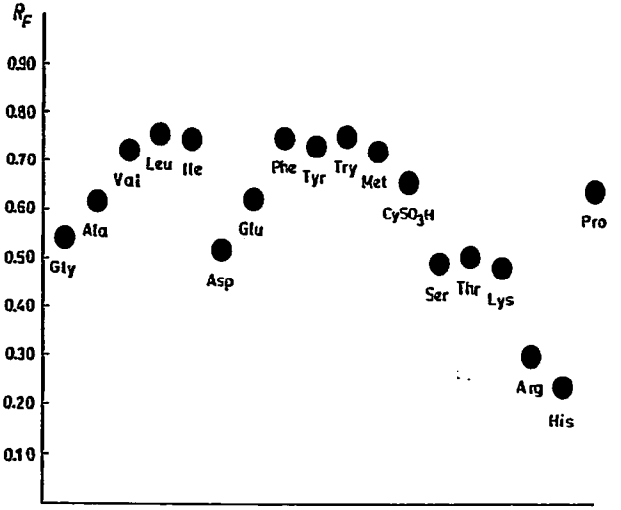


Fig. 4. TLC of hydantoins in solvent IV: *n*-butanol–acetic acid–water (15:2:5).

were not examined as their hydantoins were hydrolyzed during acidification to give aspartic and glutamic acid, respectively.

The separation of leucine and isoleucine was difficult, as in other chromatographic separations.

The limit of detection of the hydantoins with *tert.*-butyl hypochlorite was 0.2–0.5 μg, as shown in Table III. A quantitative or semi-quantitative study has not yet been carried out.

TABLE II

$R_F \times 100$ VALUES OF AMINO ACID HYDANTOINS

Results are given for three determinations with each solvent together with the average values. Solvents I–IV as listed in the text.

| *Amino acid* | *Solvent I* | | | | *Solvent II* | | | | *Solvent III* | | | | *Solvent IV* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *Av.* | *1* | *2* | *3* | *Av.* | *1* | *2* | *3* | *Av.* | *1* | *2* | *3* | *Av.* |
| Gly | 54 | 53 | 56 | 54 | 53 | 54 | 53 | 53 | 39 | 38 | 40 | 39 | 56 | 52 | 56 | 55 |
| Ala | 64 | 62 | 66 | 64 | 61 | 56 | 60 | 59 | 44 | 45 | 44 | 44 | 63 | 58 | 64 | 62 |
| Val | 75 | 75 | 78 | 76 | 72 | 68 | 71 | 70 | 48 | 56 | 56 | 53 | 70 | 71 | 74 | 72 |
| Leu | 78 | 78 | 82 | 80 | 77 | 73 | 75 | 75 | 53 | 60 | 61 | 58 | 74 | 75 | 77 | 75 |
| Ile | 78 | 78 | 83 | 80 | 78 | 70 | 74 | 73 | 52 | 60 | 59 | 57 | 75 | 74 | 75 | 75 |
| Asp | 52 | 51 | 52 | 52 | 59 | 55 | 59 | 58 | 38 | 35 | 40 | 38 | 54 | 50 | 53 | 52 |
| Glu | 53 | 52 | 55 | 53 | 59 | 57 | 64 | 60 | 39 | 35 | 37 | 38 | 62 | 59 | 62 | 61 |
| Phe | 79 | 78 | 82 | 79 | 77 | 72 | 73 | 74 | 63 | 66 | 68 | 65 | 75 | 74 | 74 | 74 |
| Tyr | 65 | 62 | 65 | 64 | 71 | 70 | 73 | 72 | 41 | 45 | 43 | 44 | 70 | 65 | 75 | 70 |
| Try | 68 | 59 | 67 | 65 | 74 | 74 | 76 | 75 | 49 | 55 | 52 | 52 | 74 | 74 | 77 | 75 |
| Met | 45 | 42 | 46 | 44 | 71 | 71 | 72 | 71 | 52 | 59 | 58 | 56 | 71 | 70 | 73 | 71 |
| CyH | 51 | 48 | 41 | 47 | 76 | 70 | 69 | 72 | 40 | 42 | 45 | 42 | 65 | 63 | 70 | 66 |
| Ser | 43 | 43 | 42 | 43 | 36 | 34 | 37 | 36 | 27 | 24 | 29 | 27 | 51 | 48 | 47 | 49 |
| Thr | 49 | 48 | 49 | 48 | 46 | 42 | 44 | 44 | 33 | 28 | 32 | 31 | 53 | 49 | 50 | 51 |
| Lys | 49 | 49 | 49 | 49 | 22 | 22 | 22 | 22 | 13 | 13 | 16 | 14 | 49 | 48 | 49 | 49 |
| Arg | 30 | 31 | 28 | 30 | 01 | 03 | 02 | 02 | 02 | 02 | 04 | 03 | 32 | 28 | 29 | 30 |
| His | 24 | 23 | 23 | 23 | 01 | 02 | 01 | 01 | 02 | 02 | 04 | 03 | 20 | 18 | 19 | 19 |
| Pro | 77 | 74 | 78 | 76 | 63 | 59 | 61 | 61 | 57 | 63 | 65 | 62 | 64 | 62 | 63 | 63 |

TABLE III

DETECTION LIMITS OF AMINO ACID HYDANTOINS USING *tert.*-BUTYL HYPOCHLORITE

| *Amino acid* | *Detection limit (μg)* | *Amino acid* | *Detection limit (μg)* |
|---|---|---|---|
| Gly | 0.2 | Try | 0.2 |
| Ala | 0.5 | Met | 0.5 |
| Val | 0.5 | $CySO_3H$ | 0.2 |
| Leu | 0.5 | Lys | 0.2 |
| Ile | 0.5 | Arg | 0.5 |
| Asp | 0.5 | His | 0.2 |
| Glu | 0.4 | Ser | 0.2 |
| Phe | 0.5 | Thr | 0.2 |
| Tyr | 0.4 | Pro | 0.5 |

When the method was applied to beef insulin, two amino acid hydantoins were detected as being N-terminal, and were identified as glycine and phenylalanine (Fig. 5).

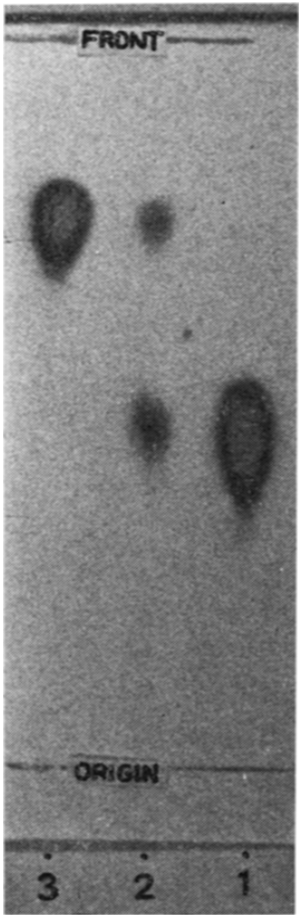


Fig. 5. TLC of N-terminal amino acids from beef insulin using solvent system II: *n*-butyl acetate–ethanol–formic acid (12:2:1). 1= Glycine hydantoin; 2= insulin; 3= phenylalanine hydantoin.

The N-terminal amino acids of glutathione (glutamic acid), ribonuclease-A (lysine), lysozyme (lysine) and trypsin (leucine) were also identified by this method.

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