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Note

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Thin-layer chromatographic separation of diphenylindonyl-substituted thiohydantoin derivatives of amino acids

In a previous paper[1] we described the preparation of 2-*p*-isothiocyanophenyl-3-phenylindone, an orange crystalline compound, for its possible use as a reagent for the determination of the amino acid sequence in peptide chains. By the interaction of this reagent with natural α-amino acids, the corresponding indonyl-substituted thiohydantoin derivatives, ITH-amino acids[2,3], which are also intensely coloured, were obtained. The purpose of the present investigations was to determine the best conditions for the direct thin-layer chromatographic separation and identification of indonyl-substituted thiohydantoin derivatives of common α-amino acids.

Experimental

Materials and reagents. Kieselgel G (Merck) containing 13 % of gypsum of particle size 13–40 μm was used on the plates. ITH-amino acids were prepared as described earlier[2,3].

For development of the chromatograms, freshly prepared solvent systems were used. The solvents were of *pro analysi* grade and were re-distilled before use. Chloroform (b.p. 59.5–61°) was purified from ethanol in the usual manner. Glacial acetic acid was treated with chromic anhydride and redistilled.

Chromatography. The standard technique of thin-layer chromatography as described by STAHL[4] was used.

R_F values were determined on plates of dimensions 100 × 140 mm with a layer thickness of 0.25 mm, 24 h after spreading the layer on the plates, in chambers saturated with the same solvent system. The ratio between the volume of the solvent system and that of the chamber was 1:30. The samples were applied in amounts of 1 μg in a 2-μl volume of acetone or methanol (for the potassium salt of ITH-cysteic acid acetic acid was used) at a distance of 1.5 cm from the lower edge of the plate and 1 cm from each other. The plate was immersed in the solvent system to a depth of 0.5 cm and the distance from the start to the front was 10 cm.

Two-dimensional chromatograms were developed on plates of dimensions 200 × 200 mm with a layer thickness of 0.5 mm in a Desaga chamber with a distance from the start to the front of 12 cm in each direction. Re-chromatography was carried out after completely removing the solvents of the previous solvent system at room temperature by evaporation.

ITH-amino acids in amounts of as little as 10^{-9} mole were visible as yellow–orange spots, indonyl isothiocyanate as a darker orange spot, bisdiphenylindonylthiourea as a pink–red spot, and monoindonylthiourea as an orange–red spot.

Results and discussion

In order to find solvent systems for the separation of ITH-amino acids on

Kieselgel G, we used the published data for the thin-layer separation of phenylthiohydantoin derivatives[4-7]. As a result of our investigations, we propose the following solvent systems as being suitable for the separation of ITH-amino acids: (A) chloroform–methanol (98:2); (B) chloroform–methanol (90:10); (C) *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20); (D) chloroform–methanol–glacial acetic acid (80:20:2).

In Table I are given the average R_F values from six determinations for 25 ITH-amino acids as well as for 2-*p*-isothiocyanophenyl-3-phenylindone, N-*p*-2'-(3'-phenylindonyl)phenylthiourea and N,N'-bis-[*p*-2'-(3'-phenylindonyl)phenyl]thiourea.

TABLE I

R_F × 100 VALUES OF ITH-AMINO ACIDS, 2-*p*-ISOTHIOCYANOPHENYL-3-PHENYLINDONE AND MONO- AND BISDIPHENYLINDONYLTHIOUREAS

ITH-derivative	R_F × 100 *in solvent system*			
	A	*B*	*C*	*D*
Alanine	26	70	28	85
α-Aminobutyric acid	34	76	41	90
Arginine	0	1	0	11
Aspartic acid	0	4	0	68
Asparagine	0	26	3	59
Cysteic acid	0	0	0	26
Glutamic acid	0	12	10	73
Glutamine	0	30	4	66
Glycine	17	62	17	82
Histidine	0	20	0	27
Hydroxyproline	11	60	8	82
Isoleucine	52	82	64	92
Leucine	54	83	65	92
Lysine	13	82	17	95
Methionine	38	79	38	90
Methionine sulphone	4	49	2	79
Norleucine	49	82	63	91
Norvaline	42	79	55	91
Phenylalanine	43	79	43	90
Proline	73	88	48	93
Serine	1	34	4	73
Threonine	3	41	8	77
Tryptophan	18	67	23	87
Tyrosine	5	46	10	83
Valine	42	79	55	91
Diphenylindonyl isothiocyanate	83	90	80	94
Monodiphenylindonylthiourea	22	70	28	85
Bisdiphenylindonylthiourea	64	89	60	96

The R_F values vary within certain limits, depending on the conditions used for the chromatography, but relative differences between them remain constant. We carried out experiments on the separation on Kieselgel G by two-dimensional chromatography of a complex mixture containing all the ITH-amino acids given in Table I except methionine sulphone, norvaline and norleucine (Fig. 1). On the start, 2 cm from both edges of the plate, was applied a mixture, containing 1 μg of each compound, dissolved in 2 μl of acetone–methanol (1:1). On the starting points I, II and III were applied mixtures of the same amounts of ITH-amino acids that separated

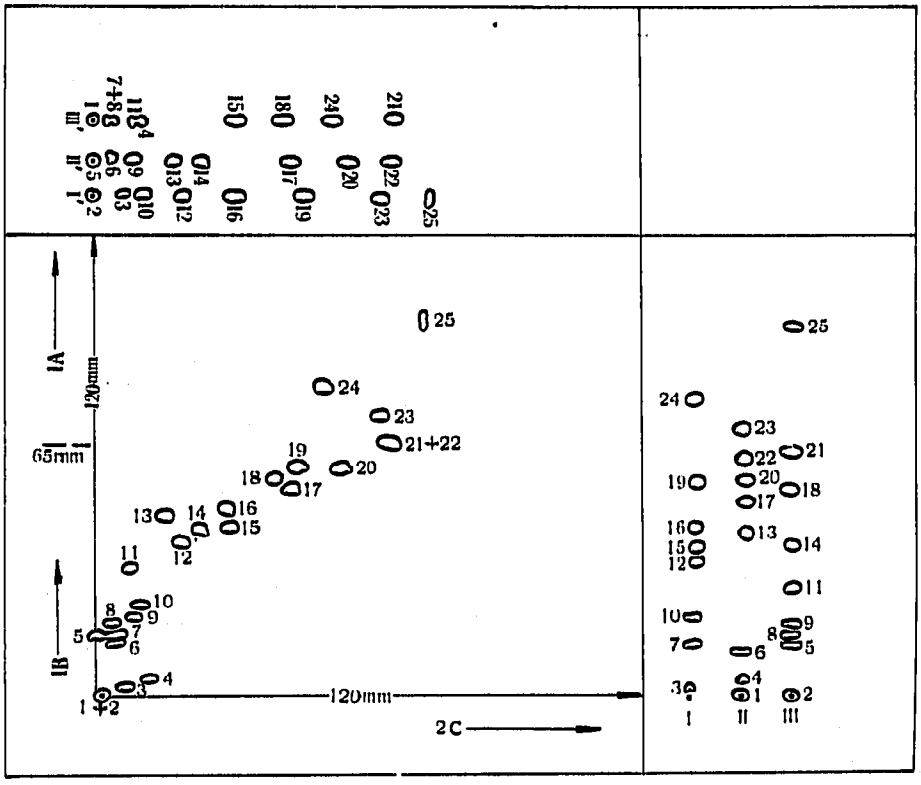

Fig. 1. Two-dimensional separation of ITH-amino acids, diphenylindonyl isothiocyanate and mono- and bisdiphenylindonylthiourea. Carrier: Kieselgel G (0.5 mm layer thickness). 1st run: solvent system B, chloroform–methanol (90:10), up to 65 mm from the starting line, and in solvent system A, chloroform–methanol (98:2). 2nd run: solvent system C, *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20). Numbers of spots: 1, ITH-cysteic acid; 2, ITH-arginine; 3, ITH-aspartic acid; 4, ITH-glutamic acid; 5, ITH-histidine; 6, ITH-asparagine; 7, ITH-glutamine; 8, ITH-serine; 9, ITH-threonine; 10, ITH-tyrosine; 11, ITH-hydroxyproline; 12, ITH-glycine; 13, ITH-lysine; 14, ITH-tryptophan; 15, monodiphenylindonylthiourea; 16, ITH-alanine; 17, ITH-α-aminobutyric acid; 18, ITH-methionine; 19, ITH-phenylalanine; 20, ITH-valine; 21, ITH-leucine; 22, ITH-isoleucine; 23, bisdiphenylindonylthiourea; 24, ITH-proline; 25, diphenylindonyl isothiocyanate.

well in solvent systems A and B (first direction). On the starting points I'; II' and III' were applied mixtures of the same amounts of ITH-amino acids that separated well in solvent system C (second direction).

It can be seen in Fig. 1 that of all the ITH-derivatives indicated, only ITH-leucine and ITH-isoleucine remain unseparated. By carrying out the separation of these derivatives in solvent system A, small but constant differences between the R_F values of both amino acids were observed, ITH-leucine being the faster. This difference was increased by developing the chromatogram in the same system on a plate that had been activated for 30 min at 80°. This method can be used to distinguish both derivatives in solvent system A, if they are not in a mixture; otherwise they give one prolonged spot.

It can also be seen in Fig. 1 that the spot of ITH-histidine is not very well

separated from that of ITH-glutamine. An additional separation of the spots of ITH-histidine, ITH-glutamine and ITH-asparagine can be achieved by re-chromatography in solvent system C in the second direction, as ITH-histidine has $R_F = 0$ in this solvent system.

The spots numbered from 1 to 10 on the two-dimensional chromatogram moved little in the second direction when solvent system C was used for development. Moreover, ITH-arginine and ITH-cysteic acid were not separated and gave one spot on the start. By using solvent system D in the second direction, these two ITH-amino acids can be separated and a more reliable identification of the remaining spots (1–10) is possible. For the two-dimensional separation of these derivatives, the combination of solvent systems B and D is suitable.

As reported earlier[2,3], these derivatives are coloured compounds (yellow, orange and red), and this colour facilitates their chromatographic separation and identification. It was shown that they could be detected directly as coloured spots on a silica gel plate in amounts of about 10^{-9} mole. The sensitivity is therefore the same as that of Edman-DNS method with phenyl isothiocyanate. But while that method requires the use of a greater amount of the initial protein, because aliquots for the determination of the N-terminal groups must be taken (20 residues, for instance, theoretically require at least $2 \cdot 10^{-8}$ mole of protein), in the present method the initial amount of protein can be of the order of 10^{-9} mole, as the cleaved product is used for the direct identification of the liberated thiohydantoin derivative. Moreover, the present method is technically very easy to carry out, as a second reagent (dansyl chloride) is not required and all the work on the determination of the N-terminal amino acids by dansylation is avoided.

Investigations aimed at determining the best conditions for the interaction of 2-*p*-isothiocyanophenyl-3-phenylindone with peptides and proteins and at increasing still more the sensitivity of the detection of the indonylthiohydantoin derivatives are at present being carried out.

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1 CH. P. IVANOV, I. N. MANCHEVA AND S. GAITANDJIEV, *C. R. Acad. Bulg. Sci.*, 20 (1967) 799.
2 CH. P. IVANOV AND I. N. MANCHEVA, *C. R. Acad. Bulg. Sci.*, 21 (1968) 785.
3 CH. P. IVANOV AND I. N. MANCHEVA, *C. R. Acad. Bulg. Sci.*, 25 (1972) 349.
4 E. STAHL, *Dünnschichtchromatographie*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1967, pp. 697–701 and 736–741.
5 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, *Experientia*, 17 (1961) 145.
6 J. O. JEPPSON AND J. SJOQUIST, *Anal. Biochem.*, 18 (1967) 264.
7 J. M. BOIGNE, N. BOIGNE AND J. ROSA, *J. Chromatogr.*, 47 (1970) 238.

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