THIN-LAYER CHROMATOGRAPHY OF DINITROPYRIDYL- AND NITROPYRIMIDYL-AMINO ACIDS

CARLO DI BELLO AND ANGELO SIGNOR
Institute of Organic Chemistry, University of Padova (Italy)
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Nearly all procedures employed for the determination of the N-terminal amino acids in peptides and proteins depend upon a reaction of an acylating agent with the N-terminal group; the linkage so formed must be more stable to subsequent hydrolysis of the peptide chain than the other linkages in the chain.

Research in this field began with the studies of SANGER in 1945[1], and the fluorodinitrobenzene (DNFB) method for the amino end-group analysis is now a well established procedure in protein chemistry. The weakest point in this method is that a noticeable destruction of the N-terminal dinitrophenyl (DNP)-amino acid occurs under the conditions of hydrolysis required to remove it from the protein (16–24 h with constantly boiling HCl).

In a previous paper[2], some new reagents which can be used for the identification and quantitative evaluation of N-terminal amino acids in peptides and proteins, were described.

Amongst the several investigated, 2-chloro-3,5-dinitropyridine and 2-chloro-5-nitropyrimidine are particularly useful. The most important feature of these reagents probably consists in a nucleophilic participation of the *aza-group* in the hydrolysis of the first peptide bond, permitting gentler conditions of hydrolysis (15–20 min in 6 *N* HCl with 30 % formic acid added) and the quantitative recovery of the derivative of any N-terminal amino acid, including proline and glycine[3].

Amongst the various methods by which the derivatives thus formed can be successfully separated and identified, paper chromatography has so far given very good results; some mixtures of our derivatives, however, have not yet been adequately resolved by this method. In particular, it was almost impossible to obtain a good resolution of the derivatives of aspartic and glutamic acids and of leucine and isoleucine respectively.

This difficulty can be overcome by regenerating the amino acid in the free form from the corresponding derivative in conc. NH_4OH at 110°[4]; however, the use of this indirect method requires a great deal of time.

In recent years, thin-layer chromatography[5,6] has proved exceptionally useful for the analysis of many types of compounds, providing a simple, rapid and sensitive method for the study of closely related substances, and it seems to be of particular value for compounds that are not readily separated by conventional chromatography.

In this paper we describe the separation of dinitropyridyl-amino acids (DNPyr-amino acids) and of nitropyrimidyl-amino acids (NPm-amino acids) by thin-layer chromatography on silica gel.

EXPERIMENTAL

Materials

Glass plates 20 cm × 20 cm × 0.4 cm. Silica gel G: E. Merck-A.G., Darmstadt. Spreader, chromatography tanks: Desaga GmbH, Heidelberg. Solvents: Merck, Fluka, Erba, specially prepared for chromatography.

Preparation of plates

Distilled water (50 ml) was added in a mortar to 25 g of silica gel G. The suspension was vigorously mixed for $1^1/_2$ min and the slurry formed was applied to five glass plates, using the Desaga spreader, to a thickness of 250 μ. The plates were left to dry in air overnight at room temperature, protected from dust.

Development

The technique of ascending chromatography was used throughout this work. The solvent systems[7] used were (minutes required for development in parentheses):

(A) Chloroform–methanol–acetic acid, 95:5:1 (30)

(B) *n*-Propanol–33 % ammonium hydroxide, 70:30 (120)

(C) Toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonium hydroxide[8], 100:30:60:60 (30)

(D) Benzene–pyridine–acetic acid, 80:20:2 (30)

(E) Chloroform–formic acid, 100:5 (20)

(F) Methyl ethyl ketone–pyridine–water–acetic acid, 70:15:15:2 (35)

The volume of solvent system used for development was always kept constant.

Chromatographic procedure

Amounts of 2–4 mg of the sample were dissolved in 2 ml of anhydrous methanol and 1μl, corresponding to 1–2μg, was applied with a micropipette on the starting points. The points were marked on a line at about 1.5 cm from the lower edge of the plate and with a distance of 1 cm between each point.

After evaporation of the solvent the plates were placed in the chromatography tank, previously saturated with the solvent vapours[9,10]. To ensure uniform saturation of the enclosed space, the walls of the tank were lined with filter paper soaked in solvent. The tank was filled to a depth of about 0.5 cm with the solvent system; the plates were placed on a glass support a little higher than 0.5 cm and allowed to saturate with solvent vapour for one hour. After saturation, development was initiated by increasing the level of the solvent to about 1 cm. When the solvent front had run 10 cm[11], the chromatoplates were removed and the solvent was completely evaporated in a current of hot air.

For two-dimensional chromatography the mixture was applied onto the diagonal of the plate, 3 cm from a corner. The plate was saturated in the solvent vapour for one hour and then developed with the first solvent system. The plate was withdrawn from the chromatography tank, dried for 30 min in an air current, placed in the second solvent system and developed at right angles to the first.

In order to obtain the maximum reproducibility of results, the layer must always be treated in exactly the same way before development in the second direction; in particular the conditions of intermediate drying must be kept strictly constant.

Detection method

Both DNPyr-amino acids and NPm-amino acids have high molar extinction coefficients[3], a factor which facilitates their micromanipulation and photometric estimation. DNPyr-amino acids are easily located either in natural light or in U.V. light. On the contrary the NPm-amino acids are not easily detected in natural light. We have therefore carried out the following chromatic reactions:

(1) The plates were sprayed with an aqueous solution of 1 % $KMnO_4$ and then with *N* HCl; the NPm-amino acids appeared as yellow spots on the deep pink background of the plate.

(2) The plates were sprayed with a solution of 8.1 g of $Hg_2(NO_3)_2$ in 100 ml of 0.5 *N* HNO_3 and then with 0.5 *N* HNO_3. After drying in a current of hot air, they were sprayed with an aqueous solution of $(NH_4)_2S$; the NPm-amino acids appeared as black spots on the dark background of the plate.

RESULTS AND DISCUSSION

This work deals with the chromatographic separation of DNPyr-amino acids and NPm-amino acids which can be extracted from the acid hydrolysate with organic solvents. Derivatives of arginine, cysteic acid and histidine, which are soluble in the aqueous layer, are easily separated even by conventional methods[3].

The R_F values of DNPyr- and of NPm-amino acids, in the solvent system used, are given in Tables I and II respectively.

TABLE I

EXPERIMENTAL R_F VALUES OF DNPyr-AMINO ACIDS

DNPyr-amino acids	*R_F values* (× 100) in solvent systems*					
	A	*B*	*C*	*D*	*E*	*F*
DNPyr-L-Asp	14	41	2	15	9	47
DNPyr-DL-Glu	25	42	—	25	19	57
DNPyr-DL-Ala	68	87	22	49	97	70
DNPyr-DL-Phe	79	93	30	51	103	72
DNPyr-Gly	45	78	14	30	48	65
DNPyr-DL-Ileu	91	92	38	82	130	79
DNPyr-DL-Leu	95	93	36	87	140	83
Di-DNPyr-L-Lys	86	94	41	47	74	84
DNPyr-DL-Ser	12	73	10	20	9	64
DNPyr-L-Pro	84	81	19	67	97	74
DNPyr-DL-Thr	21	74	20	25	97	63
DNPyr-DL-Val	88	92	32	75	9	78
DNPyr-NH_2	100	100	100	100	100	100
DNPyr-OH	20	87	11	13	13	83

* R_F values measured relative to DNPyr-NH_2.

The R_F values were measured relative to the corresponding dinitropyridyl- and nitropyrimidyl-amine. This enables us to obtain a more accurate and constant reproducibility of the experimental data, thereby compensating some factors relative to the development conditions and to the activation of the layer that are known[12] to have a great influence on the migration of the substances.

TABLE II

EXPERIMENTAL R_F VALUES OF NPm-AMINO ACIDS

NPm-amino acids	*R_F values* (× 100) in solvent systems*					
	A	*B*	*C*	*D*	*E*	*F*
NPm-L-Asp	18	33	13	20	16	43
NPm-DL-Glu	7	42	1	3	20	52
NPm-DL-Ala	57	73	8	64	82	78
NPm-DL-Phe	80	81	20	79	102	85
NPm-Gly	29	69	5	31	50	60
NPm-DL-Ileu	103	87	24	126	122	88
NPm-DL-Leu	94	88	23	132	119	88
Di-NPm-L-Lys	66	85	22	38	54	83
NPm-DL-Ser	6	62	4	14	13	56
NPm-L-Pro	96	77	12	94	87	70
NPm-DL-Thr	16	64	7	73	73	62
NPm-DL-Val	92	83	17	107	109	87
NPm-NH_2	100	100	100	100	100	100
NPm-OH	43	80	12	17	—	71

* R_F values measured relative to NPm-NH_2.

Considerable attention has been paid to improving the resolution of our compounds by one-dimensional chromatography. The results have shown that, among the many solvent systems tested, those described are capable of almost perfect separation, in a very short time, of the DNPyr- and NPm-amino acids investigated.

A diagram illustrating the patterns of one-dimensional separation is shown in Figs. 1 and 2.

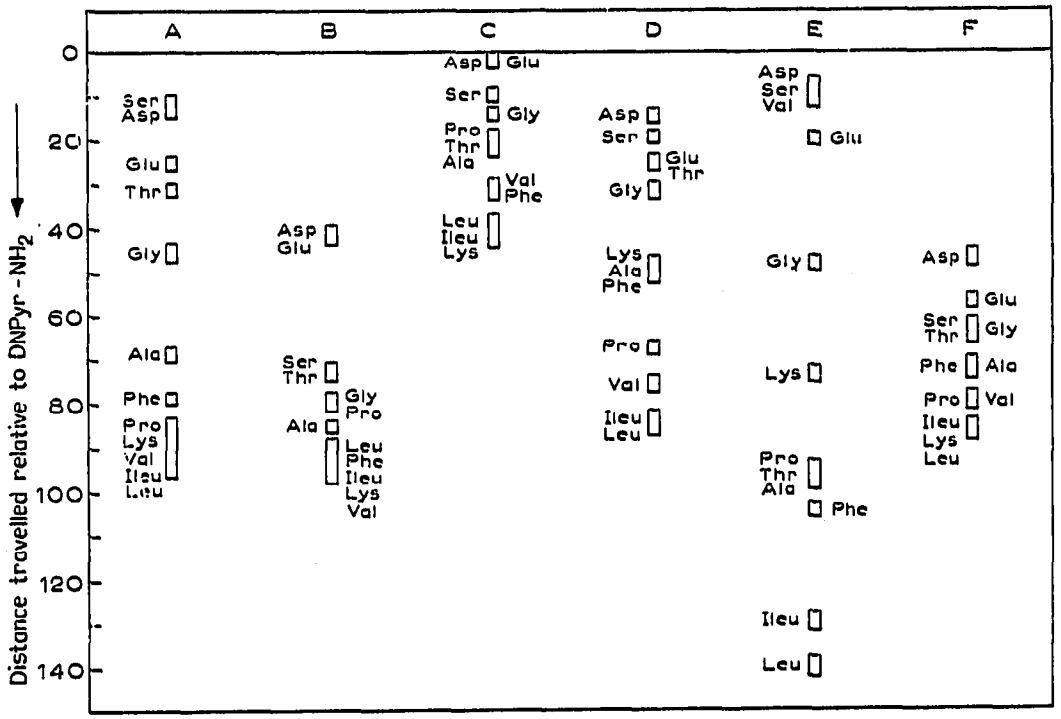

Fig. 1. Diagrammatic representation of one-dimensional separations of DNPyr-amino acids.

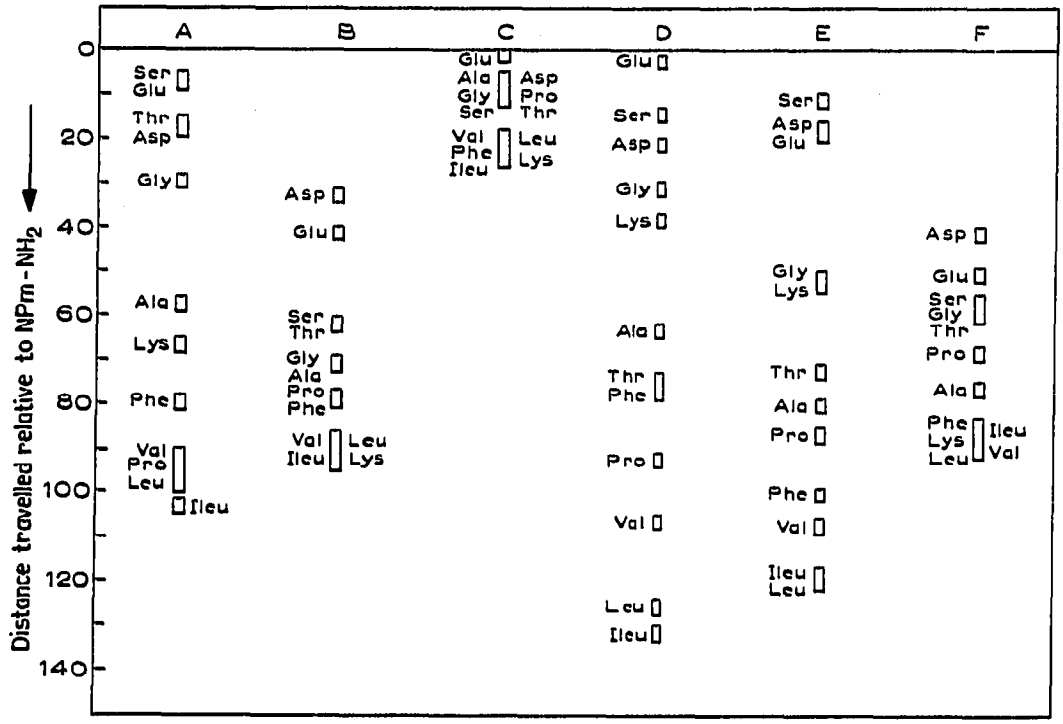

Fig. 2. Diagrammatic representation of one-dimensional separations of NPm-amino acids.

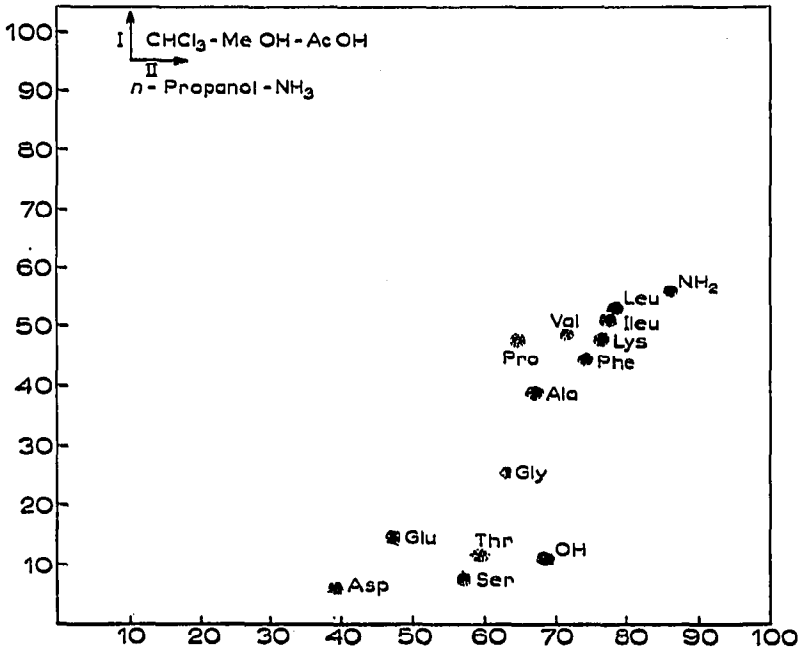

Fig. 3. Two-dimensional chromatography of a synthetic mixture of DNPyr-amino acids. Original development 10 × 10 cm.

By this method it is possible to separate the derivatives of aspartic and glutamic acid and to obtain satisfactory resolution of the derivatives of leucine and isoleucine; the latter derivatives are, however, better resolved by two-dimensional continuous chromatography[13–15].

In Figs. 3 and 4 two-dimensional separations of mixtures containing all the DNPyr- and NPm-amino acids are given.

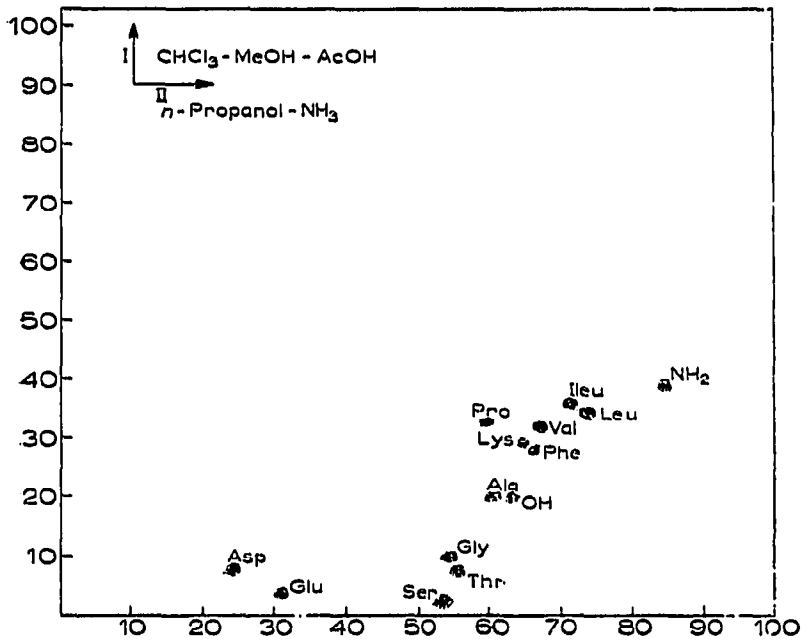

Fig. 4. Two-dimensional chromatography of a synthetic mixture of NPm-amino acids. original development 10 × 10 cm.

The preferred order of developing systems was solvent A followed by solvent B for both types of compounds. As can be seen, a good separation has been obtained both for the components of low mobility and for those of high mobility, keeping, at the same time, the distance between these two groups within reasonable limits.

SUMMARY

The separation of dinitropyridyl-amino acids and of nitropyrimidyl-amino acids by thin-layer chromatography on silica gel is described.

The advantages of this method as compared with conventional procedures are discussed.

REFERENCES

1 F. SANGER, *Biochem. J.*, 39 (1945) 507.
2 A. SIGNOR, E. SCOFFONE, L. BIONDI AND S. BEZZI, *Gazz. Chim. Ital.*, 93 (1963) 65.
3 A. SIGNOR, L. BIONDI, M. TERBOJEVICH AND P. PAJETTA, *Gazz. Chim. Ital.*, 94 (1964) 619.
4 A. SIGNOR AND L. BIONDI, *Ric. Sci.*, 34, II-A (1964) 165.
5 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, 1962.
6 K. RANDERATH, *Thin-layer Chromatography*, Verlag Chemie, Weinheim/Bergstr., and Academic Press, New York, 1963.

7 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, *Experientia*, 17 (1961) 145.
8 G. BISERTE AND R. OSTEUX, *Bull. Soc. Chim. Biol.*, 33 (1951) 50.
9 E. STAHL, *Arch. Pharm.*, 292/64 (1959) 411.
10 E. STAHL, *Pharm. Rdsch.*, No. 2 (1959) 1.
11 E. DEMOLE, *J. Chromatog.*, 1 (1958) 30.
12 M. BRENNER, A. NIEDERWIESER, G. PATAKI AND A. R. FAHMY, *Experientia*, 18 (1962) 101.
13 M. MOTTIER, *Mitt. Gebiete Lebensm. Hyg.*, 49 (1958) 454.
14 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
15 R. D. BENNETT AND E. HEFTMANN, *J. Chromatog.*, 12 (1963) 245.