POLYAMIDE LAYER CHROMATOGRAPHY OF DNP-AMINO ACIDS*

KUNG-TSUNG WANG, JOYCE M. K. HUANG AND IRIS S. Y. WANG
Department of Chemistry, National Taiwan University, Taipei, Taiwan (China)
(Received October 29th, 1965)

INTRODUCTION

Since SANGER's publication[2], fluorodinitrobenzene has been widely used as a reagent to determine N-terminal groups in peptides and proteins. Numerous techniques for the identification and estimation of dinitrophenylamino acids (DNP-amino acids) have been reviewed[3,4], including chromatography on paper, on columns of silica gel, kieselguhr, silicic acid–celite and polyamide. Recent advances have been made in partition chromatography on Hyflo Super-Cel columns[5,6], adsorption chromatography on a silica gel–celite column[7], chromatography on glass paper impregnated with silica gel[8], paper chromatography[9,10], paper electrophoresis[11] and gas chromatography of DNP-amino acid esters[12]. Thin-layer chromatographic separations of DNP-amino acids on silica gel G thin layers have been carried out by BRENNER[13–17] *et. al.* under strict conditions. In addition, their method needs a complicated B-N chamber for continuous development. The purpose of this paper is to describe a rapid chromatographic method for the analysis of DNP-amino acids on polyamide layers.

Polyamide is suitable for the chromatography of many organic compounds[18]; ENDRES[18], HILLE[19,20] and SCHWERDTFEGER[21] have had varied success in isolating DNP-amino acids on polyamide columns. Although the unique character of polyamide as a chromatographic adsorbent in columns is widely recognized, chromatography on polyamide thin layers is rare. In 1961 DAVIDEK AND DAVIDKOVA[22] prepared polyamide thin layers by spreading polyamide powder without a binder on glass plates and used it to separate antioxidants and flavonoids (see ref. 18 for review). Recent developments include the separation of quinones[23], plant tanning extractives[24] and antioxidants[25]. The chief difficulty seems to be in the preparation of durable layers.

However, WANG allowed a polyamide solution in formic acid to evaporate on a glass plate and obtained a fairly durable layer which was used to separate phenols[26]. Recent developments in the preparation of polyamide layers in our laboratory enable it to be used to separate sulfonamides[27], chloramphenicol[28] and estrogens[29]. Its application to the separation of DNP-amino acids showed many advantages over previous methods, *e.g.*, more rapid analysis, less broadening of the spots and easier handling.

We found ten solvents systems which were excellent for the separation of DNP-amino acids on polyamide layers. They can be classified into three groups

* Some of the preliminary experiments were submitted to *Nature* (see ref. 1).

according to the difference of R_F values. It is possible to combine these systems to get several combinations of solvent systems for two-dimensional chromatography.

Only seventeen DNP-amino acids are available to us at present. Further data will be reported when other DNP-amino acids have been obtained.

EXPERIMENTAL

Preparation of polyamide layer

Twenty grams of polyamide (CM 1007s, Toyo Rayon Co.) was dissolved in 100 ml of 75 % formic acid to give a homogeneous viscous solution. Fifteen ml of this solution was spread evenly on each of four glass plates (15 × 15 cm) kept horizontal in the bottom of a stainless steel chromatographic cabinet (50 × 50 × 45 cm) which was saturated with water vapor. The plates were kept in the cabinet for two days to allow slow evaporation of formic acid at 26° or at 29°. The layers were then dried in an oven at 100° for 15 min to eliminate the last traces of formic acid. The dried plates can be stored for a long period without any noticeable change. The solvent travels faster on plates prepared thus rather than by the former method[26].

Both Toyo Rayon Co.'s ε-polycaprolactam resin CM 1011 and CM 1007s have been tested. Resin CM 1011 gives a denser layer which gives nearly circular spots but requires a longer time for development which makes the spots more diffuse.

The evaporation temperature during the preparation of the polyamide layer has a profound effect on the properties of the resulting layer. A higher temperature makes the layer coarser and shortens the time for development, but some solvent systems give elongated spots on layers prepared at high temperature.

For one-dimensional chromatography, two kinds of layers were used; a CM 1007s layer made at 26° and CM 1007s layer made at 29°. All of the two-dimensional chromatograms were run on layers of CM 1007s prepared at 26°.

Reagents

All of the 19 DNP derivatives available to us were kindly supplied by Prof. T.B. Lo.

Solvents

The solvents were purified by general methods to meet the chromatographic requirements.

Chromatography

The ascending method was used. For one-dimensional chromatography, DNP-amino acids (1 μg in 1 μl of methanol) were spotted 1.5 cm from the bottom edge and the solvent was allowed to ascend a distance of 10 cm from the origin. For two-dimensional chromatography, a mixture of the 19 samples (0.5 μg each in 10 μl of methanol) was spotted on one corner 2 cm from the edges. In all development processes, there was no need to saturate the polyamide layer in advance but the chamber should be lined with filter paper saturated in solvent. Because DNP-amino acids are photosensitive, the development was run in the dark. For two-dimensional chromatography, it was necessary to carry out intermediate drying under a current of hot air for 15 min.

Detection

DNP-amino acids are visible in transmitted daylight and give dark spots in transmitted ultraviolet light. Ultraviolet contact photography has a much higher sensitivity and is the most convenient way of locating the spots and filing the results. The sensitive side of photographic paper was laid on the polyamide layer and long-wave ultraviolet light was allowed to pass through the layer on to the photographic paper for 30 sec (one-dimensional chromatogram) or 18 sec (two-dimensional chromatogram). The print was developed in the usual manner to make a chromatogram of white spots on black background. This print was used as the negative to make a chromatogram of dark spots on white background.

RESULTS AND DISCUSSION

One-dimensional chromatography

A number of solvent systems suitable for the separation of DNP-amino acids on polyamide layer are summarized in Table I together with the time required for development. They are classified into three groups according to the differences of R_F values. Table II summarizes the R_F values of DNP-amino acids in the above solvent systems.

TABLE I

SOLVENT SYSTEMS FOR DNP-AMINO ACIDS ON POLYAMIDE LAYERS

Symbol	*Group*	*Components*	*v/v*	*Time required for development (min)*	
				CM 1007s (29°)	*CM 1007s (26°)*
I	A	Benzene–glacial acetic acid	80:20	30	60
II	A	Carbon tetrachloride–glacial acetic acid	80:20	40	90
III	A	*n*-Butyl acetate–glacial acetic acid	90:10	30	60
IV	A	Diethyl ether–glacial acetic acid	90:10	15*	60
V	A	Methyl ethyl ketone–chloroform–glacial acetic acid	10:80:10	30*	60
VI	B	Glacial acetic acid–water	50:50	60	200
VII	B	90% Formic acid–water	50:50	30	60
VIII	C	*n*-Butanol–glacial acetic acid	90:10	80	200
IX	C	Dimethylformamide–glacial acetic acid–95% ethanol–water	5:10:20:20	80	180
X	C	Dimethylformamide–sulfamic acid–95% ethanol–water	5:0.5:20:20 (ml:g:ml:ml)	80	150

* Elongated spots.

Solvent IX is similar to that used in polyamide column chromatography of DNP-amino acids[18,30,31]. We found the latter equally satisfactory for polyamide layer chromatography. The only drawback was the slow rate of ascent, so we changed the solvent ratio to shorten the development time.

Solvents I–IX contain 10–20% acetic acid or formic acid, otherwise excessive

TABLE II

R_F VALUES OF DNP-AMINO ACIDS ON POLYAMIDE LAYERS

No.	DNP-derivatives	Abbreviation	Solvent system									
			I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
1	α-DNP-L-arginine	α-Arg	0.03	0.01	0.04	0.01	0.07	0.93	0.90	0.47	0.95	0.87
2	DNP-L-aspartic acid	Asp	0.07	0.03	0.11	0.10	0.05	0.50	0.55	0.12	0.30	0.48
3	DNP-DL-serine	Ser	0.09	0.03	0.21	0.17	0.10	0.56	0.61	0.20	0.44	0.54
4	ε-DNP-lysine hydrochloride	ε-Lys	0.11	0.03	0.13	0.02	0.22	0.94	0.90	0.62	0.96	0.89
5	DNP-L-glutamic acid	Glu	0.14	0.06	0.18	0.17	0.11	0.52	0.56	0.18	0.37	0.48
6	DNP-threonine	Thr	0.14	0.06	0.32	0.29	0.16	0.60	0.65	0.27	0.51	0.60
7	Bis-DNP-lysine	Bis-Lys	0.32	0.07	0.53	0.16	0.50	0.23	0.14	0.15	0.16	0.11
8	DNP-tryptophan	Try	0.28	0.09	0.47	0.39	0.31	0.24	0.12	0.23	0.30	0.26
9	DNP-glycine	Gly	0.27	0.10	0.39	0.34	0.27	0.47	0.48	0.21	0.35	0.36
10	Bis-DNP-DL-tyrosine	Bis-Tyr	0.43	0.11	0.58	0.28	0.50	0.24	0.12	0.16	0.20	0.13
11	DNP-alanine	Ala	0.44	0.22	0.55	0.63	0.46	0.52	0.49	0.33	0.42	0.48
12	2,4-Dinitroaniline	NH_2	0.48	0.23	0.75	0.91	0.74	0.59	0.49	0.64	0.65	0.49
13	DNP-L-proline	Pro	0.52	0.27	0.61	0.74	0.57	0.59	0.50	0.46	0.59	0.60
14	DNP-phenylalanine	Phe	0.52	0.31	0.56	0.70	0.54	0.37	0.23	0.33	0.38	0.39
15	DNP-DL-methionine	Met	0.52	0.31	0.55	0.64	0.54	0.47	0.41	0.32	0.39	0.46
16	DNP-DL-valine	Val	0.58	0.40	0.68	0.86	0.63	0.50	0.38	0.53	0.51	0.50
17	DNP-DL-leucine	Leu	0.64	0.49	0.71	0.87	0.66	0.47	0.32	0.53	0.48	0.48
18	DNP-isoleucine	Ileu	0.65	0.49	0.71	0.87	0.67	0.47	0.31	0.56	0.51	0.48
19	2,4-Dinitrophenol	OH	0.79	0.65	0.69	0.73	0.71	0.63	0.60	0.25	0.47	0.44

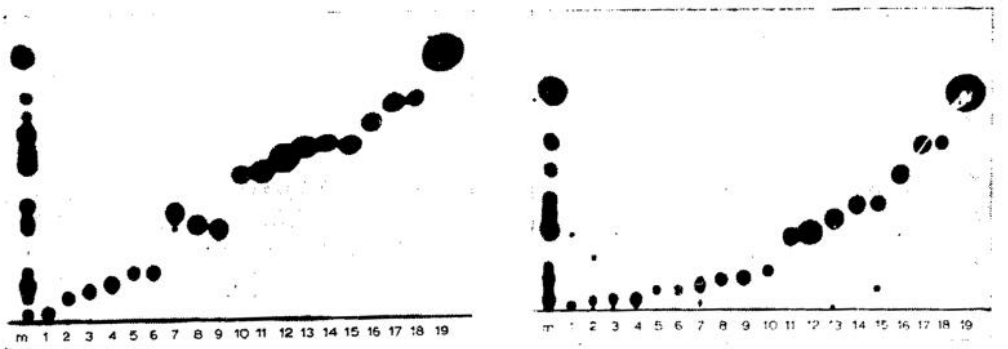

Fig. 1. One-dimensional chromatogram. Solvent: I, 60 min, 10 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 1 μg in 1 μl methanol; numbers: *cf.* Table II.

Fig. 2. One-dimensional chromatogram. Solvent: II, 90 min, 10 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 1 μg in 1 μl methanol; numbers: *cf.* Table II.

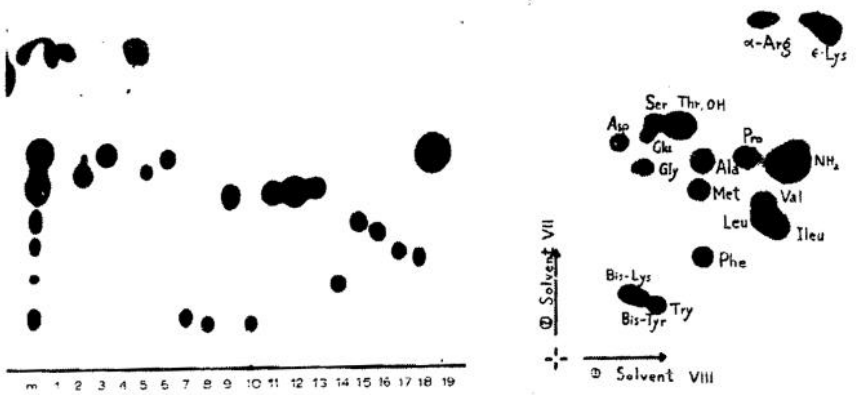

Fig. 3. One-dimensional chromatogram. Solvent: VII, 75 min, 10.5 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 1 μg in 1 μl methanol; numbers: *cf.* Table II.

Fig. 4. Two-dimensional chromatogram. Solvent: 1st dimension: VIII, 200 min, 10 cm; 2nd dimension: VII, 60 min, 11 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 0.5 μg each DNP derivative in a total of 10 μl methanol; symbols: *cf.* Table II.

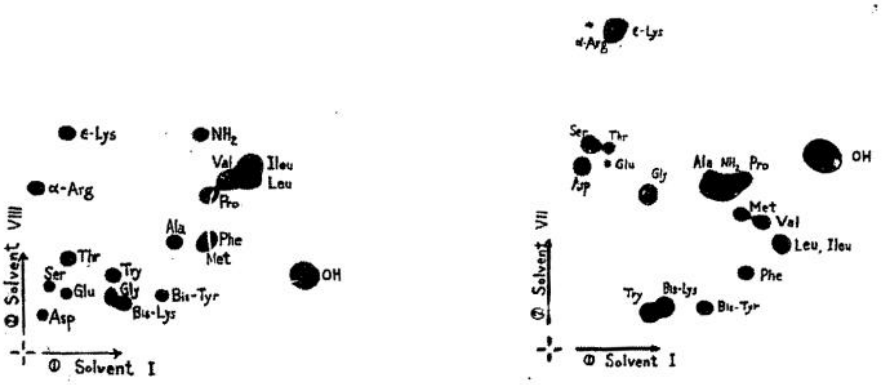

Fig. 5. Two-dimensional chromatogram. Solvent: 1st dimension: I, 60 min, 10.5 cm; 2nd dimension: VIII, 200 min, 9.5 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 0.5 μg each DNP derivative in a total of 10 μl methanol; symbols: *cf.* Table II.

Fig. 6. Two-dimensional chromatogram. Solvent: 1st dimension: I, 60 min, 10 cm; 2nd dimension: VII, 60 min, 10 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 0.5 μg each DNP derivative in a total of 10 μl methanol; symbols: *cf.* Table II.

tailing results. Higher concentration of acid gives circular spots and better distribution of R_F values but at the same time reduces the speed of ascent. In solvent X we used sulfamic acid instead of the acetic acid as in solvent IX and found that the order of R_F values was the same as in solvent IX.

We ran one-dimensional chromatograms on CM 1007s (26°) layers and CM 1007s (29°) layers. Both give similar R_F values except in the solvent systems IV and V, which give elongated spots on the CM 1007s (29°) layer but give circular spots on the CM 1007s (26°) layer.

We have tried using a basic solvent system, for example pyridine–benzene, but it did not give a good chromatogram on either CM 1007s (29°) or CM 1007s (26°) layers.

The notorious difficulty of separating leucine from isoleucine was overcome with a butanol–acetic acid (90:10) system. All of the other nine solvent systems failed to differentiate these two structurally similar compounds.

Figs. 1–3 show typical chromatograms of 19 DNP derivatives in solvent systems I, II and VII. Some samples give more than one spot after development due to the impurities present in the original sample.

In order to show the difference between the solvent systems, we numbered the DNP derivatives according to their R_F values in solvent system II.

Two-dimensional chromatography

Fig. 4 shows the separation of a mixture containing 0.5 μg of each DNP derivative using a combination of solvent systems VIII and VII. Of all the possible combinations of the above ten solvent systems, this combination gives the best distribution of spots on the chromatogram. DNP-valine, DNP-leucine and DNP-isoleucine can be separated satisfactorily. Bis-DNP-lysine and bis-DNP-tyrosine overlap to an appreciable extent. DNP-threonine and 2,4-dinitrophenol merge into one spot. The overall time required is about 2 h when the chromatogram is run on a CM 1007s (29°) layer or about 4 h when it is run on a CM 1007s (26°) layer.

Fig. 5 shows the two-dimensional chromatogram in solvent systems I and VIII. The separations of the bis-DNP-lysine–bis-DNP-tyrosine pair and the DNP-threonine–2,4-dinitrophenol pair have been achieved. But DNP-phenylalanine and DNP-methionine overlap to form an elliptical spot. This problem can be solved by running simultaneously another chromatogram in solvent systems I and VII (see Fig. 6). The overall time required is about 2 h for a CM 1007s (29°) layer or 4 h for a CM 1007s (26°) layer for the combination I–VIII. For the combination I–VII, the overall time required is about 1 h and 2 h for CM 1007s (29°) and CM 1007s (26°) layers, respectively.

ACKNOWLEDGEMENTS

We wish to thank Dr.'s Y. T. LIN and C. H. YANG for their generous encouragement and discussion. We are also indebted to Dr. T. B. Lo for his kindness in supplying the DNP-amino acids and helpful discussion. We should like to express our sincere gratitude to Dr. I. M. CHENG who provided us with the photographic equipment. Thanks are also due to Miss C. M. CHEN for technical assistance.

SUMMARY

The separation of seventeen DNP derivatives of amino acids, 2,4-dinitroaniline and 2,4-dinitrophenol by polyamide layer chromatography is investigated. Convenient procedures for the preparation of durable polyamide layers are described. A table of R_F values in ten solvent systems and ultraviolet contact photographs of three one-dimensional chromatograms and three two-dimensional chromatograms are given. The present method is better than paper or thin-layer chromatography in speed and efficiency.

REFERENCES

1 K.-T. WANG AND J. M. K. HUANG, *Nature*, 208 (1965) 281.
2 F. SANGER, *Biochem. J.*, 39 (1945) 507.
3 J. LEGGETT BAILEY, *Techniques in Protein Chemistry*, Elsevier, Amsterdam, 1962, pp. 143–169.
4 E. HEFTMANN, *Anal. Chem.*, 36 (1964) 14R.
5 A. V. PORTUGAL, R. GREEN AND T. M. SUTHERLAND, *J. Chromatog.*, 12 (1963) 183.
6 N. A. MATHESON, *J. Biochem.*, 94 (1964) 513.
7 G. MATSUDA, T. MAITA, H. TAKEI, T. MAEKAWA, T. SHIKAYA, K. MAEDA AND M. FUJIWARA, *Acta Med. Nagasakiensia*, 8 (1963) 28.
8 H. C. DESSAUER, J. G. HAMILTON AND J. K. CALVET, *J. Chromatog.*, 18 (1965) 192.
9 R. L. MUNIER AND G. SARRAZIN, *Bull. Soc. Chim. France*, (1963) 2939.
10 G. BISERTE AND Y. MOSCHETTO, *Bull. Soc. Chim. France*, (1963) 2932.
11 R. L. MUNIER AND G. SARRAZIN, *J. Chromatog.*, 12 (1963) 542.
12 J. J. PISANO, W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochem. Biophys. Res. Commun.*, 7 (1962) 82.
13 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 145.
14 D. WALZ, A. R. FAHMY, G. PATAKI, A. NIEDERWIESER AND M. BRENNER, *Experientia*, 19 (1963) 213.
15 G. PATAKI AND M. KELLER, *Helv. Chim. Acta*, 47 (1964) 787.
16 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, in A. T. JAMES AND L. J. MORRIS (Editors), *New Biochemical Separations*, Van Nostrand, London, 1964, pp. 141–156.
17 F. DRAWERT, O. BACHMANN AND K. H. REUTHER, *J. Chromatog.*, 9 (1962) 376.
18 H. ENDRES AND H. HORMANN, *Angew. Chem.*, 2 (1963) 254.
19 H. STEULE AND E. HILLE, *Biochem. Z.*, 331 (1959) 220.
20 E. HILLE, *Biochem. Z.*, 333 (1960) 269.
21 E. SCHWERDTFEGER, *Z. Tierphysiol., Tierernaehr. Futtermittelk.*, 16 (1961) 262.
22 J. DAVIDEK AND E. DAVIDKOVA, *Pharmazie*, 16 (1961) 352.
23 W. GRAU AND H. ENDRES, *J. Chromatog.*, 17 (1965) 587.
24 P. SPADLER AND H. ENDRES, *J. Chromatog.*, 17 (1965) 591.
25 J. W. COPIUS-PEEREBOOM, *Nature*, 204 (1964) 748.
26 K.-T. WANG, *J. Chinese Chem. Soc.*, 8 (1961) 241;
Y.-T. LIN, K.-T. WANG AND Y.-S. LIN, *ibid.*, 9 (1962) 68;
Y.-T. LIN AND K.-T. WANG, *ibid.*, 10 (1963) 146.
27 Y.-T. LIN, K.-T. WANG AND T.-I. YANG, *J. Chromatog.*, 20 (1965) 610.
28 Y.-T. LIN, K.-T. WANG AND T.-I. YANG, *J. Chromatog.*, 21 (1966) 158.
29 Y.-T. TUNG AND K.-T. WANG, *Nature*, 208 (1965) 581.
30 H. HORMANN AND H. V. PORTATIUS, *Z. Physiol. Chem.*, 315 (1959) 141.
31 W. GRASSMANN, H. HORMANN AND H. V. PORTATIUS, *Z. Physiol. Chem.*, 321 (1960) 120.