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DETECTION AND QUANTITATIVE MICRO-DETERMINATION OF NITRO-2-PYRIDYL AMINO ACIDS BY THIN-LAYER CHROMATOGRAPHY ON PRECOATED SHEETS

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SUMMARY

The separation of 3-nitro-2-pyridyl- and of 5-nitro-2-pyridyl-amino acids on Eastman Kodak Chromagram sheets is described, together with their detection and their quantitative spectrophotometric microanalysis. The advantages of this method over the conventional procedure are discussed.

Kinetic studies on the acid catalyzed hydrolysis of nitro-2-pyridyl dipeptides have demonstrated that the rate of hydrolysis of the peptide linkage is proportional to the concentration of the conjugate acid of the heterocyclic nitrogen; that is, the catalysis is brought about by the protonated pyridine species. The acid concentration necessary to form appreciable quantities of the kinetically important protonated intermediate is related to the basicity of the pyridyl nitrogen, and the hydrolysis of the peptide bond in these systems can be performed easily at room temperature and in dilute hydrochloric acid[1,2]. For these reasons a new approach for the determination of the N-terminal amino acids in peptides and proteins has been developed in our laboratory using as reagents 2-fluoro-3-nitropyridine and 2-fluoro-5-nitropyridine[3]. The stability of nitro-2-pyridyl amino acids under the proper hydrolytic conditions makes these compounds extremely useful for this purpose. The most important features of this new procedure are the preferential cleavage of the first peptide bond which permits extremely mild hydrolytic conditions and the facile spectrophotometric estimation of the derivatives so formed. Before one can take advantage of these desirable features a rapid and quantitative analytical method for the direct estimation of these compounds must be found. In this connection, separation by paper chromatography is not always satisfactory[3] for all the derivatives, and thin-layer chromatography with silica gel on the usual glass plates[4,5], which permits adequate separation, does not allow practical quantitative recovery of the products. Accordingly new and more satisfactory chromatographic procedures were sought.

The purpose of the present investigation was to establish accurate and reproducible chromatographic techniques for the analysis of the majority of the amino

acids resulting from the acid hydrolysis of nitropyridylated peptides and proteins. Bearing in mind the advantages of thin-layer chromatography, which offers a combination of high speed, sensitivity and powerful resolution, we have tried to improve the analytical procedure by using a thin-layer chromatography technique on Eastman precoated sheets[6,7]. In this way it was possible to use minimal quantities of sample, to recover easily the separated components by cutting out the zone of interest and, after elution, to perform the spectrophotometric determination of the derivatives. Various solvent systems were investigated: the findings are reported below.

EXPERIMENTAL

Materials

Eastman Chromagram Sheets (K 301 V). These are precoated sheets of silica gel bonded with polyvinyl alcohol to a solvent-inert support of polyethylene terephthalate. (Thickness of adsorbent layer 100–120 μ.)

Chromatography tanks. Desaga GmbH, Heidelberg.

Micropipettes. A. E. Pedersen, Denmark.

Solvents. Erba, Merck, specially prepared for chromatography.

Nitro-2-pyridylamino acids. These compounds were synthetized by coupling Fluka CHR amino acids with 2-fluoro-3-nitropyridine or 2-fluoro-5-nitropyridine. The experimental conditions used and analytical data have been described already[2].

Development

Eluent mixtures were prepared by mixing together the appropriate volumes of the components. All eluents were allowed to come to the temperature at which the chromatograms were to be eluted. The following eluents were used:

(A) Toluene–pyridine–ethylene chlorohydrin–0.8 *N* ammonium hydroxide (100:30:60:60)[9]

(B) Benzene–pyridine–acetic acid (80:20:2)

(C) Chloroform–*tert*.-amyl alcohol–glacial acetic acid (70:3:3)

(D) Chloroform–formic acid (100:5)

(E) Chloroform–methanol–acetic acid (95:5:1)

(F) *n*-Propanol–25% ammonium hydroxide (70:30)

Procedure

The sheets were activated by heating in an oven at 100° for 30 min. The samples were dissolved in anhydrous methanol and 1 μl quantities were spotted 2 cm from the lower edge and at least 2 cm from the sides of the activated sheet. The concentration of the solution spotted was varied between 0.1% and 5% w/v for the qualitative and quantitative determination respectively. The good adhesion of the adsorbent layer to the support makes it possible to use micropipettes which touch the adsorptive surface without damaging the coating. The sheets were placed in the chromatography tank for one hour and then eluted by the ascending technique at a constant temperature of 21 $\pm$ 0.5°. The length of run was standardized at 10 $\pm$ 0.5 cm; the time taken for the solvent front to travel this distance is, for each system, reported in Tables I and II.

TABLE I

EXPERIMENTAL R_F VALUES OF 3-NITRO-2-PYRIDYL AMINO ACIDS

3-Nitro-2-pyridyl	*R_F values[a] (× 100) in solvent systems (minutes required for development in parentheses)*					
	A (120)	*B (90)*	*C (90)*	*D (80)*	*E (80)*	*F (210)*
-alanine	27	76	79	72	73	83
-aspartic acid[b]	1	14	14	8	3	26
-cysteine	115	50	69	83	64	96
-glycine	17	49	58	35	44	77
-glutamic acid	1	25	38	15	10	28
-isoleucine	98	101	104	99	102	100
-leucine	100	100	100	100	100	100
-α-ε-lysine	131	85	94	47	97	99
-phenylalanine	102	85	94	95	89	98
-proline	27	96	86	76	85	73
-serine	9	27	17	6	11	64
-threonine	15	32	25	10	13	73
-valine	64	101	93	95	96	93

[a] R_F values measured relative to 3-nitro-2-pyridyl-leucine.
[b] Obtained as potassium salt.

Detection and quantitative estimation

All the compounds under investigation are coloured and appear on the chromatogram as yellow spots. The best recovery of the amounts of the separated components was performed by cutting out the spots and allowing each of them to stand in a glass tube for 15 min at room temperature with 3.0 ml of 0.1% (w/v) sodium bicarbonate solution; optical density was measured at 370 mμ for 5-nitro-2-Pyr derivatives and 420 mμ for 3-nitro-2-Pyr derivatives, employing microcells of 4 cm path-length with a Unicam model SP. 500 spectrophotometer.

TABLE II

EXPERIMENTAL R_F VALUES OF 5-NITRO-2-PYRIDYL AMINO ACIDS

5-Nitro-2-pyridyl	*R_F values[a] (× 100) in solvent systems (minutes required for development in parentheses)*					
	A (120)	*B (90)*	*C (90)*	*D (80)*	*E (80)*	*F (210)*
-alanine	39	50	58	38	47	83
-aspartic acid[b]	0	3	4	0	0	29
-arginine[b]	15	0	0	0	0	54
-cysteine	113	35	54	62	45	100
-glycine	25	23	32	12	21	76
-glutamic acid[b]	0	10	19	0	0	33
-isoleucine[b]	101	104	105	103	113	99
-leucine	100	100	100	100	100	100
-α-ε-lysine	96	23	46	4	37	104
-phenylalanine	106	71	88	92	85	99
-serine[b]	17	10	9	0	0	66
-threonine[b]	23	17	18	7	6	76
-valine[b]	66	88	90	81	95	95

[a] R_F values measured relative to 5-nitro-2-pyridyl-leucine.
[b] Obtained as potassium salt.

After subtracting the reading given by an equal area of unstained sheet, the quantitative assay of unknown amounts of nitro-2-pyridyl amino acids was obtained by reference to data obtained with known controls.

RESULTS AND DISCUSSION

Our work was performed in order to obtain the separation of each derivative by a monodimensional run, since on recovery, the losses are minimized. This method can be adapted for the quantitative determination of nitro-2-pyridyl amino acids, which can be extracted with organic solvents from acid hydrolysates of nitro-2-pyridyl proteins.

The R_F values of 3-nitro-2-pyridyl and 5-nitro-2-pyridyl amino acids are given in Tables I and II, respectively. No developing solvent could be found which would fully resolve every amino acid derivative. The results have shown that by suitable use of the solvent systems employed, a nearly complete separation of our compounds is obtained.

The R_F values measured are referred to nitro-2-pyridyl-leucine which is run simultaneously. This enables us to obtain 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[6,10] to have a great influence on the migration of the substances.

The rate of development of the type of sheet employed by us (K 301 V) is about half the rate of the plates usually employed in thin-layer chromatography on silica gel, owing to the enhanced thinness of the layer. However, the reduced thickness of the layer allows extremely small amounts of compound to be employed. Figs. 1 and 2

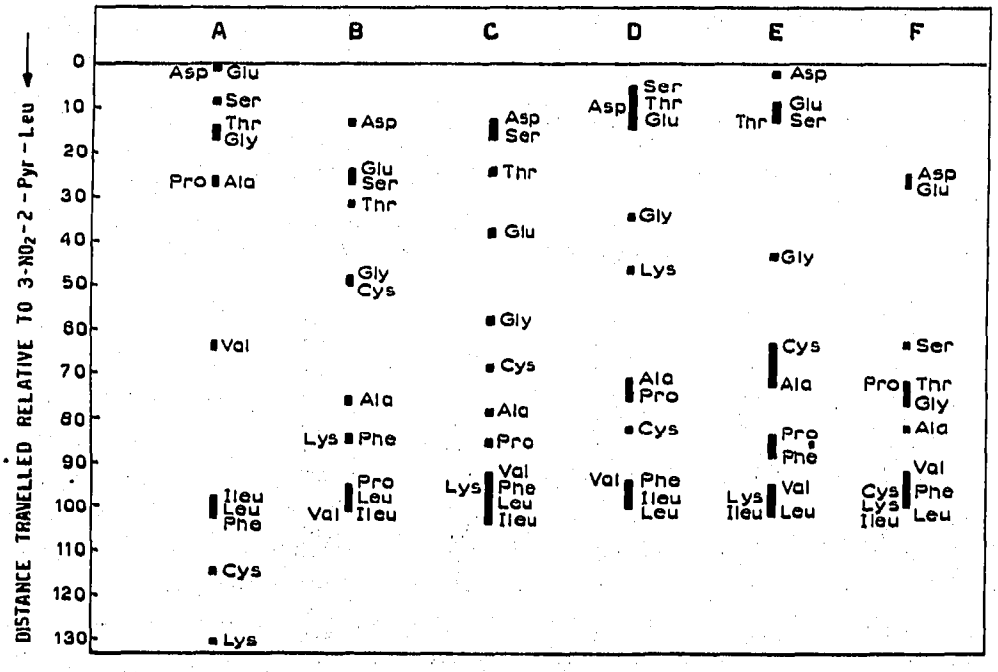

Fig. 1. Diagrammatic representation of one-dimensional separations of 3-nitro-2-pyridyl amino acids.

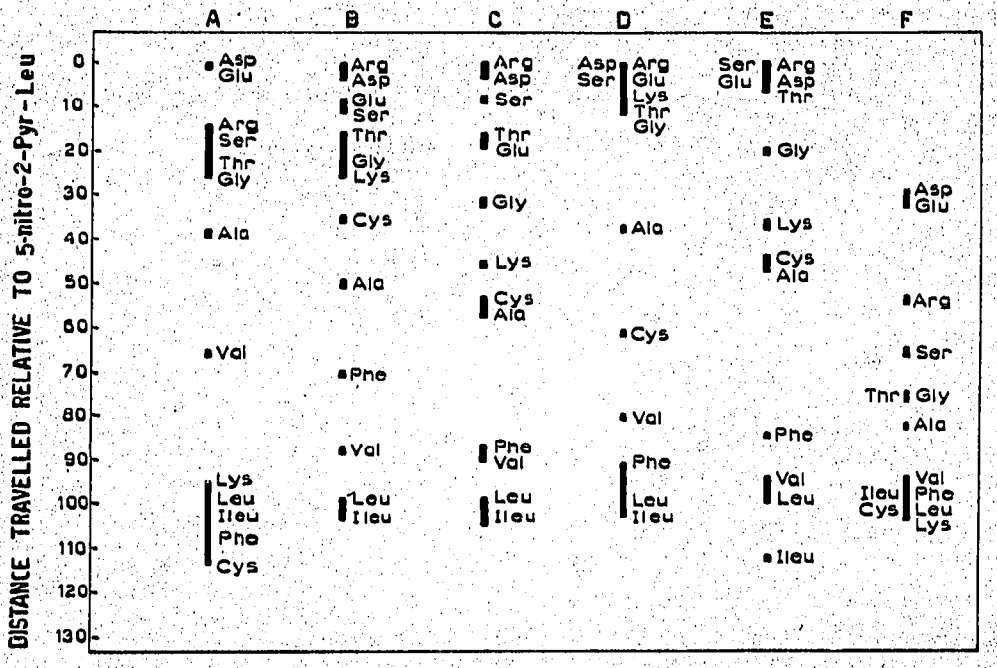

Fig. 2. Diagrammatic representation of one-dimensional separations of 5-nitro-2-pyridyl amino acids.

show a diagrammatic reproduction of actual separations obtained in representative experiments.

The solvent systems employed, apart from minor differences, show similar separating efficiencies for the various amino acid derivatives of both reagents. This made possible the normally difficult separation of both derivatives of aspartic acid from those of glutamic acid and those of serine from those of threonine.

One difficulty was encountered. While 5-nitro-2-pyridyl-leucine and 5-nitro-2-pyridyl-isoleucine could be separated, the corresponding 3-nitro-2-pyridyl derivatives could not.

In this last case as well as when a further control of the mixture is required, partially satisfactory qualitative separation and detection was achieved by hydrolyzing the derivatives to the free amino acids, and separating the latter. The hydrolysis was not complete even after 4–6 h of heating in 2 *N* NaOH at 100° in sealed tubes; the yields of the free amino acids varied from 10 to 60%.

The quantitative estimation carried out with known controls showed that the recovery for all derivatives is 90% ± 1%. This constant chromatographic loss permits one to evaluate the derivatives with high precision, provided that parallel runs of known and unknown amounts are performed and the calculations are made on the basis of the recovered known amounts.

This analytical procedure has been successfully employed in the determination of NH_2-terminal amino acids of insulin (Gly and Phe) by using either 2-fluoro-3-nitro-pyridine or 2-fluoro-5-nitro-pyridine as the reagent.

REFERENCES

1 A. SIGNOR AND E. BORDIGNON, *J. Org. Chem.*, 30 (1965) 3447.

2 A. SIGNOR, E. BORDIGNON AND G. VIDALI, *J. Org. Chem.*, 32 (1967) 1135.
3 A. SIGNOR, L. BIONDI, A. M. TAMBURRO AND E. BORDIGNON, *European J. Biochem.*, in press.
4 E. STAHL, *Dünnschicht-Chromatographie*, Springer, Berlin, 1962.
5 K. RANDERATH, *Thin-Layer Chromatography*, Verlag Chemie, Weinheim/Bergstr., and Academic Press, New York, 1963.
6 E. P. PRZYBYLOWICZ, W. J. STAUDENMAYER, E. S. PERRY, A. D. BAITSHOLTS AND T. N. TISCHER, *J. Chromatog.*, 20 (1965) 506.
7 T. C. CHU AND E. JU-HWA CHU, *J. Chromatog.*, 21 (1966) 46.
8 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, *Experientia*, 17 (1961) 145.
9 G. BISERTE AND R. OSTEUX, *Bull. Soc. Chim. Biol.*, 33 (1951) 50.
10 M. BRENNER, A. NIEDERWIESER, G. PATAKI AND A. R. FAHMY, *Experientia*, 18 (1962) 101.