IDENTIFICATION, SEPARATION, AND QUANTITATIVE ANALYSIS OF DERIVATIVES OF NUCLEOSIDES AND NUCLEOTIDES BY THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

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Thin-layer chromatography (TLC) on cellulose has been used increasingly in recent times for the analysis of the components of nucleic acids, because of its advantage over paper chromatography in speed (10-17 times greater), sensitivity, and separating power [1, 2]. A qualitative analysis by TLC requires 0.5-1.5 hr, and a quantitative analysis requires 25-26 hrs.

The TLC of nucleic acid derivatives has been used mainly for the separation of similar derivatives of different bases [2, 3] in a limited number of solvent systems. The separation of nucleosides and nucleotides in water [4], solutions of salts [2], butanol, acetic acid, and acetone [2], and the separation of bases in propanol HCl systems [3] have been reported. It has also been shown that derivatives of guanosine, diphosphates, and triphosphates are not sharply separated by TLC on cellulose [2].

We have used TLC for identification, separation (of mixtures), and quantitative analysis of 1) individual substances, 2) mixtures of various derivatives of bases of the same and different types, and 3) mixtures of guanosine mono- and polyphosphates.

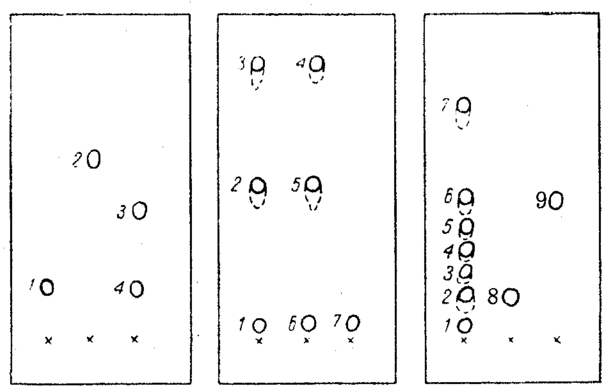

Fig. 1. Chromatograms of the separation of mixtures of uridine and guanosine derivatives:

a) Separation of uridine-2'(3')-phosphate and 2', 3'-cyclicphosphate in system 1: 1, 4) Uridine-(2')3'-phosphate (marker); 2) uridine (marker); 3) uridine-2', 3'-cyclicphosphate;

b) Separation of guanosine derivatives on cellulose impregnated with formamide in chloroform: 1) 2', 3'-O-Isopropylideneguanosine; 2) N^2-benzoyl-2', 3'-O-isopropylideneguanosine; 3) N^2, 5'-dibenzoyl-2', 3'-O-isopropylideneguanosine; 4) N^2, 5'-dibenzoyl-2', 3'-O-anisylideneguanosine; 5) N^2-benzoyl-2', 3'-O-anisylideneguanosine; 6) 2', 3'-O-anisylideneguanosine; 7) 2', 3'-O-anisylideneguanosine (marker).

c) Chromatogram of the reaction mixture from the synthesis of uridyl-(3'→5')-guanosine from 5'-O-acetyl-2'-O-tetrahydropyranyluridine-3'-phosphate and N^2-benzoyl-2', 3'-O-anisylideneguanosine in pyridine in the presence of p-toluenesulfonyl chloride (system 1): 1) Uridylyl-(3'→5')-guanosine; 2) uridine-(2')3'-phosphate; 3) uridine-2', 3'-cyclicphosphate; 4) unidentified; 5) guanosine; 6) N^2-benzoyl-2', 3'-O-anisylideneguanosine; 7) 2', 3'-O-anisylideneguanosine; 8) uridine-(2')3'-phosphate (marker); 9) guanosine (marker).

The R_f values are given in Table 1. It must be noted that in the majority of cases the R_f values and their variations are considerably greater in cellulose TLC than on paper, and depend more markedly on changes in the pH of the

Table 1

R_f Values of Nucleoside and Nucleotide Derivatives

Substance	System									
	1		2		3		4		8	
	a*	b**	a	b	a	b	a	b	a	b
Guanosine-5'-monophosphate	—	—	0.28—0.30	—	0.38	0.23[8]	—	—	0.54	0.31[7]
Morpholide of guanosine-5'-monophosphate	0.39	0.20[8]	0.45—0.48	—	0.58	0.51[8]	0.46	0.45[8]	—	—
Guanosine-5'-diphosphate	—	—	0.25	—	—	—	—	—	0.38	0.24[7]
Guanosine-5'-triphosphate	—	—	0.17—0.2	—	0.34	—	—	—	0.22—0.25	0.18[7]
Guanine	—	—	—	—	—	—	—	—	0.91	—
Morpholide of adenosine-5'-monophosphate	—	—	—	—	0.79	0.8 [8]	—	—	—	—
	1		5		6		7		8	
	a	b	a	b	a	b	a	b	a	b
Guanosine	0.41	0.37[9]	—	—	0.11	—	—	—	0.66—0.71	0.55[7]
2', 3'-O-Isopropylidene-guanosine	0.82	0.71[16]	—	—	—	—	0.09	0.08[15]	—	—
2', 3'-O-Anisylidene-guanosine	0.88	0.76	—	—	0.58	0.42[15]	0.08	0.1[15]	—	—
N^2, O^5-Dibenzoyl-2', 3'-O-isopropylidene-guanosine	0.44	—	—	—	—	—	0.92	0.89[15]	—	—

Substance	System	
	a	b
Adenosine	0.66	0.57[9]
2', 3'-O-Isopropylidene-adenosine	0.89	0.75[9]
Uridine-3', 2'-cyclic-phosphate	0.43	0.31[11]
Cytidine-3', 2'-cyclic-phosphate	0.58	0.30[12]
5'-O-Acetyluridine-2' 3'-cyclic phosphate	0.64	—
5'-O-Acetyluridine-3'-phosphate	0.32	0.1[13]
5'-O-Acetyl-2'-O-tetrahydrofuranyluridine-3'-phosphate	0.54	0.23[13]
2', 3'-O-Anisylidene-uridine	0.84	0.62[14]
2', 3'-O-Isopropylideneuridine	0.84	0.77[16]
2', 5'-O-Diacetyl-uridine-3'-phosphate	0.56	—
Uridylyl-(3'→5')-uridine	0.27	0.24[10]

Table 1 (continued)

N^2-Benzoyl-2', 3'-O-isopropylidene-guanosine	—	—	—	—	—	—	0.51	0.49[15]	—	—	Uridylyl-(3→5')-guanosine	0.09	0.08[15]
N^2, $O^{5'}$-Dibenzoyl-2', 3'-O-anisylidene-guanosine	0.42	—	—	—	—	—	0.91	0.89[15]	—	—	—	System 8	
												a	b
N^2-Benzoyl-2', 3'-O-anisylidene-guanosine	—	—	—	—	—	—	0.51	0.49[15]	—	—	Guanine	0.91	—
												System 9	
												a	b
Uridine	0.61	0.48[10]	0.66	0.47[7]	0.23	—	—	—	—	—	—		
Uridine-3'(2')-phosphate	0.17	0.12[10]	0.48	0.35	—	—	—	—	—	—	Uridine 2'(3')-phosphate	0.83	0.72[17]

* Thin-layer chromatography on cellulose

** Chromatography on paper.

Table 2

Quantitative Analysis of Nucleotide and Nucleoside Derivatives on a Thin Layer of Cellulose

No.	Analysis mixture	System	D_{260} of the solution deposited at the start	D_{260} of the eluate from the spot	Total D_{260} of the eluates of all the spots	Content of substance in the mixture, %: TLC	Content of substance in the mixture, %: Ion-exchange chromatography on DEAE-cellulose (HCO_3^-)
1	Guanine	8	3.100 3.140	0.040 0.048	3.010 2.290	1.3 2.1	—
	Guanosine			2.970 2.240		98.7 97.6	—
2	Guanosine			0.122 0.137		12.4 13.6	—
	Guanosine-5'-monophosphate	8	1.470 1.470	0.061 0.040	0.983 1.010	6.2 4.0	—
	Guanosine-5'-triphosphate			0.800 0.830		81.5 82.4	—
3	Morpholide of guanosine-5'-monophosphate			0.300 —		19.1 —	19.6
	Guanosine-5'-monophosphate	2	2.200	0.248 —	1.573	15.7 —	—
	Guanosine-5'-diphosphate			— —		— —	—
	Guanosine-5'-triphosphate			1.025 —		65.2 —	63.5
4	Morpholide of guanosine-5'-monophosphate	2	3.000	2.035 —	2.164	94.0 —	99.0
	Guanosine-5'-monophosphate			0.129 —		6.0 —	—
5	Guanosine-2', 3'-O-Isopropylidene-guanosine			0.185 0.245		10.9 13.9	—
		1	2.500		1.693 1.753		
				1.508 1.508		89.1 86.1	—
6	Uridine-2', 3'-cyclic-phosphate	1	$5.6 \cdot 10^{-2}$	5.15×10^{-2} μ mole (at λ 259 mμ)	$5.45 \cdot 10^{-2}$	94.5 —	—
	Uridine-3'-phosphate			0.3×10^{-2} μ mole (at λ 262 mμ)		5.5 —	—

solvent system, the accuracy of reproduction of the systems, the purity of the solvents, and the moisture content of the cellulose. In the separation of mixtures of nucleoside and nucleotide derivatives by TLC, we have employed various solvent systems used for paper chromatography, multiple and two-dimensional chromatography, and TLC on cellulose impregnated with solvents in the presence of markers.

For a sharp separation, in addition to the usual factors (amount of substance deposited, spot area, and the chromatography conditions), the quality of the cellulose powder is important: the method of hydrolysis, the care with which it is washed, and its moisture content. Cellulose powder prepared by the hydrolysis of filter paper with alcoholic hydrogen chloride separates a mixture of guanosine-5'-mono- and polyphosphates, a mixture of uracil and uridine, and a mixture of uridine and 2', 3'-O-alkylideneuridines better than a powder obtained by hydrolysis with hydrochloric acid. Data on the separation conditions are given in Table 2 and in Figs. 1-4.

Quantitative analysis using TLC was carried out for the following mixtures: uridine-3'(2')-phosphate and uridine-2', 3'-cyclicphosphate; guanine and guanosine; guanosine, guanosine-5'-monophosphate, guanosine-5'-triphosphate; the morpholide of guanosine-5'-monophosphate and guanosine-5'-monophosphate; 2', 3'-O-isopropylideneguanosine and guanosine; the morpholide of guanosine-5'-monophosphate and guanosine-5'-mono-, di-, and -triphosphates. When the results of the separation of the last mixture were compared with those of ion-exchange chromatography on DEAE-cellulose (HCO_3^-) in a linear gradient of triethylammonium bicarbonate, pH 7.5 [5], good agreement was found (cf. Table 2).

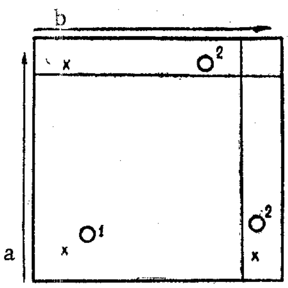

Fig. 2. Two-dimensional chromatogram. Direction a: isopropanol–ammonia–water system; Direction b: isopropanol–HCl system. 1) Urydilyl-(3'→5')-guanosine; 2) uridine-3' phosphate.

Our results show that TLC on cellulose can be used successfully for the identification, evaluation of purity, separation, and quantitative analysis of complex mixtures of nucleoside and nucleotide derivatives. In addition, for nucleoside and nucleotide chemistry, TLC on cellulose is a unique, rapid method for following reactions taking place in a complex and obscure manner or leading to unstable compounds.

Experimental

Solvents. Redistilled solvents of "pure for analysis" or "chemically pure" grades were used for preparing the systems. The aqueous ammonia was prepared by saturating distilled water with gaseous ammonia to sp. gr. 0.90. The $(NH_4)_2SO_4$ was of "chemically pure" grade and the CH_3COONH_4 of "pure" grade. The systems of solvents were as follows: 1) Isopropanol–ammonia–water (7:1:2); 2) n-propanol–ammonia–water (6:3:1); 3) isobutyric acid – 1 M ammonia – 0.1 M sodium ethylenediaminetetra-acetate (100:60:1.6); 4) ethanol – 1 M CH_3COONH_4, pH 7.5 (5:2). 5) isobutyric acid–ammonia–water (66:1:33); 6) butan-1-ol-water (85:15); 7) chloroform (for cellulose impregnated with formamide); 8) isobutyric acid–ammonia–water (57:4:39), pH 4.3; 9) isopropanol–hydrochloric acid (sp. gr. 1.19) (170:41)–water to 250 ml.

Cellulose. The cellulose powder was obtained by the hydrolysis of filter paper from the "Chistye Soli" mill by boiling it for 30 minutes with 6-10% methanolic HCl, according to a method described in [6], or in 18% hydrochloric acid.

The cellulose was washed successively with water until the reaction for chlorine ions was negative, with 3-5 volumes of 0.5% Trilon B, with 20 volumes of water, with five volumes of solvent system 1, with water until absorption at 260 mμ had disappeared, and with acetone, and was then dried in air. For the plates, cellulose with a grain size greater than 300 mesh was used and was additionally dried at 50°C and stored over $CaCl_2$. For qualitative TLC analysis, cellulose washed with hydrochloric acid, water, and acetone is suitable.

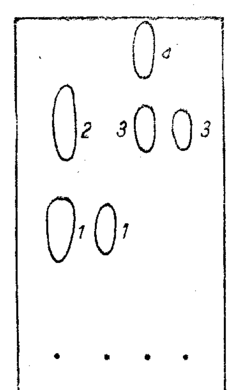

Fig. 3. Chromatogram of nucleoside derivatives in system 1): 1) Guanosine; 2) 2', 3'-O-isopropylideneguanosine; 3) adenosine; 4) 2', 3'-O-isopropylideneadenosine.

Preparation of the plates. About 7 g of cellulose was mixed with ~ 35 ml of acetone, and the pasty mass was deposited on five glass plates (20 × 10 cm), dried with shaking in a current of hot air, and used immediately. The plates can be stored for not more than a day over $CaCl_2$ without loss of their mechanical properties.

Performance of TLC. A sample of the substance (2-90 μg in 0.03-0.05 ml of solvent) was deposited on a plate with a capillary and was dried with hot or cold air. The distance from the bottom of the plate was 3 cm, from the edges 2 cm, and between the spots 2 cm. The plates were placed in the chamber at an angle of ~ 30° and were immersed in the solvent up to 1 cm below the deposited spots. After 40-90 min, the solvent had travelled 12-16 cm. After the plates had been dried, the positions of the spots were determined in UV light. (For R_f values, see Table 1).

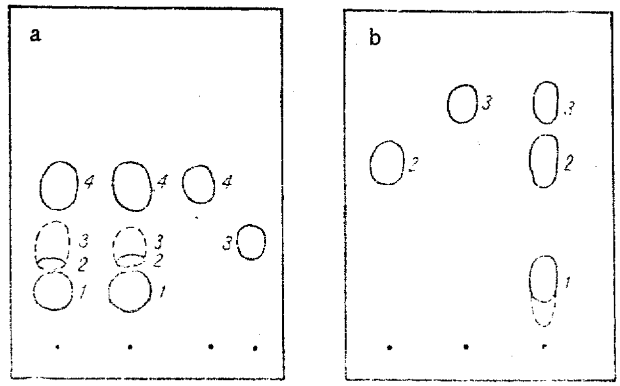

Fig. 4. Chromatograms of the separation of mixtures of derivatives of guanosine-5'-phosphates: a) Guanosine-5'-triphosphate, guanosine-5'-diphosphate, guanosine-5'-monophosphate, and the morpholide of guanosine-5'-monophosphate in system 2: 1) Guanosine-5'-triphosphate; 2) guanosine-5'-diphosphate; 3) guanosine-5'-monophosphate. b) Guanosine-5-triphosphate, guanosine-5-monophosphate and guanosine in system 8: 1) Guanosine-5'-triphosphate; 2) guanosine-5'-monophosphate; 3) guanosine.

Separation of mixtures of substances. The sample to be analyzed, containing not less than 2 mg of each substance, was deposited on a spot, with one or several known substances as markers, and chromatographed as described above. If the separation was not sharp, the plate was dried and rechromatographed in the same system. An impregnated layer was obtained by depositing cellulose powder that had been stirred in a 15% solution of formamide in acetone on the plate. The plates were used after the elimination of the acetone (Fig. 1, b).

Two-dimensional TLC. Three μg of urydilyl-(3'→5')-guanosine was deposited at a corner of a 20 × 20 cm plate 3 cm from each edge, and 3 μg each of uridine-3'-phosphate at the two neighboring corners. Chromatography was carried out first in system 1 and then at right angles in system 9 (Fig. 2).

Quantitative analysis (cf. Table 2). In quantitative TLC, after development (if necessary, chromatography repeated), the spots were outlined with a capillary and the adsorbent containing them was collected in centrifuge tubes (by scraping or suction) and weighed. The substances were eluted from the cellulose with 3 ml of water at 37° (analysis 6, Table 2) or 0.05 N hydrochloric acid at room temperature (analysis 1-5) for ~ 20 hrs. The suspension was centrifuged; the optical density of the solution was measured in comparison with the density of a solution obtained by the extraction of the same amount of cellulose, taken from the plate at the same distance from the start, in the same volume of solvent. The amount of substance in the mixture was determined as the percentage ratio of the optical density D of the spot to the total D of the spots obtained (if the substances in the mixture had similar extinctions at the wave-lengths measured) or as the percentage ratio of the micromoles at the spot to the micromolar total at all the spots (if the substances had different extinctions or the D values were measured at different values of λ).

For comparison with TLC, we used the "rapid" grade chromatographic paper of the "Chistye Soli" mill washed with 2% hydrochloric acid, water, 0.5% ethylenediaminetetraacetate, and water again; ascending chromatography was used.

Summary

The thin-layer chromatography of nucleoside and nucleotide derivatives on cellulose has been carried out, and R_f values and conditions for the separation and quantitative analysis of these mixtures by this method have been given.

REFERENCES

1. A. A. Akhrem and A. I. Kuznetsova, Thin-Layer Chromatography [in Russian], Moscow, 100, 1964.
2. K. Randerath, Dunschicht Chromatographie, Verlag. Chem., 182, 1962; Angew. Chem., 74, 484, 1962.
3. D. P. Holdgate and T. W. Goodwin, Biochim. et biophys. acta., 91, 328, 1964.

4. K. Randerath, J. Chromatogr., 6, 365, 1961; Biophys. Biochem. Res. Commun., 6, 452, 1962.
5. T. S. Lomakina and N. I. Grineva, KhPS, 4, 275, 1965.
6. E. V. Dyatlovitskaya, V. V. Voronkov, and A. D. Bergel'son, DAN SSSR, 145, 1254, 1962.
7. Ultraviolet Absorption Spectra of 5'-Ribonucleotides, Pabst Laboratories Division of Pabst Brewing Company, Wisconsin, 20.
8. J. G. Moffatt and H. G. Khorana, J. Am. Chem. Soc., 83, 649, 1961.
9. A. Hampton, J. Am. Chem. Soc., 83, 3640, 1961.
10. H. G. Khorana, J. Am. Chem. Soc., 85, 3855, 1963.
11. F. Cramer and K. H. Scheit, Angew., Chem., 74, 717, 1962.
12. E. M. Crock, A. P. Mathias, and B. R. Robin, Biochem. J., 74, 234, 1960.
13. S. Chladek, J. Smrt, and F. Štorm, coll., 27, 82, 1962.
14. J. Smrt, coll., 29, 2053, 1964.
15. S. Chladek and J. Smrt, coll., 29, 214, 1964.
16. S. Chladek and J. Smrt, coll., 28, 1305, 1963.
17. G. Wyatt, "Separation of nucleic acid components by chromatography on filter paper," in: Nucleic Acids [Russian translation], Moscow, 453, 1957.

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