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Resolution of complex purine and pyrimidine antagonist mixtures by thin-layer chromatography*

No rapid and sensitive microanalytical method has been described in the literature which permits complete resolution of complex purine and pyrimidine antagonist mixtures containing microgram amounts of individual compounds. Although WEMPEN AND FOX[1,2] have reported a paper chromatographic method for the determination of 6-fluorocytosine and a thin-layer chromatographic method and a paper electrophoresis method for the analysis of 6-fluorouracil, no R_F value has been reported. Recently, BAYER[3] reported a paper chromatographic method for the investigation of the decomposition products of 2,6-bis[bis(β-hydroxyethyl)amino]-4,8-dipiperidinopyrimido(5,4-*b*)pyrimidine. The method, however, is not applicable in the case of resolving a mixture of such compounds. In our laboratory over the past four-year period approximately 200 such compounds have been synthesized and evaluated under the auspices of the Cancer Chemotherapy National Service Center against various induced rodent tumors. It is now felt that a new sensitive and rapid chromatographic method by which complex antagonist mixtures can be separated and identified might be valuable in investigations concerning cancer research and metabolism of such antagonists in animal cells and tumor cells. This paper describes such a thin-layer chromatographic technique by which resolutions of complex purine

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and pyrimidine antagonist mixtures are achieved. The resolutions are carried out by anion-exchange chromatography on MN 300G/ECTEOLA and MN 300G/DEAE thin layers. The factors that influence the resolution are discussed in the section Results and discussion.

EXPERIMENTAL

Materials

MN 300G/DEAE, MN 300G/ECTEOLA anion-exchanger powder and MN 300 cellulose powder were obtained from Brinkmann Instruments Co., Great Neck, N.Y., U.S.A. The solvent was prepared from analytical grade N,N-dimethylformamide.

9-Ethyl-6-chloropurine was synthesized by the method of MONTGOMERY AND TEMPLE[4] and recrystallized twice from *n*-hexane to give white crystals, m.p. 81–82°. 9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]phenyl}-6-aminopurine[5], m.p. 195–198°; 9-{*p*-[N,N-bis(2-chloroethyl)amino]phenyl}-6-chloropurine[5], m.p. 135–137°; 9-{*p*-[N,N-bis(2-hydroxyethyl)amino]phenyl}hypoxanthine[5], m.p. 270–272°; 9-{*p*-[N,N-bis(2-chloroethyl)amino]phenyl}-6-hydroxypurine monohydrochloride[5], m.p. 300°; 9-[*p*-(β-hydroxyethyl)phenyl]-6-(*p*-chloroanilino)purine[6], m.p. 168–170°; 9-{*p*-[N,N-bis(2-hydroxyethyl)amino]phenyl}-6-mercaptopurine[5], m.p. 264–267°; 4-[*p*-(β-hydroxyethyl)anilino]-5-amino-6-chloropyrimidine[6], m.p. 206–208° were synthesized by the method of LIN *et al.*[5,6], recrystallized twice and dried under reduced pressure over Drierite at room temperature. Melting points were measured in a Thomas-Hoover apparatus and uncorrected.

Preparation of plates

A suspension of 15 g of MN 300 cellulose powder in 95 ml of distilled water is homogenized with a Deluxe-Liquid-Blender (Hamilton Beach Co.) for 40 sec and applied by means of an applicator (Model No. 611, Desaga Co., Heidelberg, Germany) to degreased glass plates (20 × 20 cm) to give layers 250 μ thick. A slurry of 10 g of MN 300G/DEAE or MN 300G/ECTEOLA (containing plaster of Paris) anion-exchanger powder in 95 ml of distilled water is homogenized with an electric blender for 1 min and applied by means of a Desaga-Brinkmann applicator to degreased glass plates (20 × 20 cm) to give layers 250 μ thick. The plates are separated immediately after coating and allowed to dry overnight on a horizontal support at room temperature. The plates are kept in darkness and in the cold.

Chromatography

Fresh solutions of the samples are applied on the starting line drawn 2.0 cm from the lower edge of the plate. After drying for about 3 min the chromatogram is developed ascendingly in a closed tank (23 × 23 × 5 cm) filled with solvent to a height of about 1.0 cm. The solvent front is allowed to ascend about 14 cm above the starting line and the plate is then dried in a horizontal position for about four hours. For the determination of R_F values, the chromatogram is examined under a short-wave ultraviolet lamp and the position of each spot is marked with a sharp soft pencil. A Model DB recording ultraviolet spectrophotometer from Beckman Co. was used to determine the ultraviolet absorbance (Table I) of these compounds. The development time is 40–70 min, depending upon the composition of the thin layer being used.

TABLE I

FLUORESCENT COLORS, ULTRAVIOLET ABSORPTION MAXIMA AND IDENTIFICATION LIMITS OF PURINE AND PYRIMIDINE ANTAGONISTS

Compound	*Solvent*[a]	*Fluorescent color*	*IL*[b] (μg)	*Ultraviolet absorption maxima*	
				0.1 N NaOH λ_{max} ($m\mu$)	*0.1 N HCl* λ_{max} ($m\mu$)
9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]-phenyl}-6-aminopurine	Ethanol	Light blue	2	260	268
9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]-phenyl}-6-mercaptopurine	Ethanol	Orange	2	323	310
9-[*p*-(β-hydroxyethyl)phenyl]-6-(*p*-chloroanilino)purine	Ethanol	White	2	259	253
4-[*p*-(β-hydroxyethyl)anilino]-5-amino-6-chloropyrimidine	Ethanol	Dark spot	2	321	309
9-{*p*-[N,N-Bis(2-chloroethyl)amino]-phenyl}-6-chloropurine[c]	Methanol	Greenish yellow	7	263	267
9-Ethyl-6-chloropurine	Chloroform	White	75	266	265
9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]-phenyl}hypoxanthine	Ethanol	White	1	230	262
9-{*p*-[N,N-Bis(2-chloroethyl)amino]-phenyl}-6-hydroxypurine monohydrochloride	N,N-Dimethylformamide	White	15	255	258

[a] The best solvent used to dissolve the compound for applying on chromatoplate.
[b] IL is the identification limit of the compound analyzed.
[c] 9-{*p*-[N,N-Bis(2-chloroethyl)amino]phenyl}-6-chloropurine was found active against human epidermoid carcinoma of nasopharynx and Walker carcinosarcoma 256 by the Cancer Chemotherapy National Service Center, National Institutes of Health, the U.S. Public Health Service, in March, 1964.

TABLE II

R_F VALUES OF PURINE AND PYRIMIDINE ANTAGONISTS

Thin layers: (1) MN 300 cellulose powder, 250 μ; (2) MN 300G/ECTEOLA, 250 μ; (3) MN 300G/DEAE, 250 μ. Solvent: 25% aqueous N,N-dimethylformamide (pH 7.5).

Compound	*Thin layer*[a,b]		
	1	*2*	*3*
9-Ethyl-6-chloropurine	0.79	0.85	0.86
9-[*p*-(β-Hydroxyethyl)phenyl]-6-(*p*-chloroanilino)purine	0.19	0.36	0.35
4-[*p*-(β-Hydroxyethyl)anilino]-5-amino-6-chloropyrimidine	0.76	0.76	0.63
9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]phenyl}-6-aminopurine	0.64	0.69	0.70
9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]phenyl}-6-mercaptopurine	0.62	0.25	0.17
9-{*p*-[N,N-Bis(2-chloroethyl)amino]phenyl}-6-chloropurine	0.26	0.47	0.45
9-{*p*-[N,N-Bis(2-hydroxyethyl)amino]phenyl}hypoxanthine	0.74	0.78	0.51
9-{*p*-[N,N-Bis(2-chloroethyl)amino]phenyl}-6-hydroxypurine monohydrochloride	0.54	0.58	0.42

[a] Average of five runs.
[b] The R_F values vary with the source and lot number of the thin-layer material employed.

Results and discussion

The R_F values of eight purine and pyrimidine antagonists at pH 7.5 on three different thin layers are given in Table II. Good resolutions with well formed spots were obtained by anion-exchange chromatography on MN 300G/DEAE and MN 300G/ECTEOLA thin layers using 25 % aqueous N,N-dimethylformamide as the developing solvent. The R_F values of each compound vary with the compositions of the thin layers, as shown in the last three columns of Table II. The thickness of MN 300G/ECTEOLA thin layers (containing plaster of Paris) should not be more than 250 μ. It was found that the solvent front was strongly fluorescent when the thickness of this layer was more than 250 μ, which might interfere with the fast moving compound, such as 9-ethyl-6-chloropurine. In addition to the respective R_F values, the characteristic fluorescent colors of the spots also provide good identification for each one of these compounds. The identification limits (IL), fluorescent colors and ultraviolet absorption maxima data of these compounds are given in Table I. The fluorescent colors remained stable over a period of about four months.

The method is very sensitive: 1–75 μg of each compound can be detected under the conditions used. The technique can be used for micropreparative separations. The completeness of the resolution depends not only on the composition of the mixture being analyzed, but also on the character of the thin layer.

With the application of the method described in this paper, it should be possible to detect and isolate such purine and pyrimidine antagonists in extracts from biological materials.

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