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Note

Separation of pteridines by thin-layer chromatography on combination plates

SALLY FROST and JOSEPH T. BAGNARA

Department of Cellular and Developmental Biology, University of Arizona, Tucson, Ariz. 85721 (U.S.A.)

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Pteridines, like purines, pyrimidines and other relatively small-molecular-weight compounds, are readily amenable to separation by paper and/or thin-layer chromatography (TLC)[1]. Unlike UV-absorbing compounds, such as xanthine, uric acid and others, the great majority of pteridines fluoresce when exposed to a UV light source, thus making them easy to locate on chromatograms. Moreover, their intense fluorescence allows for the detection of very small amounts from extremely dilute tissue samples.

For many years the method of choice for pteridine separations was paper chromatography[2–4]. With the advent of TLC procedures, it naturally followed that pteridines were applied to plates of cellulose, silica gel or alumina[5]. In general, cellulose TLC is extremely suitable for pteridine separations, yielding results similar but superior in resolution to paper chromatograms[1,6]. During the course of our current work on naturally occurring pteridines, we have employed TLC methods and standard solvents in an effort to achieve high resolution of pteridines from extremely heterogeneous tissue samples. The following report is a comparison of the mobility of a mixture of eight naturally occurring, authentic pteridines on thin-layer plates prepared from a combination of cellulose and silica gel as opposed to plates prepared from either silica gel or cellulose alone.

EXPERIMENTAL

Materials

Cellulose (Sigmacell, Type 100), pterin (AHP), pterin-6-carboxylic acid (AHP-6-COOH), xanthopterin (XP), isoxanthopterin (IXP), and leucopterin (LP) were purchased from Sigma (St. Louis, Mo., U.S.A.). Silica gel G (Merck, Darmstadt, G.F.R.) was supplied by Scientific Manufacturing Inc. (SMI), Emeryville, Calif., U.S.A. 7-Methyl-xanthopterin (7-methyl-XP) was synthesized as described by Elion and Hitchings[7]. Biopterin (BP) and L-*erythro*-neopterin (NP) were gifts from Dr. J. Matsumoto. All solvents used were of commercial analytical grade.

Preparation of thin-layer plates

The following preparations were found to be suitable for spreading five glass plates at a time: (1) 7.5 g cellulose in 50 ml of distilled water; (2) 15 g silica gel G in

37.5 ml of distilled water; or (3) 7.5 g cellulose and 7.5 g silica gel G in 67 ml of distilled water. Slurries were prepared by thorough mixing (60–90 sec) at low speed in a commercial blender. Plates used were 20 × 20 cm and 3 mm thick glass. Spreading of the slurries was accomplished using an SMI applicator and mounting board. Plates containing silica gel G were heat activated at 100° for 30 min immediately before use. Combination plates were activated at 100° for 10–15 min before use, while cellulose alone need not be activated. All plates were initially dried at room temperature.

Sample preparation and application

In a few drops of 1 *N* NaOH were dissolved 2 mg of each of the following pteridines: BP, NP, XP, IXP, LP, AHP, AHP-6-COOH and 7-methyl-XP. This mixture was then diluted to 100 ml with distilled water, and 20-μl samples were applied dropwise to the prepared plates using a microliter syringe. The sample spot was always centered 1 cm from the bottom of the plate and 4 cm from the left edge.

Development

Plates were developed in glass tanks equipped with metal holding stands and glass covers (SMI). Each tank contained 600 ml of one of the following solvents: (a) *n*-propanol–7% ammonia (2:1), or (b) isopropanol–2% ammonium acetate (2:1). Development was carried out ascendingly in two dimensions using solvent a for the first dimension and b for the second. Sample application and development were carried out in subdued light or total darkness to prevent degradation of photolabile pteridines. Results were viewed by placing the plates on a dark blue filter (Corning M2406) over a UV light source.

RESULTS AND DISCUSSION

Results of two-dimensional development of the eight pteridines on the three types of TLC plates are illustrated in Fig. 1. It is apparent from this illustration that combination plates provide superior resolution of this complex mixture of natural compounds. It should also be noted that all three plate types required essentially the same amount of time for two-dimensional development to proceed 10 cm from the origin in both directions.

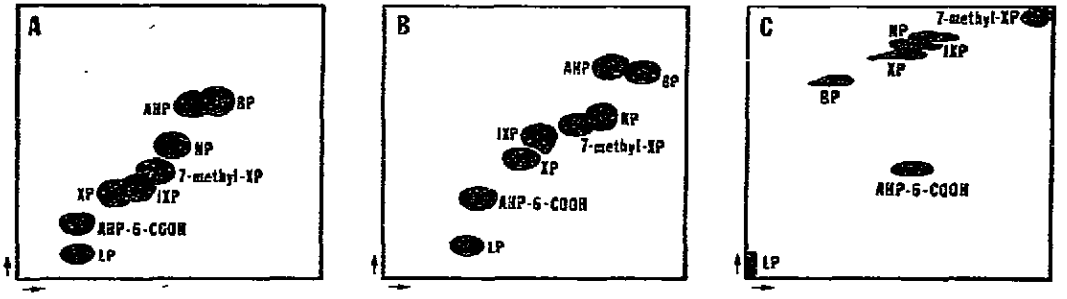

Fig. 1. Two-dimensional TLC of eight authentic pteridines on three different types of plates. The origin is the lower left corner of each illustration, and arrows indicate the direction of migration (see text for pteridine abbreviations). Plate composition: A = cellulose alone, B = cellulose and silica gel G (50:50), C = silica gel G alone

Table I presents the actual R_F values for the pteridines on the three plate types and in two different solvents. From this it is apparent that while compounds migrate much more rapidly on silica gel alone, individual spots bunch together particularly at higher R_F values. Moreover, resolution on silica gel G is consistently poor. Fluorescent spots often appear as streaks and are difficult to identify. The chromatogram illustrated in Fig. 1C was the best of ten different plates all prepared and run in the same manner. Even after this effort, biopterin could not be positively identified on any of the plates, and overall consistency of migration of specific pteridines was not good.

In contrast, cellulose alone results in fair separation and easy identification of all eight pteridines. Resolution is adequate and each pteridine migrates as a discrete and readily discernible spot. Nevertheless, some overlap occurs as a result of all eight pteridines migrating together at relatively low R_F values.

On the other hand, combination plates retain the resolution power and discrete spots characteristic of cellulose, but the silica gel allows for a wider spread of pteridines over the entire plate, resulting in little or no overlap of compounds. Fig. 2 is an example of a 70% ethanol skin extract from larvae of the Mexican leaf frog which was run on a combination plate precisely as described above for the authentic pteridines. This example best illustrates the results one can obtain using biological extracts and combination plates. Apart from some minor background contaminating spots, all fluorescing substances are clearly distinguishable and each substance migrated as a discrete spot with very little tailing. We have found that results of various biological samples on more than 50 such plates have been highly consistent, and inconsistencies were always due to insufficient mixing of the slurry prior to spreading.

Fig. 2. Two-dimensional TLC of a 70% ethanol dorsal-skin extract from young larvae of *Pachymedusa dacnicolor* on a combination plate. The preparation and chromatography of this plate was as described in the text. The ethanol extract was prepared as outlined previously[1], and 50 μl of sample was applied to the plate at the origin in the lower left corner. Arrows indicate the direction of migration.

TABLE I

R_F VALUES OF EIGHT AUTHENTIC PTERIDINES ON THREE DIFFERENT PLATE TYPES AND IN TWO DIFFERENT SOLVENTS

Solvent A: *n*-propanol–7% ammonia (2:1); solvent B: isopropanol–2% ammonium acetate (2:1).

Pteridine	*R_F values*					
	Solvent A			*Solvent B*		
	Cellulose	*Cellulose–silica gel G (50:50)*	*Silica gel G*	*Cellulose*	*Cellulose–silica gel G (50:50)*	*Silica gel G*
Leucopterin	0.07	0.09	0.07	0.15	0.22	0
Pterin-6-COOH	0.16	0.23	0.32	0.15	0.25	0.43
Xanthopterin	0.24	0.34	0.66	0.26	0.37	0.43
Isoxanthopterin	0.26	0.41	0.68	0.30	0.41	0.40
7-Methyl-xanthopterin	0.31	0.44	0.88	0.35	0.51	0.90
Neopterin	0.38	0.46	0.70	0.40	0.57	0.45
Pterin	0.50	0.61	0.58	0.56	0.60	0.23
Biopterin	0.51	0.59	?	0.51	0.68	?

CONCLUSIONS

As a final note, practical application of combination plates should be mentioned. The particular mixture of pteridines used in this study was selected because all occur in a vertebrate organism which is currently being investigated in our laboratory. In addition, this organism possesses a number of UV-absorbing substances as well as some presently unidentified fluorescing compounds, and combination plates have proved invaluable in our efforts to identify all these substances. Moreover, such heterogeneous mixtures are of frequent occurrence in invertebrates. Butterfly wings, *Drosophila* eyes and other insect organs have long been model systems for pteridine studies[6]. Thus, combination plates should prove to be a useful tool for anyone requiring excellent separation and high resolution of complex mixtures of either UV-absorbing and/or fluorescing compounds.

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