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Note

Thin-layer chromatographic separation of phosphonic acid derivatives

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We report here a convenient and sensitive thin-layer chromatographic (TLC) separation of several phosphonate compounds of current biological interest, including aminoethylphosphonic acid (ciliatine), a component of certain marine invertebrates[1]; aminomethylphosphonic acid, a plant growth regulator[2]; phosphonoacetic acid, a potent antiviral agent[3]; and methylphosphonic acid, a herbicide breakdown product found in plant tissues[4]. Ethylphosphonic acid was also included in the analysis. This TLC procedure is based on a minor modification of a solvent system developed by Libby[5], and of a phosphorus-specific visualization reagent used by Dittmer and Lester[6]. It offers a rapid procedure with a minimum number of operations, and good sample mobility, resulting in complete and reproducible resolution of all the compounds tested.

This procedure is a significant improvement over other TLC methods which are characterized by marginal resolution of phosphonic acid derivatives and/or less convenient chromatographic and visualization techniques[7–14]. Although not all the compounds tested would be expected to occur together in nature, the R_F values obtained in this study should be useful reference points on which to base TLC separations of these phosphonic acid derivatives from other materials.

EXPERIMENTAL

Materials and reagents

A plastic TLC sheet, 20 × 20 cm, precoated with a 100-μm cellulose MN-300 layer (Polygram Cell 300; Brinkmann, Westbury, N.Y., U.S.A.) is scribed to provide 2-cm wide vertical lanes. Heat activation is unnecessary. The sheet is developed in a 27.5 × 26 × 7 cm glass tank which has been lined on all sides with filter paper and pre-equilibrated for 15 min with 200 ml of developing solvent consisting of isobutanol–tetrahydrofuran–water–acetone–*p*-toluenesulfonic acid (80:60:50:10:3, v/v/v/v/w) and sealed with Saran Wrap under the lid.

The molybdenum blue visualization reagent is used to locate the phosphorus-containing solute zones and is prepared, with minor variations, by the procedure of Dittmer and Lester[6]. First two solutions are prepared. Solution I: 1 l of 25 *N* H_2SO_4 and 40.11 g of MoO_3 (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) are mixed and boiled gently until the MoO_3 is dissolved (3–4 h); the light yellow

solution is allowed to cool at room temperature overnight, during which time the color changes to light blue. Solution II: a mixture of 1.78 g of powdered molybdenum (Matheson, Coleman and Bell) and 500 ml of solution I is boiled gently for 15 min, cooled and decanted from any remaining residue. The reagent is prepared by adding equal volumes of solutions I and II to 4.5 volumes of water to form a dark green solution. Solutions I and II are stable for several months when stored in the dark, but fresh visualization reagent must be prepared weekly.

All solvents and chemicals are commercially available reagent grade materials.

Procedure

A TLC sheet, with samples spotted 2.5 cm from the bottom, is developed in the system described above until the solvent front has migrated 16 cm (approximately 2 h, 45 min). The sheet is air dried for 1 h and sprayed with the molybdenum blue reagent until it is uniformly moist. Color development is complete within 30–60 min. Phosphorus-containing spots are dark blue on a light blue background. The dark background that develops after several hours can be lightened by spraying with water. If slightly more water is added to the spray reagent to produce a yellow-green solution, the time required for full color development will be increased considerably, but the background color of the sheet will be much lighter. Convenient, permanent records are obtained by outlining the spots with a felt-tip pen on the back of the sheet and photocopying the sheet from the back side.

RESULTS AND DISCUSSION

This TLC system gave complete and reproducible resolution of ethylphosphonic acid, methylphosphonic acid, phosphonoacetic acid, aminoethylphosphonic acid, and aminomethylphosphonic acid, as shown in Table I and Fig. 1.

TABLE I

R_F VALUES OF PHOSPHONIC ACID DERIVATIVES

Compounds were spotted in 0.10 *M* aqueous stock solutions. Means and standard deviations (S.D.) are based on 15 determinations.

Compound	*Formula*	*μg spotted*	*μl spotted*	$R_F \pm$ *S.D.*
Ethylphosphonic acid	$CH_3CH_2PO_3H_2$	22.0	2.0	0.78 ± 0.02
Methylphosphonic acid	$CH_3PO_3H_2$	38.0	4.0	0.71 ± 0.02
Phosphonoacetic acid	$HO_2CCH_2PO_3H_2$	56.0	4.0	0.62 ± 0.03
Aminoethylphosphonic acid	$NH_2(CH_2)_2PO_3H_2$	25.0	2.0	0.34 ± 0.03
Aminomethylphosphonic acid	$NH_2CH_2PO_3H_2$	22.2	2.0	0.21 ± 0.02

The molybdenum blue reagent was a convenient and sensitive means of visualizing the aforementioned phosphonic acid derivatives. Distinct blue spots were obtained with 0.2–0.4 μmoles of material, but spots were detectable with as little as 0.02 μmoles of phosphonate. On an equimolar basis, methylphosphonic acid and phosphonoacetic acid produced spots of approximately half the visual intensity of those produced by the other compounds.

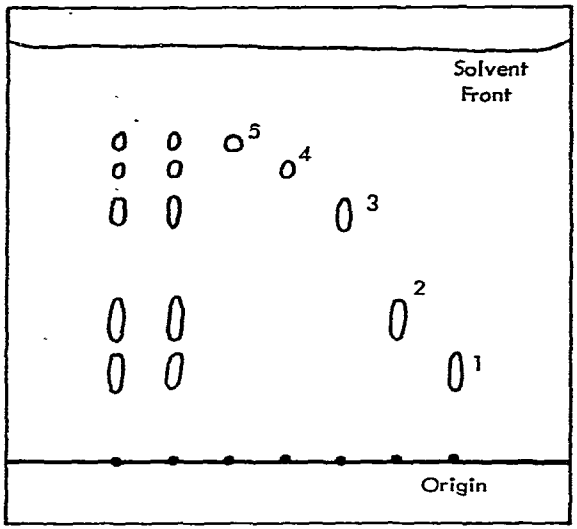

Fig. 1. TLC separation of phosphonic acid derivatives. 1 = Aminomethylphosphonic acid; 2 = aminoethylphosphonic acid; 3 = phosphonoacetic acid; 4 = methylphosphonic acid; 5 = ethylphosphonic acid.

The *p*-toluenesulfonic acid remaining on the plate after development and drying prevented the characteristic amino-specific color reaction of ninhydrin with the aminoalkylphosphonic acids. After spraying a plate with ninhydrin and heating at 100° for 30 min, all sample spots remained colorless on a light brown background.

Separation of the five phosphonic acid derivatives was complete and reproducible with this TLC system, and we obtained rapid sensitive visualization of the phosphonate spots with the molybdenum blue reagent. This system has been used successfully in our laboratory to detect impurities (approximately 20%) in 25-μg samples of aminomethylphosphonic acid preparations.

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