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Note

Thin-layer chromatography of phosphonic acids

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The phosphonic acids described here are essentially hydrolysis residues of intermediate synthesis compounds or in some cases from their highly toxic anticholinesterase products. Although the original esters are readily determinable in trace quantities by gas chromatography[1] or chemical ionization mass spectrometry[2], the acids must be extracted and derivatized to be effectively determined by these methods.

We have developed a thin-layer chromatography (TLC) method that allows the detection and identification of a variety of phosphonic acids on the basis of R_F and relatively specific visualization tests.

The method development discussed here evolved from a required capability for additional confirmation in the detection and identification of various labile diester and haloester phosphonates. In principle, the residual phosphonic acids could be evidence of pre-existing diester or haloester compounds that had been present in environmental or biological media. For best separation of our phosphonic acids a cellulose chromatoplate and a developing solvent of 1 *M* ammonium hydroxide–*n*-butanol–*n*-propanol (1:3:1) were found to be most favorable. A variety of detection (or visualization) systems, all for the microgram and lower levels, are reported. The phosphonic acids included in this study are itemized in Table I.

EXPERIMENTAL

Apparatus and reagents

Glass plates (50 × 200 mm or 200 × 200 mm) were coated with a 0.5 mm thick layer of cellulose MN 300, and a Desaga thin-layer applicator (Brinkmann Westbury, N.Y., U.S.A.) .The plates were allowed to air dry and then were stored over silica gel until used. No activation of adsorbent was necessary. Thin-layer chromatograms were developed with a solvent system of 1 *M* aqueous ammonium hydroxide–*n*-butanol–*n*-propanol (1:3:1). The solvents and other compounds were of reagent grade and required no further purification.

Reagents for visualization of developed chromatograms included a modified Hanes and Isherwood reagent for phosphorus[3], a horse serum cholinesterase preparation and indoxyl acetate substrate[4,5] for phosphonic acids, as well as for true inhibitors; a modified Dragendorff procedure for amines; and a nitropyridine disulfide method for thiols[6]. The phosphorus reagent was prepared by dissolving 1 g of am-

TABLE I

R_F VALUES OF SELECTED PHOSPHONIC ACIDS

Acid	*Abbreviation*	R_F	*Sensitivity threshold (μg)*			
			Molybdate reagent (blue spots)	*Cholinesterase reagent (white-on-blue spots)*	*Dragendorff reagent (orange-red/ orange spots)*	*Nitropyridine disulfide (yellow spots)*
Pinacolyl methylphosphonic	PMPA	0.75	1	5	$>10^3$	$>10^3$
S-2-Diisopropylaminoethyl methylphosphonothioic	DPAESMPA	0.72	1	0.05	6.5	10^3
Cyclohexyl methylphosphonic	CHMPA	0.67	0.5	5	$>10^3$	$>10^3$
Ethyl methylphosphonothiolic	EMPSA	0.57	0.5	5	$>10^3$	0.5
Cyclopentyl methylphosphonic	CPMPA	0.55	0.5	5	$>10^3$	$>10^3$
Isopropyl methylphosphonic	IMPA	0.44	0.5	5	$>10^3$	$>10^3$
Methylphosphonofluoridic	MPFA	0.31	0.5	1	$>10^3$	$>10^3$
Methylphosphonic	MPA	0.0	0.5	5	$>10^3$	$>10^3$

monium molybdate in 40 ml of water, followed by 3 ml of concentrated hydrochloric acid, 5 ml of 70% perchloric acid and diluted under chilled conditions to 100 ml with acetone. The light-yellow colored reagent was stable for 1 to 2 weeks when stored in the dark. The enzyme solution was Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) horse serum cholinesterase made up as 1 mg/ml in 0.05 *M* tris-(hydroxymethyl)aminomethane(Tris buffer); and the substrate, 15 mg of indoxyl acetate (Mann Labs., New York, N.Y., U.S.A.) in 5 ml of absolute ethanol. The indoxyl acetate solution was mixed just prior to use with a solution of 4 ml of 0.05 *M* Tris buffer, 5 ml of 2 *M* sodium chloride, 0.2 ml of 1 *M* calcium chloride and 2.8 ml of distilled water. The Dragendorff reagent was composed of 1.7 g of bismuth subnitrate, 20 g of tartaric acid and 80 ml of distilled water, to which was added a solution of 16 g of potassium iodide in 40 ml of water. The mixture was heated carefully until solution was complete. On cooling, some crystals were formed. This solution (reagent A) was stable indefinitely when kept in the dark. As a working solution for spraying, a 5 ml aliquot of the clear mixture of stock solution was dissolved daily with 10 g of tartaric acid in 50 ml of distilled water (reagent B). Nitropyridine disulfide, the reagent for thiols, was procured as a 0.03% in acetone aerosol spray from Newcell Biochemicals, Berkeley, Calif., U.S.A. For the thiol test a chromatographic development tank served as a chamber containing just enough concentrated hydrochloric acid to maintain a saturated acid atmosphere.

All of the phosphonic acids reported here with one exception, were synthesized by the Chemical Branch, Research Division, Chemical Systems Laboratory. S-2-Diisopropylaminoethyl methylphosphonothioic acid (DPAESMPA) was prepared by the authors through the hydrolysis of a prepared sample of bis(S-2-diisopropylaminoethyl) methylphosphonothioate and subsequent clean-up.

Procedures

The 50 × 200 mm cellulose chromatoplate was scribed 100 mm above the point of sample application as a horizontal break. Vertical scribing allowed up to five separate chromatography channels separated by parallel breaks in the adsorbent. This vertical discontinuity prevented overlapping of developing samples and allowed selective masking of plate areas for separate sprays of different detector solutions on the same plate. To determine the amount of unknown liquid (aqueous or other) sample to apply to the chromatoplate, aliquots of 1 μl, 5 μl, and 10 μl were applied to a test plate.

The 50 × 200 mm plate developing chamber was maintained at 23° to 25° and contained 20 ml of the 1 *M* ammonium hydroxide–*n*-butanol–*n*-propanol solution. This amount of developing solvent produced a desired depth of 10 mm. The chamber was lined with filter paper or filter paper wick (50 × 150 mm) to aid in saturating the chamber atmosphere. Samples were applied as water, methanol or chloroform solutions of the acids or esters and by means of micropipets or drawn out tubes. If particular acids were suspected, known samples of these were pipetted as potential references on a parallel channel. Development in this system required approximately 90 min for a 100 mm frontal movement.

Detection of the acids was performed as follows. The chromatoplate was air dried in a fume hood until no trace of an *n*-butanol odor was detected (approximately 30 min). An exposed portion of the plate was sprayed with the modified molybdate

reagent and while still wet was irradiated using a 254 nm ultraviolet source for 3 to 5 min. The plate channel was then further exposed to air and light for 1 to 2 h to assure complete color development. The phosphonic acids appear as blue spots on a white background.

Detection of the acids with the cholinesterase reagent was performed as follows. The previously masked channels, or preferably those on a fresh chromatoplate, were then sprayed with the enzyme solution in Tris buffer and the plate was incubated at room temperature for 20 min. The incubated plate was sprayed immediately after dilution of the indoxyl acetate reagent. The phosphonic acids appear as white spots on a blue background in approximately 5 min with DPAESMPA being the most sensitive.

DPAESMPA could also be detected by use of the Dragendorff reagent via the amine moiety of the compound. The unmasked plate was sprayed with reagent B (diluted Dragendorff) and a pink to red color at R_F 0.72 indicated the presence of DPAESMPA as opposed to pinacolyl methylphosphonic acid (PMPA) (R_F 0.75 by molybdate).

To detect as well as distinguish between phosphonic and phosphonothiolic acids, the nitropyridine disulfide reagent was sprayed on another unexposed channel and the chromatoplate was placed into the hydrochloric acid vapor chamber for several minutes for color development. This allowed the discrimination between cyclopentyl methylphosphonic acid (CPMPA) (R_F 0.55) and ethyl methylphosphonothiolic acid (EMPSA) (R_F 0.57). The thiolate appears as a stable yellow spot on a white background and is detectable down to 0.5 μg.

RESULTS AND DISCUSSION

Initial attempts to effectively chromatograph the various acids were made on silica gel plates and with various solvents and mixtures thereof. Solvents used included methanol, chloroform, 1 *M* ammonium hydroxide, *n*-propanol, ethanol, 1,4-dioxane, acetone, 2-propanol, and *n*-butanol. At best, only partial resolution was obtained with even the best of combinations. The work of Plapp and Casida[7] on the paper chromatography of hydrolysates from organophosphate pesticides appeared to most closely approximate our type of problem.

The cellulose chromatoplate and the 1 *M* NH_4OH–*n*-butanol–*n*-propanol (1:3:1) combination was selected after having tested also methanol, ethanol, dioxane and 2-propanol separately, as mixtures with one another, and with NH_4OH.

Samples of the acids up to 10 mg/ml in water were applied to the chromatoplates as 1 μl spots and developed separately to obtain their average R_F under the procedural conditions. Ten such spots were used for each R_F determination. The standard deviation was found to be less than 0.05 R_F units under the conditions of minimum spot blossoming. The average R_F values found for the acids, the visualization methods, and the sensitivity thresholds are shown in Table I.

Mixtures were made of equal volumes of the acid solutions. Aliquots in the range of 1 to 4 μl were applied to cellulose plates and the development allowed to proceed. After air drying, the molybdate reagent was sprayed onto the plates leaving some of the channels masked. With the exception of the compounds that were within 0.03 R_F units of one another, definitive spots were apparent. Even those that showed

overlapping still indicated the presence of at least two acids. Another developed channel was unmasked and sprayed with the cholinesterase reagent and subsequently, after incubation, with the indoxyl acetate substrate. At a level of 5 μg or higher per spot the acids showed the positive test of white on blue, but more as enzyme denaturants. DPAESMPA, the only true anticholinesterase among the compounds, was detectable at a level of 50 ng or higher. Similar observations had been made in our laboratory for parathion *versus* some of its acid decomposition products. Our experience over the years has been to distinguish between denaturation and inhibition by sensitivity of the test on the plate and the relative degree of reversibility.

The nitropyridine disulfide spray was applied to another previously masked channel and the plate placed into the hydrochloric acid vapor chamber. This produced a definitive yellow, EMPSA spot (R_F 0.57) which by molybdate had been detected as the upper of two overlapping spots, the other being CPMPA (R_F 0.55).

With the employment of TLC and the visualization methods, sufficient definition was obtained on the chromatoplates to give reasonable certainty of the presence of all of the acids in the mixture. Where fusion of one spot into another was evident, the selective detection systems aided in proving the presence of both compounds.

Attempts to extract the alkali salts of the phosphonic acids into chloroform prior to chromatography failed because their partition coefficients were highly favorable to the water. When a very minimal amount of hydrochloric acid was added to the sample solution with adjustment to approximate pH 4, extraction into chloroform of all acids with the exception of methylphosphonic acid (MPA) was quantitative. The addition of sodium chloride solution aided also, as a salting out process. Concentration of MPA in water through heating presented no problem since it is the most stable of the phosphonic acids.

Another chromatographic system tested here (also on a cellulose chromatoplate) was based on a paper chromatographic study made by the authors as an in-house report[8]. In this research, a butanol–acetic acid–water system was found to be very effective for separating phosphonic acids as well as basic (amino compounds) components related to some intact phosphonates. When checked against the cellulose TLC plate, blossoming of the phosphonic acid spots was significantly more evident thus providing poorer separations. The solvents were also more persistent thus affecting some of the visualization reactions, especially the enzyme method. Similarly, the same solvents when used on a silica gel plate produced sluggish separation, and poor visualization tests.

In one attempt at a reversed-phase type system using a C_{18} silica gel, we were less than successful mainly because the aqueous reagents did not effectively wet or penetrate the non-polar adsorbent.

High-performance liquid chromatography employing a cellulose column and an eluent system of *tert.*-amyl alcohol–1 *M* NH_4OH (7:1) had proved to be effective for separating a variety of phosphonic acids[9]. However, sensitivity was limited to that of the existing refractive index detectors (> 20 μg of compound). The TLC visualization systems described here give not only better sensitivity but also greater specificity of detection.

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