notes on methodology

Oxalate–silica gel thin-layer system for free 2-hydroxy fatty acids and for fatty acyl coenzyme A

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Summary The 2-hydroxy fatty acids tend to yield streaks in thin-layer chromatography on silica gel plates. If potassium oxalate is included with binder-free silica gel, good spots are obtained. Similar difficulties are found in paper chromatography of the fatty acid derivatives of coenzyme A, especially with long-chain acids. The same thin-layer system gives good spots with these compounds.

Supplementary key words 2-hydroxystearoyl coenzyme A · 2-hydroxylignoceroyl coenzyme A

The 2-hydroxy fatty acids can be separated readily from nonhydroxy fatty acids by TLC on silica gel with a variety of acidic solvents, such as hexane–ether–acetic acid, but good spots are obtained with the polar acids with very small samples only. Streaking or trailing or other distortions appear when heavier, more readily visualized spots are applied. A series of tests with a variety of solvents did not help. A similar problem with phosphoinositides (which can be conceived of as being hydroxy acids) was solved by incorporating potassium oxalate into the TLC silica gel (1). The theory behind this idea was that traces of calcium in silica gel interfere with the migration of the acids by forming salts, and that oxalate should bind the calcium competitively. The 2-hydroxy fatty acids form chelates readily and might be expected to meet similar interference.

A test of the oxalate TLC plate with chloroform–methanol–water 45:45:15 yielded excellent spots with h18:0 acid at load levels up to 140 μg (Fig. 1). A comparative plate made without oxalate yielded bad streaks with the same solvent. The more commonly used solvent system, hexane–ether–acetic acid, yielded trailing spots with some material at the origin. Satisfactory spots were obtained in the oxalate system with fatty acids h10:0 up to h24:0.

Abbreviations: TLC, thin-layer chromatography.

TABLE 1. Distribution of radioactivity on TLC plate after chromatography of fatty acyl coenzyme A compounds

Region Counted	Coenzyme A Derivative			
	18:0	h18:0	24:0	h24:0
	%			
Between spot and solvent front	1.7	4.7	2.3	5.1
Acyl CoA spot	96.5	93.4	95.7	92.7
Between spot and origin bottom	1.8	1.9	2.0	2.2

Data shown are percentages of total activity recovered. The plate was prepared as in Fig. 1 and was run ascending. The acyl CoA spots were located by radioautography, and the three TLC zones were scraped into counting vials. The powders were counted by liquid scintillation, using water and Beckman Bio-Solv (5). The acyl CoAs were prepared chemically (4), starting with ^{14}C-labeled fatty acids. A 4 mM solution was made by suspending the acyl CoAs in water and adjusting the pH to 5 with 0.1 M sodium hydroxide, using nitrogen for agitation. Contaminating free fatty acid was removed by partitioning in the system of Folch, Lees, and Sloane Stanley (6). The lower layer was washed with methanol–water 1:1, and perchloric acid was added to the pooled upper layers (from which the chloroform was removed by bubbling with nitrogen briefly) to precipitate the acyl CoA.

The specific role of the oxalate ion was indicated by substituting KCl for potassium oxalate. This produced the same streaks seen in Fig. 1, middle plate. Tests with other silica gels, more highly purified than the one used here and in Ref. 1, also showed the need for oxalate. Additional work is needed to confirm the hypothesis (1) that the oxalate acts by binding calcium.

The solvent system used in Fig. 1 moved the nonhydroxy fatty acids close to the solvent front. For better visualization of these acids we use a less polar solvent, chloroform–methanol–water 72:36:4. The R_F value for the hydroxy acid is 0.33, and the value for nonhydroxy acid is 0.70. The shape of the spots is a little sensitive to the water content (or silica gel batch) and a slight adjustment of the percentage of water in the developing medium might be advisable.

The inadvertent use of a slightly impure batch of h18:0 for Fig. 1 illustrates the effectiveness of the oxalate plate in separating the impurities.

In a study of the formation of ceramides from fatty acyl CoA compounds, we found a similar streaking problem with the paper chromatographic systems ordinarily used for palmitoyl CoA (3, 4). When the less soluble derivatives were examined (24:0 CoA and h24:0 CoA), we found marked retardation and elongation of the spots, even with small samples. Attempts to use more lipophilic solvents gave disappointing results. Since TLC of acyl CoA might be expected to show interference by metal contaminants, due to the phosphate and hydroxyl groups, the oxalate plate should prove useful.

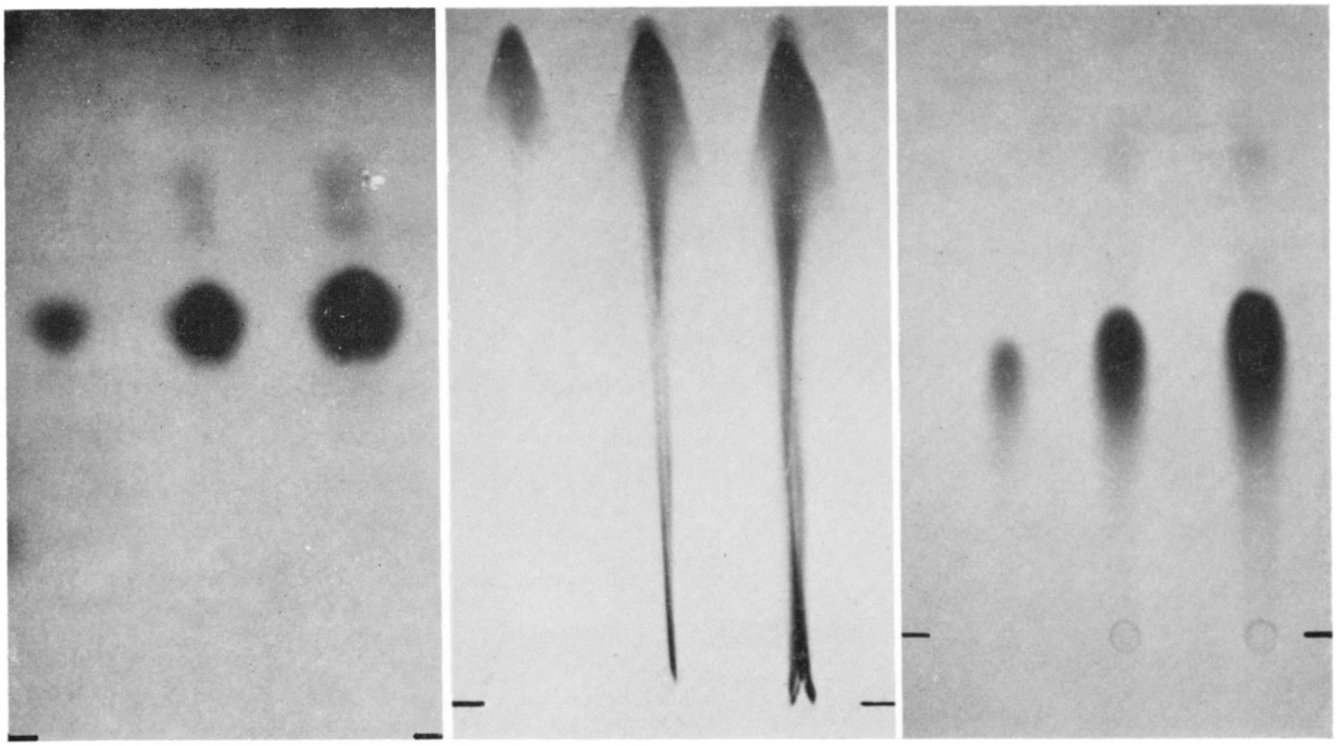

FIG. 1. Thin-layer chromatograms with 2-hydroxystearic acid. The spots on each plate contain (from left to right) 20, 80, and 140 μg. The left plate was made with potassium oxalate (1.75 g), silica gel H (E. Merck/Brinkmann, 88 g), water (175 ml), and methanol (1 ml). After spreading, the plates were air-dried with a fan, run in a chloroform bath, air-dried, and activated at 120°C for 60 min. (If stored before use, they were reactivated as before.) Development was with chloroform–methanol–water 45:45:15. The center plate was made in a similar fashion, except that oxalate was omitted. The plate on the right was made with silica gel PF-254 (same supplier) and was developed with hexane–ether–acetic acid 60:40:4. The coatings on all plates were 0.5 mm thick. The spots were detected with a copper–phosphoric acid charring spray (2). The plates were sprayed carefully, to avoid disintegration of the coating, and heated at 160°C for 45 min. To prevent cracking, we start with a cool oven and let the plate cool in the oven.

A test with labeled acyl CoA compounds, using chloroform–methanol–water 45:45:15, yielded well-shaped spots with an R_F of 0.37 (Table 1). When ammonia was included in the solvent (1), a slight amount of free stearic and lignoceric acid was seen near the solvent front. In the case of the hydroxy acyl CoA compounds, about 28% of the applied compound appeared as free fatty acid. Evidently the thiol ester bond is somewhat labilized by the 2-hydroxyl group. Even in the absence of ammonia, a slight amount of cleavage of the derivatives seems to occur (Table 1), possibly due to the slight alkalinity of potassium oxalate.

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