CHROM. 5526

Color detection of bile acids in thin-layer chromatography

The mobilities of deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid) and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanic acid) on thin-layer chromatography (TLC) are generally so similar that it is not easy to identify them by their R_F values. Color reagents have been applied and found to be useful in such a situation[1–5]. Sulphuric acid[4], perchloric acid[6], antimony(III) chloride[4,7], anisaldehyde[3], and molybdophosphoric acid[8,9] have all been used to visualize the bile acids after their development by TLC. We wish to report our experience with a 1:1 mixture of ceric ammonium sulphate and molybdenum solution, which appears to have advantages over the others reported previously.

Materials and methods

Standard bile acids and their methyl esters were obtained from Applied Science Laboratories, Inc., Calbiochem, and Supelco, Inc. The purity of the standards was tested by TLC and gas–liquid chromatography (GLC) before use. The chemicals and solvents used were of reagent grade. TLC was done on Silica Gel H in the solvent system isooctane–ethyl acetate–acetic acid–*n*-butanol (10:5:1.5:1.5) (ref. 10). After development, the plates were dried in hot air and sprayed with the color reagent using Desaga TLC spray apparatus connected to compressed air. The plates were heated in an oven at 110–130° for 10–15 min and the colors were observed in visible light.

Ceric ammonium sulphate reagent was prepared as follows: 10 g of ceric ammonium sulphate were powdered and ground into a paste with 17.5 ml of concentrated sulphuric acid and the grinding was continued as 20 ml of distilled water were added carefully on the sides. The resultant mixture was made up to 100 ml with distilled water and filtered. Molybdenum solution was made by the standard method[11].

A 1:1 mixture of ceric ammonium sulphate reagent and molybdenum solution was prepared for these investigations. Reagents B and C as shown in Table I were prepared fresh and used according to the published methods[8,9].

Results

10 μg each of cholic, chenodeoxycholic, deoxycholic, and lithocholic acids dissolved in 10 μl of ethanol were applied as spots on thin-layer plates and sprayed with the three reagents under identical conditions. The colors given by them are listed in Table I. The colors given by the other reagents reported in the literature are also shown in Table I for comparison.

Mixtures of bile acids (10 μg in 10 μl of ethanol) were applied with a micropipette on a 0.5-cm line at a distance of 3 cm from the bottom of the plates. After development in isooctane–ethyl acetate–acetic acid–*n*-butanol (10:5:1.5:1.5) (ref. 10), the plates were dried and sprayed with reagent A (Table I). Chenodeoxycholic and deoxycholic acids gave distinctly different colors with this reagent and the colors were identical to those obtained with the undeveloped spots on thin-layer plates. The colors appeared after heating the plates and did not fade for several days.

To test the lower limits of detection, mixtures of different amounts of four bile acids (1, 2, 5, 10 and 20 μg) in 10 μl of ethanol were applied on 0.5-cm lines and the

TABLE I

COLORS GIVEN BY DIFFERENT SPRAY REAGENTS WITH BILE ACIDS

	Spray reagent	*Cholic acid*	*Chenodeoxycholic acid*	*Deoxycholic acid*	*Lithocholic acid*	*Ref.*
A	1:1 mixture of reagent C + molybdenum solution made as per ref. 11	Brown	Bluish black	Brownish yellow	Chocolate brown	SUNDARAM AND SODHI
B	10% solution of molybdophosphoric acid in ethanol	Greenish blue	Bluish black	Blue	Blue	8
C	5 g phosphomolybdic acid in 100 ml acetic acid + 5 ml concentrated sulphuric acid	Dark blue	Royal blue	Dark blue	Pale blue	9
D	Iodine followed by a solution of 0.15 g benzoic acid in 0.15 ml concentrated sulphuric acid + 20 ml acetic acid	Brownish yellow	Brown	Deep yellow	Light brown	5
E	0.5 ml anisaldehyde in 1 ml concentrated sulphuric acid + 50 ml acetic acid	Purple	Blue	Brown	Bluish green	3
F	15 ml concentrated sulphuric acid in 85 ml *n*-butanol	Yellow	Greyish green	Yellow	Purple	4
G	20 g antimony trichloride in 50 ml *n*-butanol mixed with 10 ml sulphuric acid + 20 ml acetic acid	Yellowish green	Greenish yellow	Yellow	Pinkish purple	4
H	5 ml sulphuric acid + 95 ml acetic anhydride	Yellow	Greyish green	Yellowish brown	Purple	4
I	2 g ferric chloride in 83 ml *n*-butanol mixed with 15 ml concentrated sulphuric acid	Greenish black	Purplish black	Brown	Purplish black	4

thin-layer chromatograms were developed. The plates were sprayed with the reagent and heated. Amounts as low as 1 μg each in the mixture of bile acids were clearly detectable. Moreover, the differences in the colors given by chenodeoxycholic and deoxycholic acids were easily differentiated from each other. Reagent A (Table I) gave a bluish black color with chenodeoxycholic acid and a brownish yellow color with deoxycholic acid.

Discussion

The reagent suggested by KRITCHEVSKY *et al.*[3] gives as good a differentiation of color between chenodeoxycholic and deoxycholic acids as the one suggested by us. However, in their case, it is necessary for the reagent to be made fresh immediately before use. The spray reagent suggested by GOSWAMI AND FREY[5] is also adequate except for the fact that it requires exposure to iodine vapours before the reagent is sprayed. The spray reagents used by ANTHONY AND BEHER[4] are not useful in differentiating clearly between chenodeoxycholic and deoxycholic acids. The reagent examined by us gave excellent differentiation between the colors given by chenodeoxycholic and deoxycholic acids. Amounts as low as 1 μg were easily detected and were also well differentiated by their specific colors. The colors did not fade for several days. The reagent itself is stable at room temperature for a period of weeks. It is not viscous and can be sprayed efficiently. When sprayed on thin-layer plates, it interacts only with the bile acids so that the background remains clear white. (If excess reagent is used, the background becomes pale yellow or blue.)

These studies were supported by grants from the Medical Research Council of Canada and from the Canadian and Saskatchewan Heart Foundations.

Medical Research, Department of Medicine, G. S. SUNDARAM
University of Saskatchewan, Saskatoon (Canada) H. S. SODHI

1 E. STAHL, *Arch. Pharm.*, 292 (1959) 411.
2 P. ENEROTH, *J. Lipid Res.*, 4 (1963) 11.
3 D. KRITCHEVSKY, D. S. MARTAK AND G. H. ROTHBLAT, *Biochemistry*, 5 (1963) 388.
4 W. L. ANTHONY AND W. T. BEHER, *J. Chromatogr.*, 13 (1964) 567.
5 S. K. GOSWAMI AND C. F. FREY, *J. Chromatogr.*, 47 (1970) 126.
6 S. HARA AND M. TAKEUCHI, *J. Chromatogr.*, 11 (1963) 565.
7 M. D. SIPERSTEIN, S. M. HAROLD, J. L. CHAIKOFF AND W. G. DAUBEN, *J. Biol. Chem.*, 201 (1954) 181.
8 D. KRITCHEVSKY AND M. R. KIRK, *Arch. Biochem. Biophys.*, 35 (1952) 346.
9 T. USUI, *J. Biochem. (Tokyo)*, 54 (1963) 283.
10 G. S. SUNDARAM, H. SINGH AND H. S. SODHI, *Clin. Chim. Acta*, 34 (1971) 425.
11 J. C. DITTMER AND R. L. LESTER, *J. Lipid Res.*, 5 (1964) 126.

First received March 25th, 1971; revised manuscript received June 29th, 1971