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CLASS SEPARATION OF BILE LIPIDS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A thin-layer chromatography technique is described that permits separation of each class of bile lipid, such as cholesterol, free (unconjugated) bile acids, glycine- and taurine-conjugated bile acids and phospholipids, in a single run. The use of silica gel G–aluminium pre-coated sheets facilitates further processing, such as the extraction *in situ* of each class of separated bile lipids for determination by conventional methods.

INTRODUCTION

The increased interest in the pathophysiology of bile acid metabolism in recent years necessitates an exact knowledge of the composition of bile acids as they occur in their natural state. During their enterohepatic circulation bile acids are present mainly as free (unconjugated) and as glycine- and taurine-conjugated acids. The physicochemical states of these different forms affect their physiological functions (such as micelle formation and intestinal absorption[1–3]). Thus, development of a simple method of separation of each bile acid group in its natural state is desirable. For such a group separation, thin-layer chromatography (TLC)[4–17], column chromatography[18], paper electrophoresis[19], cellulose acetate electrophoresis[20] and column chromatography on DEAP[21] or PHP[22] Sephadex LH-20, with stepwise elution, have been used. The last-named technique gives a clear-cut separation of each group, but is not applicable to large numbers of samples. In our hands, the TLC methods so far proposed are not satisfactory; either good separation of free from glycine-conjugated bile acids[17] is not achieved, or multiple runs are needed. In the present communication, a TLC method is described that permits clear-cut separation of free (unconjugated), glycine-conjugated and taurine-conjugated bile acids in a single run, *i.e.*, the complete separation of lithocholic, deoxycholic, chenodeoxycholic and cholic acids, and their glycine and taurine conjugates, as groups, which has hitherto been difficult to accomplish. The use of silica gel G pre-coated on aluminium sheets facilitates further processing, such as the *in situ* extraction of each separated group of bile acids for subsequent determination with 3α-hydroxysteroid dehydrogenase or by gas–liquid chromatography (GLC) or high-performance liquid chromatography (HPLC).

Further, this TLC technique also separates cholesterol and phospholipids, an important feature[23,24] for finger-printing bile lipid composition in studies on gallstone formation.

MATERIALS AND METHODS

Human gallbladder bile was obtained by aspirating gallbladder contents with a syringe during laparotomy. The reference compounds used were cholesterol (Applied Science, State College, Pa., U.S.A.) and chenodeoxycholic, deoxycholic and lithocholic acids (California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A.). The cholic acid was obtained from General Biochemicals (Chagrin Falls, Ohio, U.S.A.) and was recrystallized twice according to the method of Hofmann[25] (m.p. 198°). Lecithin, L-α-dipalmitoyl phosphatidylcholine (synthetic) was purchased from Sigma (St. Louis, Mo., U.S.A.). Pre-coated silica gel sheets (TLC aluminium sheets pre-coated with silica gel 60; 20 × 20 cm; layer thickness 0.2 mm) were purchased from E. Merck (Darmstadt, G.F.R.) and were cut into pieces 13 cm wide and 15 cm long. Two longitudinal lines were drawn 0.5 cm from the edges (to prevent "edge" effects), and the plates were used immediately after activation at 120° for 1 h. All solvents used were of reagent grade and were redistilled in glass apparatus before use. Anisaldehyde (reagent grade) was purchased from Wako Pure Chemicals, (Tokyo, Japan). Anisaldehyde–sulphuric acid reagent was prepared by the addition of 0.1 ml of anisaldehyde to a mixture of 10 ml of acetic acid and 0.2 ml of sulphuric acid[26]. A 0.5-ml portion of bile containing 15 to 100 μmoles of bile acid, 2 to 6 mg of cholesterol and 5 to 15 mg of phospholipids was pipetted, with swirling, into a 10-ml volumetric flask about two-thirds filled with ethanol, and the mixture, after having been brought to boiling on a water bath, was left overnight at room temperature. The mixture was then made up to volume and filtered through paper (Toyo Roshi 5A, Toyo Roshi Tokyo, Japan; comparable to Whatman No. 43). An aliquot of 150 μl of the filtrate was loaded (with a Hamilton microlitre syringe, No. 1725) on to the pre-coated sheet as a band 8 cm long, 2.5 cm from each side edge and 1.0 cm from the bottom edge under a stream of warm air to minimize broadening of the loading zone. Then 20 μl of the standard solution (containing 200 μg of cholesterol and 200 to 300 μg each of cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic and lithocholic acids and their glycine and taurine conjugates, and 500 μg of lecithin) were applied as a band of length 1 cm sited 1 cm from each side edge (hereinafter called the reference lanes). The plate was developed at room temperature in a pre-saturated chamber lined with filter paper that had been soaked with the solvent system to be used for 2 h. The solvent system was isopropanol–isooctane–dioxane–acetic acid (7:10:6:2, v/v), which permitted separation of cholesterol, free (unconjugated) bile acids, glycine- and taurine-conjugated bile acids and phospholipids (in that order from the solvent front) as shown in Fig. 1. When the solvent front had ascended 12 cm from the bottom, after about 90 min, the plate was taken out and air-dried, and the reference lanes were cut off with scissors, sprayed with the anisaldehyde–sulphuric acid–acetic acid reagent and heated for about 5 min at 120° for colour development. The boundaries between cholesterol and lithocholic acid, cholic acid and glycolithocholic acid, glycocholic acid and taurolithocholic acid, and taurocholic acid and the phospholipids remaining on the loading zone were marked

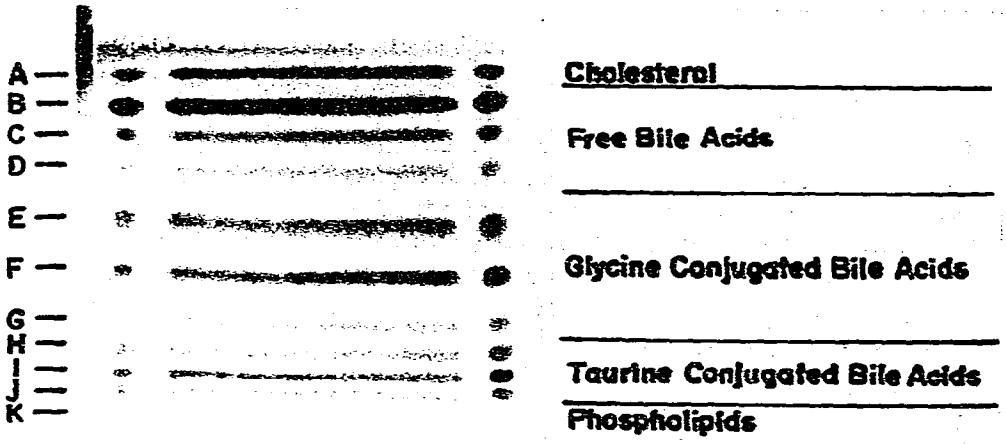

Fig. 1. Group separation of bile lipid classes by TLC under conditions described in the text; developing time, 1.5 h. A, Cholesterol; B, lithocholic acid; C, chenodeoxycholic acid; D, cholic acid; E, glycolithocholic acid; F, glycochenodeoxycholic acid; G, glycocholic acid; H, taurolithocholic acid; I, taurochenodeoxycholic acid; J, taurocholic acid; K, lecithin. Glycodeoxycholic, glycoursodeoxycholic, taurodeoxycholic and tauroursodeoxycholic acids ran together with the corresponding glycine and taurine conjugates of chenodeoxycholic acid.

by drawing lines between the corresponding boundaries on both reference lanes across the centre lane to demarcate the zones for cholesterol, free (unconjugated) bile acids, glycine-conjugated bile acids, taurine-conjugated bile acids and phospholipids.

TABLE I

R_F VALUES OF BILE LIPIDS

R_F 1.00 = 13 cm.

Compound	R_F *value*
Cholesterol	0.94
Free bile acids	
Lithocholic acid	0.86
Deoxycholic acid	0.81
Chenodeoxycholic acid	0.79
Ursodeoxycholic acid	0.76
Cholic acid	0.71
Glycine-conjugated bile acids	
Glycolithocholic acid	0.57
Glycodeoxycholic acid	0.44
Glycochenodeoxycholic acid	0.43
Glycoursodeoxycholic acid	0.39
Glycocholic acid	0.28
Taurine-conjugated bile acids	
Taurolithocholic acid	0.21
Taurodeoxycholic acid	0.14
Taurochenodeoxycholic acid	0.14
Tauroursodeoxycholic acid	0.13
Taurocholic acid	0.08
Phospholipid (lecithin)	0.00

RESULTS

By using the solvent system isopropanol–isooctane–dioxane–acetic acid (7:10:6:2) and pre-coated silica gel sheets, each bile lipid class, *i.e.*, cholesterol, free (unconjugated) bile acids, glycine-conjugated bile acids, taurine-conjugated bile acids and phospholipids, could be clearly separated (see Fig. 1). The separations hitherto most difficult to achieve in the same run, *i.e.*, those of cholesterol from lithocholic acid, cholic acid from glycolithocholic acid, and glycocholic acid from taurolithocholic acid were successfully accomplished (see Table I). Better separation was obtained by applying an ethanol extract of bile rather than bile itself to the silica gel. Separations of human, dog and ox gallbladder bile lipids are shown in Fig. 2.

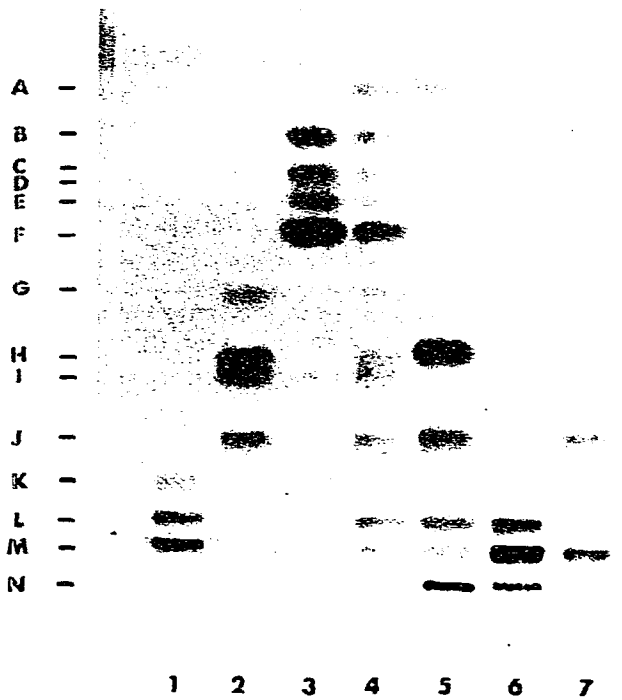

Fig. 2. Group separation of mixed standard, human gallbladder bile, dog gallbladder bile and ox gallbladder bile. 1, Taurine-conjugated bile acid; 2, glycine-conjugated bile acid; 3, free bile acid; 4, mixture of free with taurine- and glycine-conjugated bile acids and cholesterol; 5, human gallbladder bile; 6, dog gallbladder bile; 7, ox gallbladder bile. A, Cholesterol; B, lithocholic acid; C, deoxycholic acid; D, chenodeoxycholic acid; E, ursodeoxycholic acid; F, cholic acid; G, glycolithocholic acid; H, glycodeoxycholic acid; I, glycochenodeoxycholic and glycoursodeoxycholic acids; J, glycocholic acid; K, taurolithocholic acid; L, taurodeoxycholic, taurochenodeoxycholic and tauroursodeoxycholic acids; M, taurocholic acid; N, phospholipid.

DISCUSSION

For the development of a simple and accurate method of finger-printing bile lipid composition, the combined use of such sensitive techniques as TLC, the 3α-hydroxysteroid dehydrogenase method for bile acid analysis, GLC and HPLC is mandatory. Preliminary group separation of bile lipids has presented difficulties

because of the presence of several different classes of compounds, *i.e.*, neutral steroid, acidic, and amphoteric. The use of TLC with successive developments with a combination of solvent systems[27], and with a solvent system specific for the purpose, has been reported[17]. Although the former method gives a fair separation of each class of lipids, it is time-consuming. On the other hand, the latter technique is not satisfactory because of the difficulty in separating glycocholic acid from taurolithocholic acid. With the solvent system described here, separations of cholesterol from lithocholic acid, cholic acid from glycolithocholic acid, and glycocholic acid from taurolithocholic acid have been accomplished in a single run. Although it was not possible to separate sulphated bile acids from the unsulphated compounds in this system, the sulphated species are present in bile only in small quantities[28]. The reason for the better and reproducible separation obtained by chromatographing an ethanol extract rather than bile itself is not clear, but the water contained in bile may interfere with the separation by modifying the composition of the developing solvent. Further, the use of an ethanol extract gives a more representative bile lipid composition than when bile is used. The presence of cholesterol-containing precipitate in some biles[29] makes it difficult to obtain a representative sample when only a small aliquot is taken directly for TLC. The use of silica gel G pre-coated aluminium sheets in the present method has two advantages: (a) good reproducibility is attained in each run, thus making more accurate the use of the reference lanes in marking the boundary between each zone and thereby avoiding the use of iodine in locating the bands (use of iodine is best avoided, as iodine is an oxidizing agent and its removal often necessitates heating the plate, which makes extraction of the lipids more difficult); and (b) it makes the extraction of the lipids after TLC much easier than when conventional glass plates coated with silica gel are used. Each zone can be cut into strips of size convenient for extraction in a small test-tube, thus circumventing the cumbersome scraping of silica gel from the plate.

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