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# NOVEL THIN-LAYER CHROMATOGRAPHIC SEPARATION AND SPECTROFLUOROMETRIC QUANTITATION OF LITHOCHOLIC ACID

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## SUMMARY

Chromatographic separation of lithocholic acid from a mixture of four unconjugated bile acids was achieved in 5 sec. The system consisted of Gelman ITLC type SG chromatography sheets, Seprachrom miniature chromatography chambers, an isooctane–isopropyl ether–acetic acid (2:1:0.04, v/v/v) solvent system and a 5% $H_2SO_4$ in methanol detection spray. The fluorescent spots were quantitated by direct scanning of the chromatograms with a Farrand Mark I spectrofluorometer. Maximum excitation and emission wavelengths were 375 and 436 nm, respectively. Accurate measurement of lithocholic acid was performed within the range of 25 to 175 ng.

## INTRODUCTION

Lithocholic acid (LC) (3$\alpha$-hydroxy-5$\beta$-cholanic acid) has been isolated in free and conjugated forms from human bile[1,2], feces[3,4], serum[1,5–9] and urine[10]. Sophisticated multi-step purification techniques have been developed for quantitative separation of LC and other bile acids from biological material[1–6,9,10]. Thin-layer chromatography (TLC) has frequently been employed as a preliminary separation technique[2,3,5,9,11–14]. However, present procedures for the quantitative measurement of free and/or conjugated LC and other bile acids are laborious, time consuming, and involve highly sophisticated instrumentation, *e.g.* liquid scintillation counter[7,8], gas–liquid chromatograph[1–3,5,15], gas–liquid chromatograph–mass spectrometer[13,14] and computerized gas–liquid chromatograph–mass spectrometer combination[6,10].

Many TLC procedures[2,11,12,15–23] have separated free and/or conjugated forms of LC from a bile acid mixture. Although some procedures have achieved separation on silica gel impregnated glass micro-fiber sheets, direct quantitative measurement of the separated bile acids was not reported[20–23].

In this paper, a novel 5-sec TLC separation of LC is reported. Sensitive, quantitative measurement of the fluorescent spots is performed by direct scanning of the chromatograms with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment.

## EXPERIMENTAL

### *Materials*

Chenodeoxycholic (CDC), deoxycholic (DOC) and lithocholic (LC) acids were purchased from Supelco (Bellefonte, Pa., U.S.A.). Cholic acid (C) (purity 99–100%) and 5β-cholanic acid were obtained from Sigma (St. Louis, Mo., U.S.A.)

Certified grades of 2,2,4-trimethylpentane (isooctane) and diisopropyl ether (isopropyl ether) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Glacial acetic acid and concentrated sulphuric acid were obtained from Canadian Industries (St. Boniface, Canada). Absolute ethanol was purchased from Standard Chemical Co. (St. Boniface, Canada). Reagent-grade methanol was from Canadian Chemical Supplies (Winnipeg, Canada).

Seprachrom miniature chromatography chambers and 20 × 20 cm Instant TLC sheets type SG (ITLC type SG) were supplied by Gelman (Ann Arbor, Mich., U.S.A.). A Gem hand-punch (McGill, Marengo, Ill., U.S.A.) containing a round 3.0-mm die was employed to excise chromatography discs. Bile acids were applied to chromatographic discs with a 10-μl Hamilton syringe from Hamilton (Reno, Nev., U.S.A.).

Chromatography sheets were heated in a Thelco Model 18 oven from Precision Scientific (Chicago, Ill., U.S.A.). The developed chromatograms were scanned with a Farrand Mark I spectrofluorometer (Farrand Optical, Mount Vernon, N.Y., U.S.A.) equipped with a thin-layer scanning attachment and a magnetic xenon arc stabilizer. The instrument was modified by shielding the lamp housing and the photomultiplier with Nu-metal. A Topaz Series 73000 Model L1, a.c. line regulator (Topaz Electronics, San Diego, Calif., U.S.A.) was employed to regulate the line voltage. The intensity of the lamp was reduced by inserting a 60-mesh wire gauze brass screen (Canadian Laboratory Supplies, Winnipeg, Canada) in front of the quartz window at the exit port of the lamp housing. The reduced light intensity prevented the otherwise frequent breakage of the excitation filter. The Color Specification numbers for the excitation and emission filters were 7-54 and 3-73, respectively. The excitation slit widths were 5 nm and the emission slit widths were 10 nm. The intensity of the reflected fluorescence was recorded with a Farrand Model 100 strip-chart recorder (Model SR-204, Heath, Benton Harbor, Mich., U.S.A.). Area measurements were performed with a Koizumi compensating polar planimeter, purchased from Reliable Drafting Supplies (Regina, Canada).

### *Methods*

*Preparation of chromatography materials and bile acid standards.* ITLC type SG chromatography sheets were cut to size (6.5 × 9.9 cm) to fit the Seprachrom chromatography chambers. The miniature chromatography sheets were heated in a 100° oven for 1 h. Activated sheets were stored above silica gel in a desiccator.

A stock solution containing C, CDC, DOC and LC was prepared by adding 25.0 mg of each bile acid to a 50-ml volumetric flask which was filled to volume with absolute ethanol. A working standard was prepared by adding an appropriate volume of the stock solution to a 10-ml volumetric flask, to produce a standard containing 200 ng of each bile acid per μl of absolute ethanol. Sample application was performed by the procedure of Popowicz[24]. Blank chromatography discs were excised from an

activated miniature chromatography sheet with a Gem hand-punch. Holes were punched 7 mm from the bottom and at 13-mm intervals from the left edge of the sheet. A 1 $\mu$l volume of the 200-ng bile acid standard was applied with a Hamilton 10-$\mu$l syringe to each of four blank chromatography discs. The prepared discs were reinserted into the chromatography sheet.

The chromatography solvent system was prepared fresh prior to use. A 2-ml volume of isooctane, 1.0 ml of isopropyl ether and 0.04 ml of glacial acetic acid were pipetted into a test tube. The solvent system was mixed and transferred into a Sepra-chrom chromatography trough. A chromatography sheet containing bile acid standard discs was inserted into the chromatography chamber and the solvent was allowed to migrate for 5 sec. The chromatogram was immediately removed, air dried for 5 min, and oven dried for an additional 5 min. The chromatogram was sprayed with 5% $H_2SO_4$ in methanol[25] and placed in a 95° oven for 20 min. Fluorescent bile acid spots were observed under long-wave UV light. The fluorescence intensity of the LC spots was measured with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. Maximum excitation and emission wavelengths were determined. The chromatogram was scanned across the origin, the LC spot and the solvent front. The above procedure was performed repeatedly to evaluate reproducibility. The procedure was similarly performed for 10-, 20- and 30-sec solvent migration times. For the 30-sec. migration, the origin was located 13 mm from the bottom of the chromatography sheet.

*Lithocholic acid concentration study.* A bile acid stock solution was prepared by adding 25.0 mg of each bile acid to a 25-ml volumetric flask which was brought to volume with absolute ethanol. A stock solution of internal standard was prepared by adding 62.5 mg of 5$\beta$-cholanic acid to a 50-ml volumetric flask which was brought to volume with absolute ethanol. Working standards were prepared by adding appropriate volumes of the bile acid stock solution and the internal standard stock solution to a series of 5-ml volumetric flasks to produce eight bile acid standards ranging from 25 to 200 ng/$\mu$l of absolute ethanol, each standard containing 5$\beta$-cholanic acid at a concentration of 500 ng/$\mu$l. A 1-$\mu$l volume of each of the standards was applied to blank chromatography discs. Chromatographic separation and visualization were performed as described above. A 30-sec solvent migration time was employed to accommodate introduction of the internal standard. Chromatograms were scanned with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. The LC spot was employed to maximize the excitation and emission monochromators. The intensity of the reflected fluorescence was recorded on a Farrand strip-chart recorder. Peak areas were measured by planimeter. A duplicate set of analyses was performed with freshly prepared stock standard solutions.

## RESULTS AND DISCUSSION

The Kelsey and Sexton[23] chromatography solvent system was tested and modified to achieve optimal separation of LC from a mixture containing four unconjugated bile acids. The new solvent system consisted of isooctane–isopropyl ether–acetic acid (2:1:0.04, v/v/v). Rapid chromatographic separation was obtained by positioning the bile acid standard discs within 7 mm from the bottom end of the ITLC type SG miniature chromatography sheet. Excellent chromatographic separation of

LC was achieved in as little as 5 sec (see Fig. 1). It should be noted that splashing of the solvent system must be avoided. However, to attain greater separation distances and improved reproducibility, the standard discs were positioned within 13 mm of the bottom edge of the sheet and a 30-sec solvent migration time was employed. Solvent front migration was approximately 23 mm from the origin. Under the experimental conditions of this laboratory, the $R_F$ value for LC was 0.49. The unseparated CDC, DOC and C bile acid fraction remained near the origin, below $R_F$ 0.13. Each of the aforementioned $R_F$ values represents an average of twenty results.

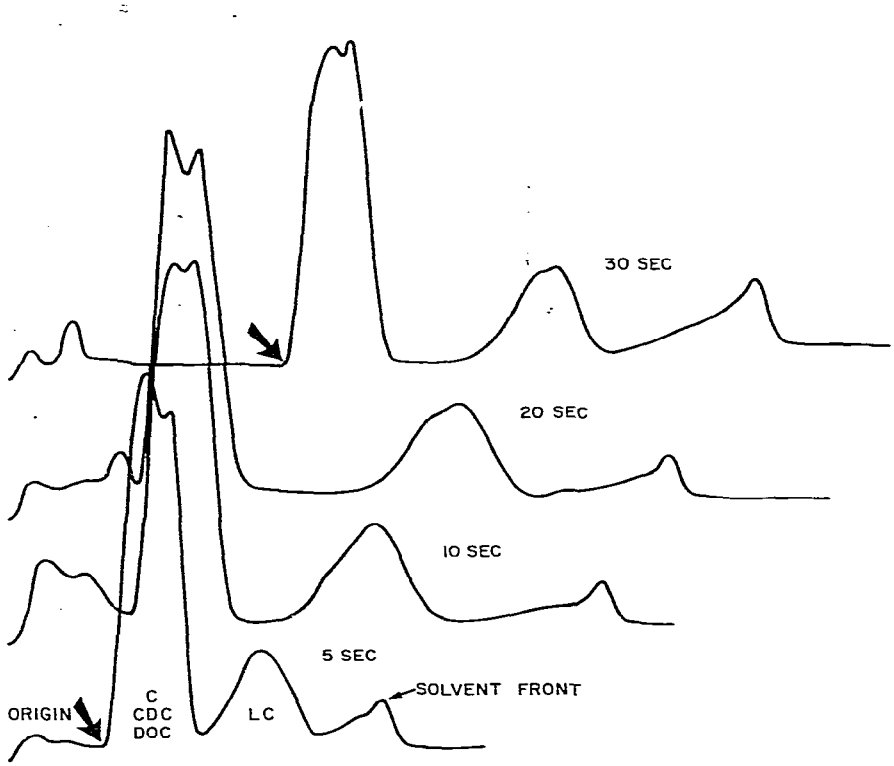


Fig. 1. Spectrofluorometer scans depicting the reflected fluorescence intensity of the bile acid spots. Chromatogram solvent migration times were 5, 10, 20 and 30 sec. Refer to text for complete details.

Heating of the ITLC type SG chromatography sheets was not essential for optimal separation of LC. However, heat-activated chromatograms were observed to produce more uniformly shaped LC spots. The chromatograms were scanned with a Farrand Mark I spectrofluorometer equipped with a thin-layer scanning attachment. The damping mode was not employed. The excitation and emission maxima for the LC fluorophore were 375 and 436 nm, respectively. The wavelength accuracy of the Mark I spectrofluorometer was better than 0.5 nm, as determined with a Farrand wavelength calibration unit. Although the ITLC type SG chromatography sheets have an irregular surface appearance, a very high signal-to-noise ratio was observed

when the intensity of the reflected fluorescence of the chromatogram was recorded (Fig. 1). Peak area was plotted *versus* quantity of LC (Fig. 2). A linear relationship was observed for LC within the range of 25 to 175 ng.

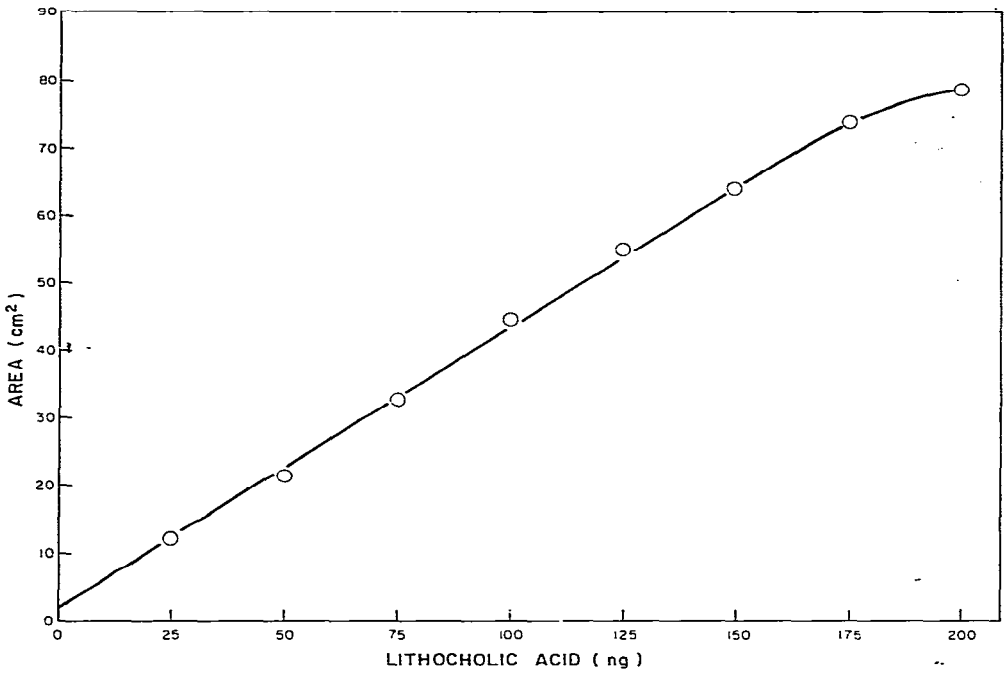


Fig. 2. Lithocholic acid standard curve. The results presented represent an average of two samples at each concentration studied. Refer to text for complete details.

A value of 4.2% was calculated for the coefficient of variation of the 150-ng standard. The average variation calculated from the duplicate results of the linear portion of the LC calibration curve (Fig. 2) was 4.2%. However, it should be emphasized that variations in sample application, detection spraying and oven temperature must be minimized. In general, improved reproducibility has been achieved with less effort for systems employing a ratio procedure, *e.g.* tri-/di-hydroxy bile acids ratio[26] and lecithin/sphingomyelin ratio[27].

The internal standard, 5$\beta$-cholanic acid, has been employed for the analysis of bile acids by gas–liquid chromatography[28]. However, attempts to employ 5$\beta$-cholanic acid as an internal standard in the presently developed ITLC separation were only partially successful. The 5$\beta$-cholanic acid was observed to migrate near the solvent front, $R_F$ 0.88. Solvent front variation was observed to interfere with accurate measurement of the 5$\beta$-cholanic acid spot. Although very reproducible results have been achieved (Fig. 3), sheet-to-sheet solvent front variations have also been observed.

The xenon arc lamp of the Mark I spectrofluorometer was stabilized with a magnetic arc stabilizer. The spectrofluorometer was further modified by shielding

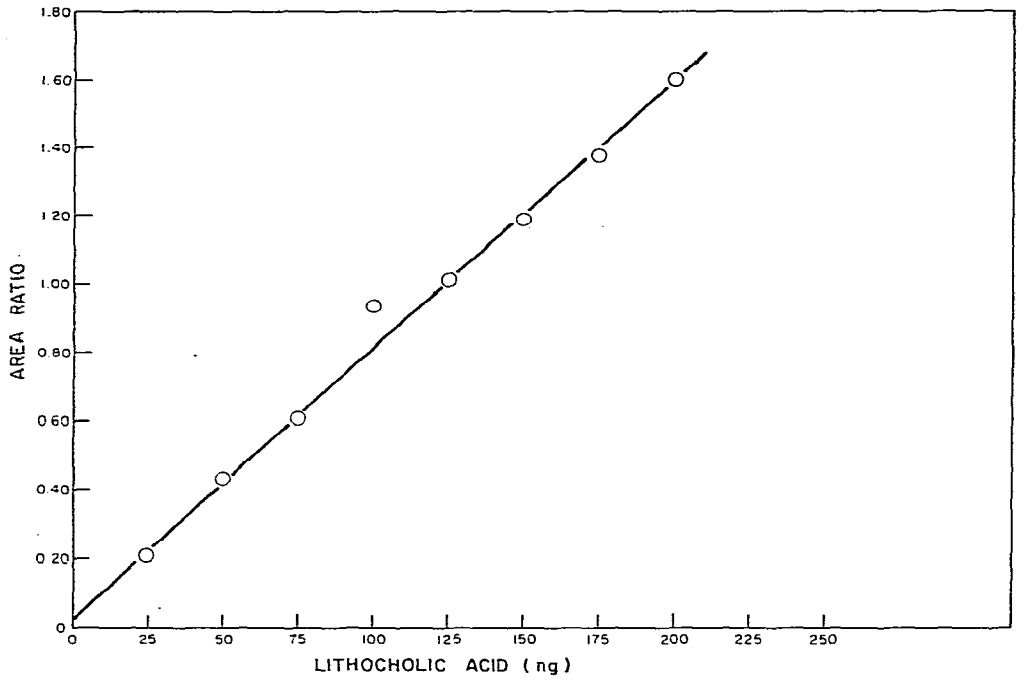


Fig. 3. Lithocholic acid standard curve obtained by area ratio (peak area for lithocholic acid/peak area for 5$\beta$-cholanic acid). Refer to text for complete details.

the xenon arc lamp and the photomultiplier tube with Nu-metal. A Stabiline voltage stabilizer was employed to overcome voltage line fluctuations. Under our operating conditions, spectrofluorometer drift was approximately $\pm 1\%$ per 12 h. Furthermore, day-to-day variation of the xenon arc lamp intensity was compensated by adjusting the spectrofluorometer to a Pyrex standard.

The fluorescence intensity of the LC spots was observed to be stable for over 6 h. A gradual decrease of 3.5% was calculated for measurements taken at 12 and 24 h for the 150-ng LC spot. On occasion, diffuse spot formation and decreases in fluorescence intensity have been observed. Similar results have been observed after splashing the solvent system. As such, it is recommended that splashing be avoided and chromatography discs not be placed closer than 1.0 cm from the bottom edge of the chromatography sheet.

ITLC coupled with direct spectrofluorometric quantitation has made possible the development of a rapid, simple, and accurate procedure for the measurement of nanogram quantities of lithocholic acid.

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