Journal of Chromatography, 277 (1983) 321—327
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1775

Note

Rapid quantification on Chromarods of cholesterol, total bile salts and phospholipids from the same microliter sample of human gallbladder bile

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(First received January 24th, 1983; revised manuscript received April 13th, 1983)

A number of procedures exist for the analysis of bile, each involving a separate assay for the quantification of the three major bile components: cholesterol, bile salts and phospholipids. The major analytical methods include gas—liquid chromatography (GLC) and colorimetric assays for cholesterol [1—3], GLC and enzymatic assays for total bile acids [4, 5], and the determination of total phosphorus to quantify phospholipids [6, 7]. Though the process of bile lipid analysis has been automated [8, 9], each component must still be assayed separately.

A rapid technique, that couples thin-layer chromatography (TLC) with flame ionization detection (FID), is available for the quantification of lipid [10]. A number of investigators have quantified the response of cholesterol, phospholipids, and bile acids using this method with Chromarods (ref. 10 and references therein, and ref. 11). We wish to report a rapid technique for determining the concentration of these three components in human bile utilizing a single assay with a two-stage TLC system on Chromarods.

0378-4347/83/$03.00

MATERIALS AND METHODS

Chemicals

Cholesterol [5(6)-cholesten-3-ol], glycocholic acid (GC) (sodium salt), and bilirubin were purchased from Sigma (St. Louis, MO, U.S.A.) and found to be 99% pure by thin-layer chromatography (TLC). The A grade sodium salts of glycochenodeoxycholate (GCDC), taurodeoxycholate (TDC), and taurocholate (TC) were purchased from Calbiochem (La Jolla, CA, U.S.A.) and used without further purification. Egg phosphatidylcholine (PC) was prepared according to a method by Singleton et al. [12] and was found to be > 99% pure by TLC. Glass distilled solvents were of pesticide grade quality or better.

Preparation of standards and human bile for Iatroscan TH-10

A representative bile standard mixture (BSM) was prepared according to the method of Beke et al. [13] which consisted of a mixture of four bile salts in the following percentages: GCDC 44.9%, TC 17.9%, TDC 27.3%, and GC 9.9% (w/w). This mixture was combined with cholesterol and egg PC in chloroform—methanol (1:1) to produce a standard containing 1 μg/μl of each. This solution was serially diluted (0.25—8.0 μg) and applied to each Chromarod in 5-μl aliquots with a 5-μl microcapillary pipette (Arthur H. Thomas, Philadelphia, PA, U.S.A.) for maximum reproducibility [14].

Fresh human bile was obtained from patients undergoing elective cholecystectomy and was homogenized with a Brinkman polytron (Brinkman Instruments, Westbury, NY, U.S.A.) controlled by a Kinematic rheostat (Westbury, NY, U.S.A.) at a setting of 4 for 2 min. It was then divided into 1-ml aliquots and stored at −20°C. Aliquots (100 μl) of bile taken with a 100-μl capillary pipette (Fisher Scientific, Pittsburgh, PA, U.S.A.) were diluted 1:10, 1:15, 1:20, 1:25, or 1:30 with chloroform—methanol (1:1) then centrifuged at 2120 *g* in a clinical centrifuge for 2—5 min to sediment the bile proteins. An aliquot of the clear supernatant was removed from each and diluted 1:4 with chloroform—methanol (1:1) and 5 μl were spotted per Chromarod.

TLC of human bile, standard and bilirubin

Bile was diluted 1:10 with chloroform—methanol (1:1), centrifuged as above and 25 μl were spotted with a 25-μl capillary pipette (Fisher Scientific) on Silica gel 60 TLC plates (E. Merck, Darmstadt, F.R.G., distributed by Bodman Chemicals, Doraville, GA, U.S.A.) along with 50 μl (300 μg) BSM and 25 μl of a saturated bilirubin solution [2 mg bilirubin in 2 ml chloroform—benzene (1:1)].

Solvent systems for TLC and Iatroscan assays

TLC plates were run in a 20 × 26 × 9.5 mm glass chamber (Supelco, Bellefonte, PA, U.S.A.). Chromarods were run in small glass chambers [19 × 17.5 × 9.5 mm (Supelco)]. Paper wicks, made from Whatman 3 MM chromatography paper (VWR Scientific, Atlanta, GA, U.S.A.) and cut to fit the back chamber wall of each type of glass chamber, effected chamber saturation.

Two solvent systems were utilized for bulk component separation. Prior to running Chromarods with these systems, samples were focused to 1 cm above

the origin chloroform–methanol (1:1) and dried between systems according to the method of Harvey and Patton [14]. The first system consisted of chloroform–light petroleum–methanol–acetone (60:20:10:10) and the solvent front was run 8–10 cm (time, 30 min). Acetone–water (50:50) comprised the second system, the solvent front being run to 5 cm (time, 15 min). Each system was used for up to three consecutive analyses.

Operating conditions for Iatroscan TH-10

Samples for TLC–FID analysis were run in the Iatroscan TH-10 analyzer (Iatron Laboratories, Tokyo, Japan; marketing consultants Newman and Howell, Winchester, U.K.) on new type S Chromarods (Iatron Laboratories) and were activated by scanning through the hydrogen flame (30 sec per scan). After overnight hydration, as described by the manufacturer, rods were activated by scanning through the hydrogen flame (30 sec per scan). Integration was performed by a Hewlett-Packard 3390A integrator (Avondale, PA, U.S.A.) set at a threshold of 3 and 0.01 peak width. Hydrogen gas flow-rate to the flame ionization detector was 170 ml/min from a pressure of 0.8 kg/cm^2 and air flow-rate was 2 l/min. Scanning speed of Chromarods was 30 sec per scan and chart speed was 6 cm/min.

Statistical analysis

Statistical studies of correlations and differences between means were performed according to the method of Bailey [15]. The lipid concentrations of native bile were calculated by employing molecular weights of 498.9, 778.0, and 386.6 for bile salts, phospholipids, and cholesterol, respectively.

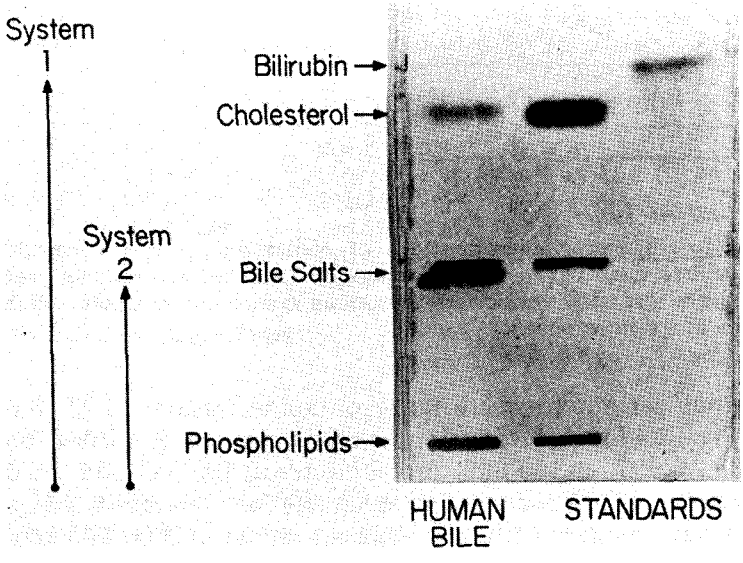

Fig. 1. Separation of bilirubin, cholesterol, bile salts and phospholipids by two solvent systems. System 1: chloroform–light petroleum–methanol–acetone (60:20:10:10) developed to 10 cm. System 2: acetone–water (50:50) developed to 5 cm. Spots visualized by charring with 40% sulfuric acid at 120°C for 5 min.

RESULTS

The separation of cholesterol, bile salts and egg PC by TLC is illustrated in Fig. 1. Neutral class components such as cholesterol and bilirubin migrated with the solvent front in the first system leaving bile salts and phospholipids 1 cm above the origin. In the second system only bile salts traveled with the solvent front, the polar phospholipids remained immobile. A similar procedure with acetone was utilized by Gentner et al. [16] for the separation of phospholipids and neutral lipids in milk.

Bile components (cholesterol, bile salts, phospholipids) were quantified from the area of three distinct peaks in the chromatogram. No quantifiable response occurred for bilirubin at physiological concentrations. All rods were repeatedly scanned (re-scanned) until no residual sample remained (2—3 times). Peak areas from consecutive scans were summed in order to calculate the total sample concentration. Above 3—4 μg per rod, cholesterol and phospholipids required two scans while 4.6 ± 1.29% and 1.2 ± 0.36% bile salts remained after the second and third consecutive scan.

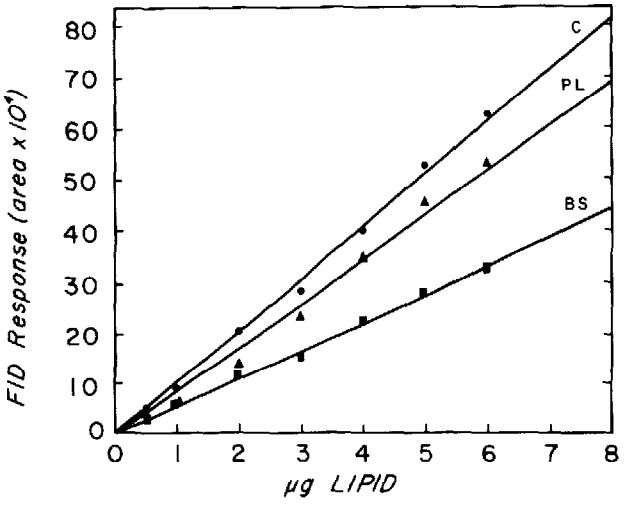

Fig. 2. Standard curve for increasing concentrations of cholesterol (•——•), egg PC (▲——▲), and a bile standard mixture (■——■). The sum of the initial and residual peak areas from consecutive scans was used to calculate the overall sample concentration. Each point represents the mean of six measurements.

A standard curve was generated over a range of concentrations (0.25—8.0 μg) as shown in Fig. 2. Linear regression analysis produced correlation coefficients of 0.998, 0.997 and 0.999 for cholesterol, egg PC and BSM indicating a high degree of reproducibility over the concentration range employed. Sample chromatograms of human bile are shown in Fig. 3. Variations in the quantities of cholesterol and phospholipid are evidenced by the changes in peak area. Results of bile analysis are shown in Table I. Each bile sample was assayed six times at three different dilutions in order to generate the data in Table I.

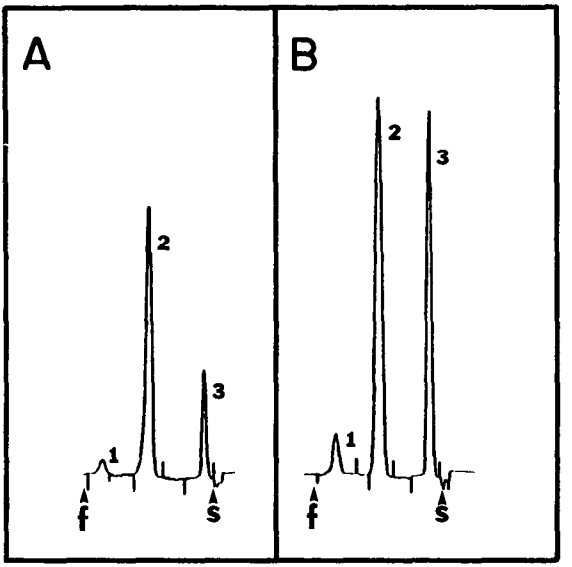

Fig. 3. Representative chromatograms of human bile. Trace A is from a sample with no visible gall stones. Trace B, with cholesterol type gallstones. Peaks: 1 = cholesterol, 2 = bile, 3 = phospholipids; f = solvent front, s = start.

TABLE I

BILIARY LIPID COMPOSITION

The data are means ± S.D. for six samples at three concentrations.

Sample	Composition (mmole/l)			Mole percent		
	Cholesterol (C)	Bile salts (BS)	Phospholipid (PL)	C	BS	PL
1	14.5 ± 1.62	206.6 ± 6.65	35.5 ± 1.51	5.7	80.5	13.8
2	7.0 ± 2.60	152.7 ± 3.03	19.1 ± 4.18	3.9	85.4	10.7
3*	18.0 ± 2.53	224.4 ± 9.88	66.2 ± 5.52	5.8	72.7	21.5
4	7.3 ± 2.24	66.2 ± 3.87	17.0 ± 1.56	8.1	73.1	18.8
5	24.7 ± 1.00	213.8 ± 14.00	72.0 ± 10.43	8.0	68.9	23.1
6	15.0 ± 0.45	140.5 ± 8.74	39.5 ± 2.58	7.7	72.0	20.3

*With cholesterol gallstones.

DISCUSSION

The procedure described here allows for the simultaneous analysis of the three major lipid components of bile. Ten samples could be examined in less than 1 h and only a small volume of bile is required. Since the actual amount of bile added per rod was between 0.045 μl (1:10 dilution) and 0.025 μl (1:30), smaller volumes could be sampled (< 50 μl bile) though care must be taken to minimize solvent evaporation.

The dilution range (1:10 to 1:30) was chosen due to the small amount of cholesterol and the large amount of bile salt in a sample. At the lowest concentration of cholesterol (7 mmole/l) only 0.125 μg were actually placed on a rod. Such a value approaches the detection limits of the system [13]. It was also desirable to keep the number of re-scans to a minimum. Re-scans were necessary for quantitative results since most of the samples, even at the highest dilution, contained overloading amounts of bile salt. Rods scanned over 170 times exhibited neither broadening nor tailing of peaks.

The composition of human bile varies depending on the physiological status of the gall bladder and bile duct, diet, sampling time, and the general health of the individual [17, 18]. The actual component percentages shown here are well within known ranges for individuals with and without gallstones [19, 20]. Cholesterol gallstone formation is thought to be caused by the supersaturation bile with cholesterol. According to Carey and Small [21] the total lipid concentration plus the bile salt:phospholipid ratio are the predominant determinants physiologically for cholesterol solubilization.

The present technique produced a low degree of variation per sample component over a wide range of clinical and standard concentrations. Current methodologies for the analysis of bile lipids utilize a series of time-consuming assays. Quantification of bile lipid classes by TLC—FID on Chromarods greatly simplifies the analysis of this important hepatic secretion.

ACKNOWLEDGEMENTS

We are grateful to H. Rodger Harvey for the purification of egg phosphatidylcholine and Pam Bruce for excellent technical assistance. This research was supported by United States Public Health Service Grants AM 01076 and AM 27304 awarded to J.S.P.

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