QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF FREE AND CONJUGATED CHOLIC ACID IN HUMAN BILE AND DUODENAL CONTENTS*

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Methods for the quantitation of free and conjugated bile acids by paper chromatography have been described in detail by SJÖVALL[1]. These methods have the advantage of making multiple determinations practical but require a time-consuming preliminary washing of the papers with a variety of solvents prior to chromatography. The methods are specific and sensitive in that each bile acid is measured spectrophotometrically at an optimal time and temperature after reaction with 65 % (w/w) sulfuric acid. For cholic, glycocholic, and taurocholic acids these conditions were defined as a reaction at room temperature (20°) for 60 min with the optical density being read at 320 mμ.

Quantitative thin-layer chromatography of bile acids was described by GÄNSHIRT *et al.*[2] in 1960 and elaborated on by FROSCH AND WAGENER[3,4]. By using several solvent systems, they were able to separate and quantitate mixtures of several pure bile acids. This approach also was applied to biologic material by FROSCH AND WAGENER[5]. In these methods, the spots containing bile acid were scraped from the plates directly into 65 % sulfuric acid and, in the case of cholic, taurocholic, and glycocholic acids, the mixture was heated to 60° for 60 min to develop the color. After removal of the silica by centrifugation, the optical density was measured at 385 mμ against a blank obtained by heating a similar amount of plain silica gel with 65 % sulfuric acid. GÄNSHIRT *et al.*[2] stated that quantitation at the peak of 320 mμ which is obtained without heating was not feasible because of the high background. It is of interest to note that, when these authors as well as FROSCH AND WAGENER[3] compared the extinctions of unchromatographed and chromatographed free and conjugated cholic acid standards, the extinction coefficients at 385 mμ were higher after chromatography even though measurements after chromatography were done with a blank to correct for the silica. (Usually, one would expect chromatography to result in a loss of material and a lower optical density.) They described the solutions as having a yellowish green color. It is possible that this effect might be due to a modification of the sulfuric acid reaction by interfering substances, such as iron, as described by ERIKSSON AND SJÖVALL[6] and WATANABE[7] for paper chromatography.

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An improved method for identification and quantitation of bile acids by thin-layer chromatography is described in which the silica gel is subjected to a purification step.

PROCEDURES AND RESULTS

Methods

Purification of silica gel G. The silica gel is allowed to stand in diluted sulfuric acid (1:1) for 5 to 6 h. After dilution with glass-distilled water, the mixture is filtered by suction with a Büchner funnel and the silica is washed several times with glass-distilled water. The silica is then allowed to stand in diluted hydrochloric acid (1:1) overnight. After dilution with glass-distilled water, the mixture is again filtered and washed with glass-distilled water until the washings are neutral to litmus paper. The silica is dried in an oven at 110° for 24 to 48 h and then passed through a No. 60 brass screen. This material is then used to make thin-layer plates in the usual manner[8]. The silica gel on 20 × 20 cm plates was divided with a pencil into nine strips 2 cm wide as shown in Fig. 1.

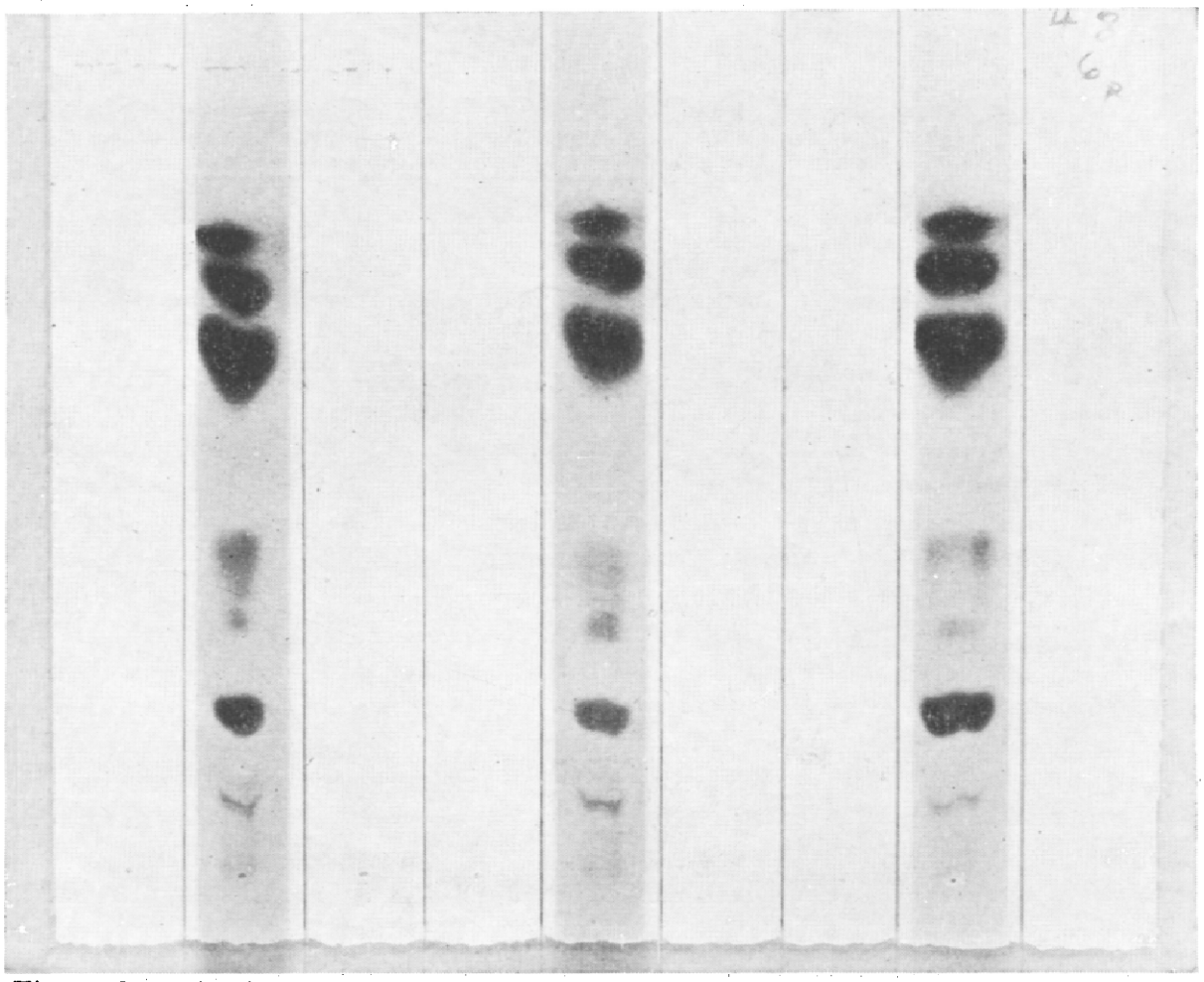

Fig. 1. Quantitative thin-layer chromatography of taurocholic acid. Taurocholic acid is the first major spot from the bottom. The dihydroxytaurine conjugates are just above this and the three major spots at the top in order are glycocholic acid, glycodihydroxy acids, and free acids with the solvent front. Solvent system: butanol–acetic acid–water (10:1:1).

Preparation of samples. Samples of bile or duodenal contents are extracted with 4 volumes of methanol–acetone (1:1). The mixture is heated to 37° for 1 to 2 min to precipitate the proteins, the mixture is centrifuged, and the supernate is concentrated with an airstream at room temperature. Usually, the extract from 5 to 10 ml of duodenal contents is concentrated to a volume of 0.5 ml. We attempt to adjust the concentration so that 25–75 μg of bile acid is applied to the plates in a volume of 5 or 10 μl.

Application of samples. A 5-μl, uniform-bore, sharply pointed micropipette proved to be the most satisfactory. Suction on the pipettes is controlled with a mercury manometer (the suction is regulated by turning a large thumbscrew which compresses the mercury reservoir and thus controls the level of mercury in the manometer). The plates are placed on a laboratory jack and cautiously elevated to make contact with only the initial portion of the fluid emerging from the pipette, so as not to disrupt the silica surface. Accurate volumes can be measured and a fairly uniform flow obtained. The spots are gently dried in an unheated airstream. Appropriate amounts of the sample are applied 1.5 cm from the lower edge of the plate on eight of the strips. The remaining strip is used as a blank. In addition, 15 or 25 μg of standard bile acid is applied to the origin of one or two strips as internal standards (see strips in Fig. 1).

Solvent systems. The use of a separate solvent system for each bile acid being quantitated was found to be more satisfactory than the attempt to separate all the major bile acids by a single system. For taurocholic acid we use butanol–acetic acid–water (10:1:1, v/v) as described by GÄNSHIRT and co-workers[2]. For glycocholic acid, isoamyl acetate–propionic acid–propanol–water (4:3:2:1, v/v) as described by HOFMANN[9] is used. For free bile acids, either of two systems is used: (1) isooctane–ethylene chloride–glacial acetic acid (2:1:1, v/v) modified from that described by HAMILTON[10], or (2) isooctane–ethyl acetate–glacial acetic acid (5:5:1, v/v) as descrbed by ENEROTH[11]. The ethylene chloride is purified as described by SJÖVALL[1] and the butanol is redistilled. All other solvents are analytical grade. After equilibrating the tanks for 1 h, chromatograms are developed by an ascending technique at room temperature*.

Quantitation. After development of the chromatograms, strips 2, 5, and 8 are stained with 15 % phosphomolybdic acid in ethanol and heated at 110° for 2 to 3 min to locate the bile acids. The corresponding areas of the unstained strips are removed individually with a sharp razor blade and transferred to test tubes by using weighing papers. Four milliliters of 65 % sulfuric acid is added to each silica sample from strips 3, 4, 6, 7, and 9 and mixing is accomplished quickly with a Vortex mixer. The tubes are allowed to stand for 15 min and then are centrifuged for 60 min at 2,700 r.p.m. in a refrigerated centrifuge at 20°. The colorless supernates are poured into 1-cm cells and read, against a blank of 65 % sulfuric acid, at 320 mμ in a Beckman DU spectrophotometer. The optical density of the chromatography blank (column 9) is subtracted from the other readings to obtain the net extinction. In an attempt to correct for any possible interference from other substances in biologic material, a modification of the technique described by SJÖVALL[1] was used: 80 % ethanol is added to the silica from strip 1 containing the bile acid spot and, after

* The development times are: taurocholic acid, 5–8 h; glycocholic acid, 4–6 h; free bile acids, $1^1/_2$–$2^1/_2$ h.

centrifugation, the extinction of the supernate is determined against a similar supernate prepared from an equivalent amount of silica from strip 9 (the blank strip). This correction is usually negligible. The sulfuric acid blank has an optical density ranging from 0.010 to 0.035 and varying with each batch of washed silica.

Hydrolysis. For determination of the total trihydroxy bile acid as free cholic acid, 0.1 to 0.2 ml of the concentrated methanol–acetone extract is hydrolyzed by adding 2 ml of 2 *N* NaOH and heating in a pressure cooker at 15 lb./sq.in. for 3 h. After cooling, 1 ml of glass-distilled water is added and the pH is rapidly reduced to below 3.5 with concentrated hydrochloric acid. The bile acids are then extracted, in a glass-stoppered tube, with three 2 ml portions of ethyl ether previously washed with 1% ferrous sulfate in 1 *N* HCl to remove any peroxides. The ether layer is separated by aspiration. The combined extracts are taken to near dryness with an airstream at room temperature and the residue is redissolved in 0.2 ml of 95% ethanol. Five or 10 μl of this solution is spotted on the plates.

Verification of method with standards

Synthetic taurocholic acid standard was generously supplied by Dr. H. L. MASON of the Mayo Clinic. Glycocholic acid standard was obtained from Sterling Winthrop Research Institute and purified by thin-layer chromatography, eluted from the silica with ethanol–acetic acid (10:1), and recrystallized from hot ethanol by addition of ethyl acetate. Except for the presence of barely detectable amounts

TABLE I

CHROMATOGRAPHY OF BILE ACID STANDARDS

Bile acid	*Sample* (μg)	*Optical density*		*Recovery* (%)	*Mean recovery*
		Direct	*After chromatography*		
Taurocholic acid	25	0.198	0.167	84.5	
		0.190	0.162	85.2	
	50	0.370	0.350	94.5	
		0.380	0.345	91.0	
	75	0.558	0.525	94.0	
	100	0.740	0.690	93.0	90.4
Glycocholic acid	25	0.235	0.197	83.0	
		0.240	0.203	84.5	
	50	0.480	0.405	84.5	
		0.495	0.420	85.0	
	75	0.760	0.610	80.0	
		—	0.622	82.0*	
	100	0.945	0.810	85.8	83.4
Cholic acid	10	0.085	0.081	95.0	
		0.083	0.076	91.5	
	20	0.170	0.144	84.7	
		0.168	0.143	85.0	
	30	0.250	0.224	89.6	
		0.254	0.218	85.8	
	40	0.330	0.296	89.0	
		—	0.300	91.0*	89.0

* Recovery calculated on basis of single direct value available.

of deoxycholic acid in the cholic acid standard (from Eastman Organic Chemical Co.) and trace amounts of free cholic acid in the taurocholic acid standard, the standards migrated as single spots in the systems described. They gave linear extinctions from 10 to 100 μg and characteristic spectra with 65 % sulfuric acid.

Standards were chromatographed and quantitated by the described method, and recoveries were compared with equal amounts of standard bile acid pipetted directly into 65 % sulfuric acid (Table I). Recoveries averaged 89.0 % for cholic acid, 83.4 % for glycocholic acid, and 90.4 % for taurocholic acid.

Application of method to biologic materials

Multiple samples of duodenal contents were obtained by intubation. Methanol–acetone extracts of these were analyzed for taurocholic and glycocholic acids, and a portion of each extract was subjected to hydrolysis for analysis of cholic acid. All results were calculated as millimoles per liter of extract so that the concentrations of conjugated cholic acid before hydrolysis could be compared with the concentrations of free cholic acid following hydrolysis. A few examples of the type of data obtained in such analyses are shown in Table II. The results in the entire series of samples analyzed are summarized in Tables III and IV. The mean percent difference of duplicate measurements from the mean was 2.3 % (range 0.0 to 12.3 %) for taurocholic acid, 1.1 % (range 0.0 to 9.7 %) for glycocholic acid, and 1.5 % (range 0.0 to 6.3 %) for cholic acid. The recoveries of conjugated cholic acid as free bile acid after hydrolysis ranged from 72.2 to 115 % (see Table III for means). Mean recoveries of internal standards after thin-layer chromatography are shown in Table IV; ranges were 71 to 105 % for taurocholic acid, 75 to 104 % for glycocholic acid, and 82 to 107 % for cholic acid.

DISCUSSION

The use of thin-layer chromatography for quantitation of bile acids makes it possible to do multiple determinations, to utilize relatively rapid rates of chromatography, and to analyze small samples of biologic material. It appears that the improvements described (the silica purification step and the technique for applying the samples) have increased the accuracy and reproducibility of quantitation by thin-layer chromatography. As a result of the silica purification step, no separate elution step is required and, in addition, the color can be developed at room temperature for reading at 320 mμ. At this peak, the optical density obtained from a given amount of standard is almost twice that obtained from the same amount of standard heated to 60° for 60 min and measured at 380 mμ[6].

With our technique, the optical density of the silica blank when compared to 65 % sulfuric acid is very small (0.010 to 0.035). Other workers[2–4], using unpurified silica gel and measuring optical density at or near 385 mμ, have not compared their silica blanks with sulfuric acid. Also, they have found that given amounts of standards give higher optical densities after than before chromatography, even though it is well known that losses commonly occur with most chromatographic methods. With our method, the recoveries with standards alone are equivalent to those with internal standards in samples of biologic material. These recoveries remain essentially unchanged over the range of sample sizes studied. It should be emphasized that we

TABLE II

EXAMPLES OF RESULTS OF ANALYSIS OF DUODENAL CONTENTS

Sample	*Taurocholic acid (TC)*			*Glycocholic acid (GC)*			*Cholic acid (C) (after hydrol.)*			$\frac{C}{TC+GC} \times 100$	*Ratio,* $\frac{GC}{TC}$
	*mmoles/l**		*Recovery (%)*	*mmoles/l**		*Recovery (%)*	*mmoles/l**		*Recovery (%)*		
	Anal.	*Mean*		*Anal.*	*Mean*		*Anal.*	*Mean*			
1	8.00			23.2			26.9			86.5	2.9
	8.02	8.01	87.8	22.9	23.0	83.8	26.6	26.8	88.5		
2	1.28			11.6			11.3			87.6	9.2
	1.26	1.27	84.8	11.7	11.7	90.2	11.4	11.4	84.5		
3	6.20			20.1			23.6			89.1	3.2
	6.32	6.26	90.8	20.4	20.3	87.0	24.7	23.7	98.6		
4	6.65			41.9			46.6			95.8	6.3
	6.57	6.61	83	41.9	41.9	87.5	46.3	46.5	—		
5	1.09		84	1.58		104	2.64			98.2	1.4
	1.11	1.10	82.5	1.58	1.58	103	2.58	2.63	—		
							2.68				
6	1.61		100	16.1		93.6	15.1			85.4	10.6
	1.47	1.54	98	16.5	16.3	102.5	14.5	15.2	99.4		
							15.9				

* Calculated on the basis of the volume of the original methanol–acetone extract. Because all subsequent manipulations are done on aliquots derived from this extract, results of different analyses are comparable for any one sample. Wide fluctuations in volume and water content of the duodenal samples themselves make it impossible to compare absolute values from one sample to another.

TABLE III

RECOVERY OF FREE CHOLIC ACID (C) AFTER HYDROLYSIS OF SAMPLES CONTAINING TAUROCHOLIC (TC) AND GLYCOCHOLIC (GC) ACIDS

No. of samples	*Conc. of cholic acid (mmoles/l)*	$\frac{C}{TC + GC} \times 100$
5	0– 4.99	97.4
6	5– 9.99	91.0
10	10–19.9	93.7
13	20–29.9	92.6
6	30–39.9	91.9
4	>40	97.0
Total 44		Mean = 93.6 S.D. = 8.6

TABLE IV

RECOVERY EXPERIMENTS WITH INTERNAL STANDARDS

Bile acid	*No. of samples*	*Concentration (mmoles/l)*	*Mean recovery (%)*
Taurocholic acid	4	0–0.99	88.4
	9	1–1.99	87.6
	6	2–2.99	84.5
	6	3–3.99	89.3
	3	4–4.99	87.3
	2	5–5.99	83.8
	4	6–6.99	86.2
	2	>7	86.8
Total	36	Mean ± S.D.* =	87.0 ± 6.8
Glycocholic acid	8	0–4.99	91.8
	4	5–9.99	90.7
	8	10–14.99	88.1
	4	15–19.99	94.8
	7	20–24.99	87.0
	2	25–29.99	85.8
	2	30–34.99	78.6
	1	35–39.99	95.2
	1	>40	87.5
Total	37	Mean ± S.D.* =	84.2 ± 6.5
Cholic acid	3	0– 9.99	96.8
	5	10–19.99	90.2
	4	>20	93.0
Total	12	Mean ± S.D.* =	92.8 ± 8.3

* Means calculated on 36, 37 and 12 individual recoveries, respectively.

used standards pipetted directly into 65 % sulfuric acid for this comparison and did not just compare internal standards with chromatographed standards. If this were done, our recoveries would be nearly 100 %.

Since the recoveries of internal standards of all three bile acids are roughly equivalent, the effect of hydrolysis is reflected by the ratio of the free cholic acid after hydrolysis to the sum of the conjugates prior to hydrolysis. It can be seen that the ratio is fairly consistent although, on the average, some loss occurs. In a recent paper, SANDBERG and co-workers[12] pointed out that this loss can be reduced if the hydrolysis is done in nickel crucibles rather than in glass tubes as was done here.

The ratio of glycine conjugates to taurine conjugates was quite variable, although there was a tendency toward consistency in each patient on multiple samples. This confirms the finding of SJÖVALL[13]. In some instances, oral administration of magnesium sulfate was used to stimulate the flow of bile. At times this caused taurocholic acid to form a double spot on the chromatograms. Addition of magnesium sulfate to a sample which showed the usual single spot for taurocholic acid also produced a double spot. The magnesium sulfate did not have a detectable effect on any of the other separations. Fig. 2 shows the separations obtained, with mixtures of bile

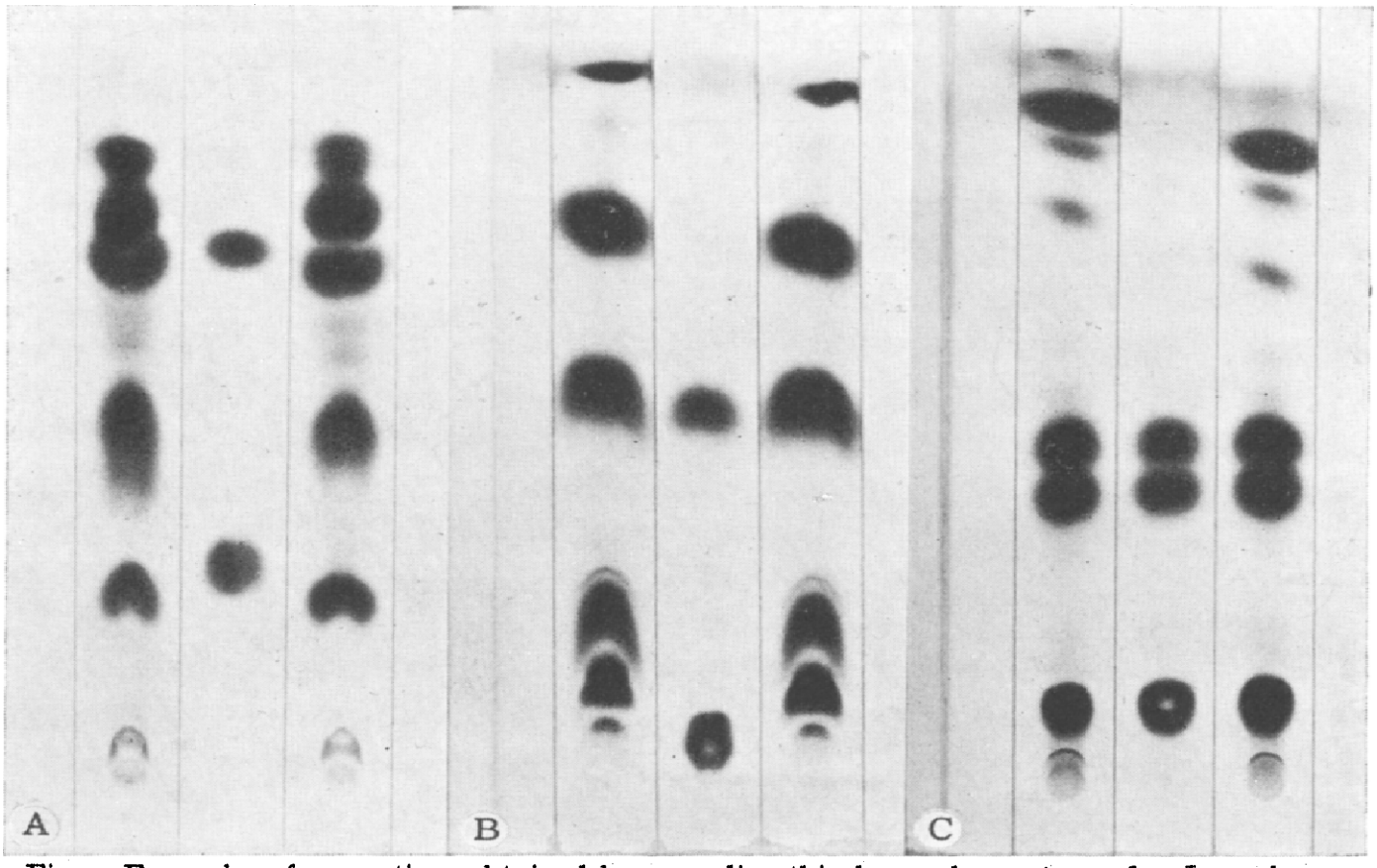

Fig. 2. Examples of separations obtained by ascending thin-layer chromatography. In each case, extract of duodenal contents is on the left, standards in the center, and extract and internal standards on the right. (A) Butanol–acetic acid–water (10:1:1). Standards in order from origin: taurocholic and glycocholic acids. (B) Isoamyl acetate–propionic acid–propanol–water (4:3:2:1). Standards in order from origin: taurocholic and glycocholic acids. (C) Isooctane–ethyl acetate–acetic acid (5:5:1). Standards in order from origin: cholic, chenodeoxycholic, and deoxycholic acids.

acid standards and with duodenal contents, by the solvent systems used. These separations are similar to those reported with the use of unpurified silica G.

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SUMMARY

An improved method for the quantitation of conjugated and free cholic acid by thin-layer chromatography has been described. The major changes involve preliminary purification of the silica and a special technique for applying the samples to the plates. This method has been applied to human duodenal contents.

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