QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF CHENODEOXYCHOLIC ACID AND DEOXYCHOLIC ACID IN HUMAN DUODENAL CONTENTS*

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We previously described a method[1] for the quantitation of free and conjugated cholic acid in bile and duodenal contents by using thin-layer chromatography (TLC). However, the quantitative separation of the dihydroxycholanic acids, chenodeoxycholic acid (CDC) and deoxycholic acid (DC), from a mixture was particularly difficult. We shall show that, despite the contiguity of the spots for CDC and DC on TLC plates, sufficient separation can be obtained to permit the quantitative determination of both of these bile acids.

METHODS

Preparation of plates and samples

Silica gel G and standard equipment for TLC were used. The commercial silica gel G was washed in sulfuric and hydrochloric acids as described previously[1]. Pure CDC and DC were supplied by Dr. H. L. MASON, Section of Biochemistry, Mayo Clinic, and were dissolved in ethanol for use as standards. Samples of duodenal contents were obtained by intubation. A methanol–acetone (1:1, v/v) extract was prepared with 4 volumes of methanol–acetone for each 1 volume of sample. After centrifugation, the supernate was concentrated in a stream of air. An aliquot of the concentrated extract was submitted to alkaline hydrolysis in 2 ml of 2 *N* NaOH for 3 h at 15 p.s.i. in a pressure cooker. After acidification with concentrated HCl the free bile acids were extracted three times with 3-ml portions of ethyl ether. The ether extracts were taken to near-dryness in an airstream and the residue was redissolved in 0.1 to 0.2 ml of 95 % ethanol. Appropriate aliquots of this preparation, usually 5 to 25 μl, were used for TLC as previously described[1].

Solvent systems

Two systems were used: isooctane–ethylene chloride–acetic acid (2:1:1) as modified from the system described by HAMILTON[2] and isooctane–ethyl acetate–acetic acid (5:1:1) as described by ENEROTH[3]. The latter system was used primarily

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because of the slightly better separation that could be obtained. The tanks were equilibrated with the solvents for 1 h prior to chromatography. The solvents were allowed to ascend approximately 18 cm on the 20 cm plates; this required approximately 2 h.

Identification and quantitation of CDC and DC

One column on each plate was sprayed with 15 % phosphomolybdic acid in 95 % ethanol, which stained the bile acids dark blue after heating. The remainder of the plate was carefully sprayed with water until the areas that contained bile acids stood out as light spots against the darker, wet background. The locations of the CDC and DC spots were identified and a fine, interrupted, dividing line was drawn between the CDC and DC areas. Then the plates were dried for a few minutes at 100°. The CDC and DC spots identified by water spraying were scraped off the plates

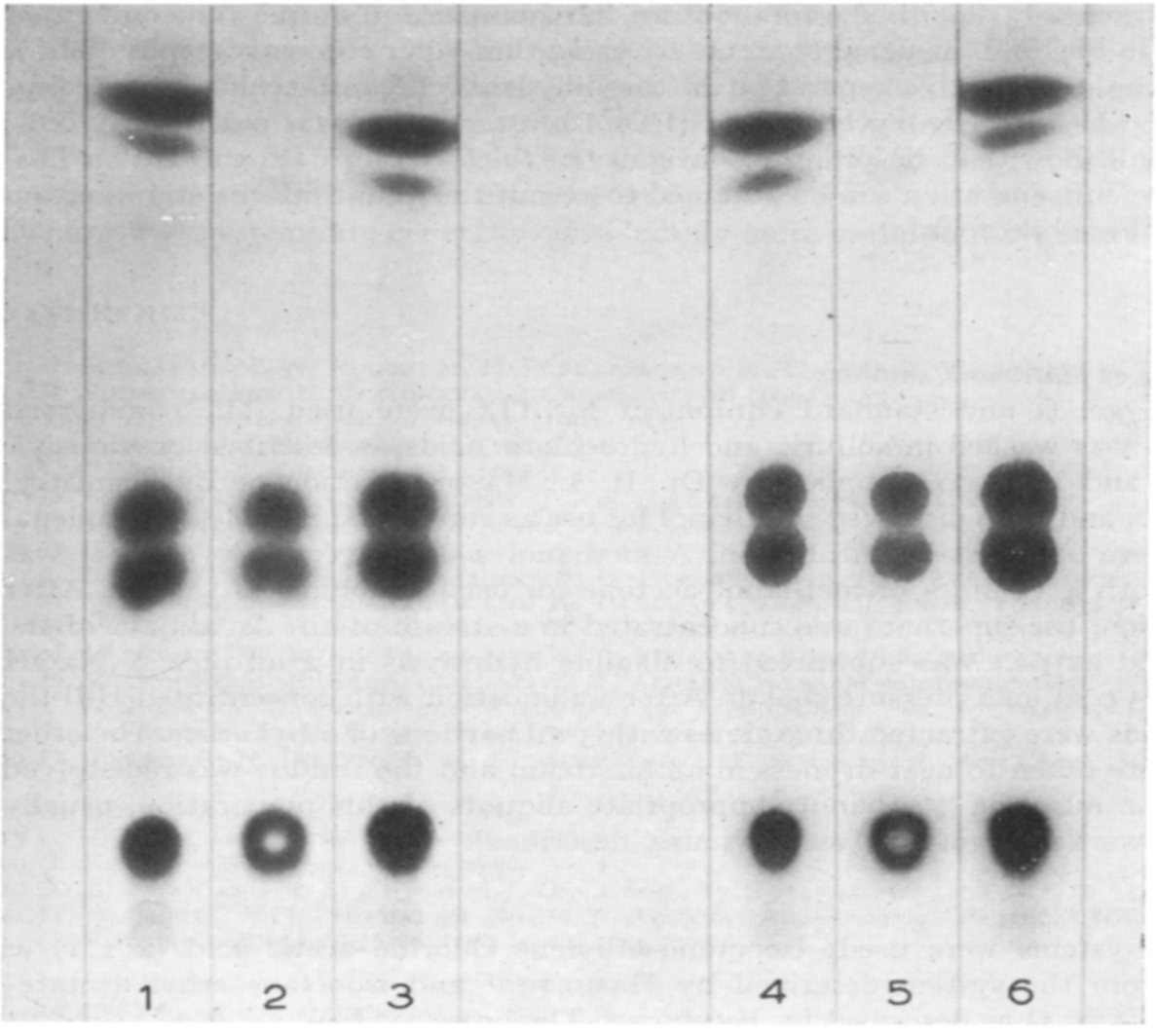

Fig. 1. Thin-layer chromatogram of free bile acids, developed in isooctane–ethyl acetate–acetic acid (5:5:1), from a mixture of standards and from hydrolysates of bile extracts. Columns 1 to 3 were stained immediately after development; columns 4 to 6 were stained after water spraying. Columns 2 and 5 represent the mixture of standards; columns 1 and 4, hydrolyzed bile extract; columns 3 and 6, bile extract plus standard bile acids. Standards on columns 2 and 5 from below upward are cholic acid, chenodeoxycholic acid, and deoxycholic acid.

separately and transferred to test tubes. The amount of bile acid present in each spot was determined spectrophotometrically in 65 % (w/w) sulfuric acid according to the method of ERIKSSON AND SJÖVALL[4] as modified for TLC by GÄNSHIRT and co-workers[5]. Four milliliters of 65 % sulfuric acid was added to the test tube and mixed with a Vortex mixer. After incubation for 60 min at 60° and centrifugation for 60 min at 20° and 3,000 r.p.m., the clear, slightly yellow supernate was poured into 1 × 1 cm cuvettes and read against a blank of 65 % sulfuric acid, in a Beckman DU spectrophotometer. CDC was measured at 380 mμ and DC, at 385 mμ[4]. An equivalent area of silica gel from a blank column of the plate was similarly treated and the optical density of this was subtracted from the sample readings to obtain the net extinctions.

Isotope techniques

Multiple samples of duodenal contents were obtained from persons who had received tracer doses of either ^{14}C-labeled cholic acid alone or ^{14}C-labeled cholic acid and tritiated CDC (cholic acid-carboxyl-^{14}C and randomly tritiated CDC, prepared by the method of WILZBACH, were supplied by Tracerlab, Waltham, Massachusetts). The CDC-^{3}H was purified by TLC. The administration of ^{14}C-labeled cholic acid to humans results in the appearance of DC-^{14}C in bile. Thus, bile extracts contained DC-^{14}C as well as cholic-^{14}C acid and CDC-^{3}H. After identification of CDC and DC on the TLC plates by spraying with water, the area containing each radioactive bile acid was scraped into plastic counting vials and the amount of radioactivity associated with either CDC or DC was assessed by liquid-scintillation spectrophotometry in a Packard Tri-Carb counter, by a modification of the techniques described by SNYDER AND STEPHENS[6] for labeled fatty acids and tripalmitin. A 4 % solution of Cab-O-Sil in a liquid scintillator described by KINARD[7] was used as scintillating solution; the silica gel from the plates was suspended in this solution by vigorous shaking. Tritium and ^{14}C were counted simultaneously. Silica gel exerted no significant quenching effect[6], in contradiction to the report of EKDAHL and associates[8] who found 10 % quenching with unwashed silica. The relative amounts of the two isotopes present in each sample were calculated by the discriminator-ratio method according to KABARA and co-workers[9].

RESULTS

DC and CDC standards each migrated as single spots on chromatograms. Fig. 1 shows the degree of separation that was obtained, both from mixtures of standards and from extracts of bile. After spraying with water, the edge of each spot was more sharply outlined than it was before spraying. On the other hand, the faint halos around the spots suggest that some minor spreading and diffusion of substances does occur.

When anisaldehyde in sulfuric and glacial acetic acids was used as a stain for bile acids[10], CDC and DC appeared as distinctly different colors, both in visible light and in ultraviolet light, and the different colors were sharply separated from each other. This separation remained sharp when the plates were sprayed with water prior to the staining with anisaldehyde and was especially distinct when viewed under ultraviolet light.

To verify the method of quantitation, various amounts of pure CDC and DC

were measured before and after TLC. Fig. 2 demonstrates that the bile acids followed Beer's law within the concentration ranges studied. The slope of the plot was less steep after TLC; however, linearity remained.

Table I summarizes recovery studies of standards chromatographed as pure substances (singly or combined) or as internal standards added to hydrolyzed bile extracts. Recovery of standards averaged 82 % for DC and CDC with similar values

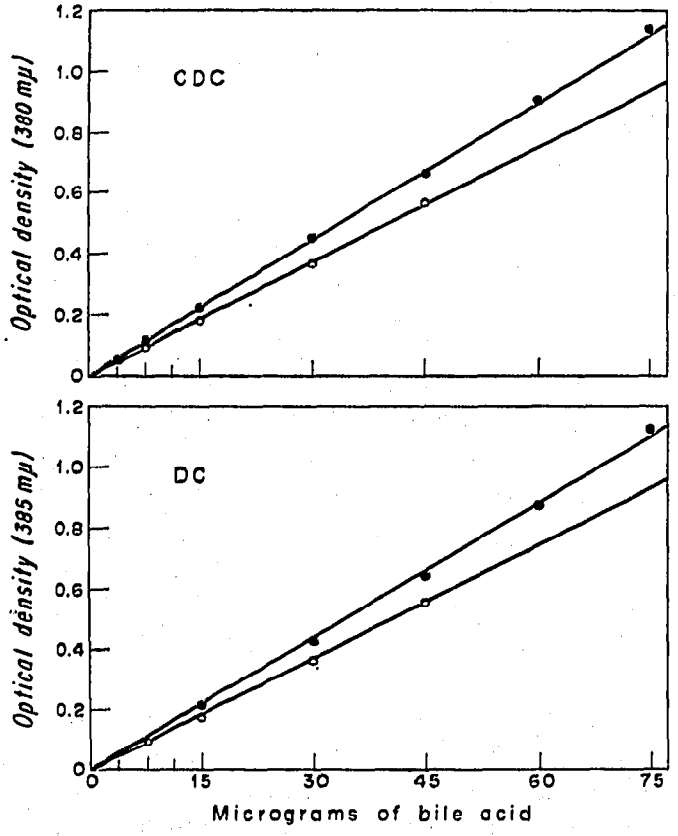

Fig. 2. Standard curves for deoxycholic acid (lower panel) and chenodeoxycholic acid (upper panel) in 4 ml of 65 % sulfuric acid without TLC (solid circles) and following TLC in the presence of silica gel (open circles).

for internal standards. The standard deviations for internal standards were somewhat larger because of the additional variability of the amount of bile acid present in the hydrolyzed extracts.

Studies with varying ratios of CDC and DC in the mixture, ranging from 1:3 to 3:1, resulted in essentially the same percentage recovery for each of the constituents. Only when the amount of one bile acid to be chromatographed was less than 5 μg did the identification by spraying with water become difficult.

CDC and DC were determined in duplicate in 68 samples of hydrolyzed bile extracts; the variation between duplicates is summarized in Table I.

The use of bile acids labeled with ^{14}C and ^{3}H permitted precise evaluation of cross contamination of the bile acids during TLC. After the chromatographic separation of CDC and DC, the total radioactivity of both spots was determined. The contamination of one spot with ^{14}C or ^{3}H counts from the adjacent spot was expressed as a percentage of the total ^{14}C or ^{3}H count associated with the bile acid of the adja-

TABLE I

RESULTS OF RECOVERY AND REPRODUCIBILITY STUDIES

System	*Chenodeoxycholic acid (CDC)*		*Deoxycholic acid (DC)*	
	No. of samples	*Mean ± S.D. (%)*	*No. of samples*	*Mean ± S.D. (%)*
Recovery of pure standards*	26	82.1 ± 4.2	23	82.7 ± 4.8
Recovery of internal standards*	32	82.2 ± 7.9	18	84.8 ± 7.0
Variation between duplicate determinations**	68	2.1 (0–7.1)***	68	2.6 (0–10.5)***

* In each case, 15 μg of CDC or DC was added.
** Calculated as the deviation of the mean from the lower value in duplicate runs.
*** Range of values.

cent area (Table II). Cross contamination was least when the absolute counts for ^{14}C and ^{3}H were in the same order of magnitude. When the disparity between radioactivity in the two bile acids became great, the calculated value for cross contamination tended to be greater for one of the bile acids.

TABLE II

STUDIES OF CROSS CONTAMINATION*

	No. of samples	*Mean ± S.D. (%)*
Contamination of CDC by DC (1 isotope only)	10	8.5 ± 2.9
Contamination of CDC by DC (2 isotopes)	40	7.9 ± 5.2
Contamination of DC by CDC (2 isotopes)	40	9.4 ± 4.6

* Contamination is expressed as percentage of counts found to be associated with the area of CDC when DC was labeled, or vice versa, following separation by TLC.

DISCUSSION

In an excellent review, SJÖVALL[11] discussed the different approaches used for the separation and quantitation of bile acids. GÄNSHIRT and co-workers[5], who first used TLC for the quantitative determination of bile acids, could not quantitate CDC and DC from a mixture because of inadequate chromatographic separation of these two bile acids. Although more suitable solvent systems have been developed in recent years[2,3] to separate these two bile acids, the difference in the R_F values on thin-layer chromatograms remained small.

FROSCH AND WAGENER[12] bypassed this difficulty by scraping off CDC and DC as a combined spot and using a specific color reaction[13,14] for each of the acids. Thus, they were able to use TLC to quantitate each bile acid in the presence of the other from mixtures of pure bile acids and also from duodenal contents[15]. However, a number of factors make it desirable to separate the bile acids before quantitation is carried out. These include the lack of absolute specificity of the color reagents[11] and the modification of the absorption spectra by the presence of silica gel which makes it impossible to measure these colors at their peak optical densities.

In a previous report[1] we noted that commercial silica gel G contains impurities which interfere with the quantitation of bile acids in 65 % sulfuric acid. This proved to be particularly true for CDC and DC. Only when the silica gel was treated by a specific washing procedure before being made into plates could we obtain reproducible results and linear extinctions in the concentration ranges studied. The recovery of standards is quite constant and compares favorably with the results obtained previously[1] for taurocholic, glycocholic, and cholic acids.

The reasons for the consistent 15 to 18 % loss of internal standard are not fully understood. Migration losses during chromatography do not appear to be a significant factor because the same magnitude of loss was noted when standard bile acids were spotted on a TLC plate and the spotted areas were transferred directly to test tubes without chromatography. Studies of the supernate obtained after centrifugation of a mixture of silica and 65 % sulfuric acid revealed the presence of a Tyndall effect and fluorescent properties in this fraction. The Tyndall effect suggests that a colloidal suspension of silica particles remains in the supernate. The Tyndall effect and the fluorescence could interfere with the spectrophotometric determination of the bile acids, but, ordinarily, the silica blank would be expected to correct for this. The addition of more silica gel to the supernate after the color reaction of the bile acids in sulfuric acid had reached its end point did not result in further decreased recoveries. When standard bile acid was pipetted into 65 % sulfuric acid, the characteristic color allowed to develop, and then more silica added and the mixture centrifuged, there was no additional loss of optical density in the supernate. Incomplete elution of the bile acids from the silica gel may be a reason for the losses. However, since these losses are linear in the ranges studied (Fig. 2), they are of no significance as far as the reliability of the method is concerned. It should be emphasized that the recoveries and the low optical densities of the silica blank were consistent only when the duration of the centrifugation of the sulfuric acid suspension was extended to 60 min at 2,700 to 3,000 r.p.m.

Water was used by GÄNSHIRT and co-workers[5] as a spray reagent to locate the different bile acids on the plates. Compared to other means of identification, water has a distinct advantage in that the same spot can subsequently be used for quantitation. It was not possible to locate and identify the CDC and DC spots accurately by staining adjacent strips or columns because of the inevitable small fluctuations in the R_F values between different columns even on the same plate. The detection of as little as 5 μg of bile acid by water spraying is unique for free acids. Larger amounts of conjugated acids are necessary for detection by this technique.

The anisaldehyde stain[10] and the studies with labeled CDC and DC support the feasibility of the approach described. As a consequence of virtually eliminating interfering substances from the silica, the presence of silica became less of a problem and measurements could be carried out at the peak of the absorption spectra. In 68 samples of duodenal contents the ratio between CDC and DC was usually close to 1, with a range of 1:2 to 3:1. In no instance did this range present a problem as far as identification on the plates was concerned.

FROSCH AND WAGENER[15] used the specific color reactions for CDC and DC for the quantitation of the dihydroxycholanic acid conjugates as well as for the free CDC and DC. At present, no solvent systems are known for separation of either the glycine or the taurine conjugates of CDC and DC by TLC. Attempts were made to

quantitate the conjugated dihydroxycholanic acids by a two-step chromatographic procedure. The conjugates were isolated from bile extracts by TLC in suitable systems and identified as previously described[1]. The combined taurine-conjugate spot and the combined glycine-conjugate spot were scraped into separate test tubes and submitted to hydrolysis in the presence of the silica gel. Each hydrolysate was chromatographed again as described above and an attempt was made to quantitate the resulting free bile acids separately. Reproducible results could not be obtained and the recoveries of standards did not exceed 65 %. This is most likely due to the very small amounts (10 to 50 μg) of conjugates subjected to hydrolysis. It has been pointed out that[11,16], when very small amounts of bile acids are hydrolyzed in glass tubes, a significant amount of bile acid material sticks to the glass or may even form silicate complexes. A loss of this type may be enhanced in the presence of silica. When large amounts of conjugated bile acids were hydrolyzed, recoveries of standards after hydrolysis and TLC were only a few per cent lower than after TLC alone. For this reason, whenever possible we used aliquots of bile extract containing at least 1 mg of conjugated bile acid material for hydrolysis.

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SUMMARY

A method is described for quantitating chenodeoxycholic acid and deoxycholic acid in a mixture after separating them by thin-layer chromatography. The procedure involves the preliminary purification of the silica and the identification of the bile acids on thin-layer plates by water spraying. The feasibility of the method and the reproducibility of the results have been demonstrated by special staining techniques and radiotracer studies. The method was applied to human duodenal contents.

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