Analysis of bile acids in conventional and germfree rats

D. Madsen, M. Beaver, L. Chang, E. Bruckner-Kardoss, and B. Wostmann

University of Notre Dame, Notre Dame, Indiana 46556

Abstract The well-known bile acid analysis technique used by us and others (Grundy, Ahrens, and Miettinen. 1965. *J. Lipid Res.* **6:** 397–410) does not allow for the detection of hyodeoxycholic acid, a product of quantitative importance in rodent feces. Using updated methodology, it was established that hyodeoxycholic acid and ω-muricholic acid, both apparent conversion products of β-muricholic acid, occur in appreciable amounts in intestinal contents and feces of conventional Wistar type Lobund rats. In conventional rats, these bile acids comprise about 50% of fecal bile acids; they are not found in intestinal contents or feces of germfree rats. Others have demonstrated that hyodeoxycholic acid is formed by combined action of gut flora and liver.

A new method for the separation of conjugated and free bile acids in biological samples was developed. Results with this method confirmed the total conjugation of bile acids in the germfree rat, and the almost total deconjugation that takes place in the cecum of the conventional rat.

Supplementary key words hyodeoxycholic acid • ω-muricholic acid • keto bile acids • conjugated bile acids • gas–liquid chromatography • thin-layer chromatography

Bile acids are formed in the liver from cholesterol. These primary bile acids are then modified in various ways by gut bacteria to secondary bile acids. The array of primary and secondary bile acids found especially in lower intestine and feces may present problems in qualitative detection. One of the most comprehensive procedures published to date for the identification of bile acids is that of Grundy, Ahrens, and Miettinen (1). This methodology has been used in our laboratory (2, 3) but, in its original form, it does not allow for the detection of hyodeoxycholic acid (HDC). This bile acid is found in considerable amounts in the feces of Lobund/Wistar conventional rats, as is the secondary trihydroxy bile acid, ω-muricholic acid (ω-MC). We report here analysis of conventional and germfree intestinal and fecal bile acids using a procedure modified primarily to detect and quantitate HDC, and a new technique for separation and determination of free and conjugated bile acids.

MATERIALS AND METHODS

Animals

All rats used were 3–6 month-old males of the Lobund/Wistar strain (LOB:(WI)). Germfree rats were housed in flexible plastic isolators according to accepted procedures (4). Conventional animals were maintained under otherwise similar conditions in the temperature and humidity-controlled open animal house. Rats were maintained on wire screen-bottoms during fecal collection, and coprophagy was not prevented. Typically a six rat-day sample was collected for analysis. All colony production had been on commercial diet L-485 (5); but starting at least two weeks before experimental periods, all rats were fed heat-sterilized L-488 (6).

Sample preparation and analysis

The procedures for analysis of bile acids were basically those of Grundy et al (1), with modification. Feces were homogenized in 50% ethanol and made to a convenient volume. Intestinal contents were collected as desired in saline and made to volume with ethanol at a final concentration of 50%. Bile was diluted with an equal volume of 95% ethanol, and aliquots were taken for analysis.

For calculation of recovery, [^{14}C]cholic acid was added to each sample (generally 20 ml) before further processing. Glass boiling beads and then 1.0 ml of 10 N NaOH per 10 ml of sample were added. After refluxing for 1 hr, the sample was cooled and neutral sterols were extracted with hexane.[1] Saponification of the aqueous phase was carried out for 3 hr at 252°F (15 psi) followed by acidification and extraction of bile acids with chloroform. After evaporation of solvent, bile acids were methylated by standing in 10 ml of 5% acetyl chloride in methanol for 16–18 hr. The methylating solution was removed with a rotary evaporator, and bile acids were redissolved in 0.5 ml of chloroform–methanol 2:1.

Bile acids were next applied to a TLC plate[2] and a spot

Abbreviations: Trivial names of bile acids in the text refer to hydroxy-substituted 5β-cholanic acids, as follows: HDC, hyodeoxycholic, 3α,6α; chenodeoxycholic, 3α,7α; deoxycholic, 3α,12α; cholic, 3α,7α,12α; α-muricholic, 3α,6β,7α; β-MC, β-muricholic, 3α,6β,7β; ω-MC, ω-muricholic, 3α,6α,7β; HC, hyocholic, 3α,6α,7α; and LC, lithocholic, 3α. Derivatives with keto or mixed hydroxyl and keto functions are designated by positions of the functions. TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TFA, trifluoroacetate; and TMS, trimethylsilyl.

[1] All organic solvents were high purity grade, and were redistilled in this laboratory before use.

[2] TLC plates: Silica gel G., 250 μ thick, on 20 × 20 cm glass plates; Analtech Laboratories, Newark, Del.

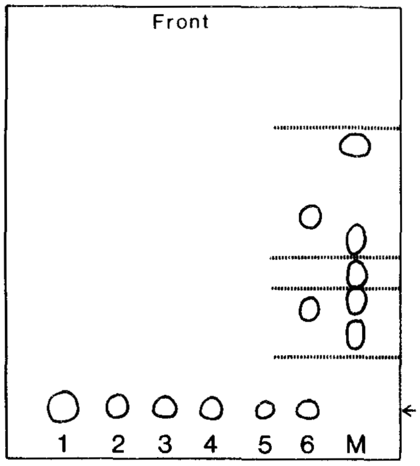

Fig. 1. Representative thin-layer chromatogram of standards of conjugated bile acids and methyl esters of bile acids. Developing solvent: chloroform–acetone–methanol 70:25:5. Arrow denotes position of the origin. Silica gel G, 250 μm thick. 1, Taurocholate; 2, taurochenodeoxycholate; 3, taurodeoxycholate; 4, glycocholate; 5, glycochenodeoxycholate; 6, in ascending order: glycodeoxycholate, ω-muricholate, and deoxycholate; M, mixture of methyl esters of the following bile acids, in ascending order: cholate, β-muricholate, hyodeoxycholate, chenodeoxycholate, and lithocholate. The broken lines indicate the areas removed for analysis by GLC (see text).

standard of methyl lithocholate (LC) was placed next to the origin. The bile acids were then separated from faster moving (R_f = 0.65) fatty acids using a solvent system of chloroform–benzene 1:1 (TLC-I). After development, the plate was sprayed with Kellogg reagent (7) and the spots were visualized with long-wave UV light. The entire origin and the area up to 1.0 cm above the LC standard were collected.

After elution from the gel with acetone, bile acids were streaked on a second plate with spot standards of LC and β-muricholic acid (β-MC)[3] next to the origin. This plate was developed in a bath of isooctane–isopropanol–acetic acid 100:60:1 (TLC-II), which separates the bile acids from most of the pigments present in large intestinal and fecal samples. (Other biological samples such as bile or small intestinal contents may be handled without using this solvent system.) The bile acids were eluted as before from a zone bounded by methyl-LC on the top and methyl-β-MC on the bottom.

Total bile acids were then applied to a third (or second) TLC plate. Spot standards contained β-muricholic acid, cholic acid, hyodeoxycholic acid, deoxycholic acid (and/or chenodeoxycholic), and lithocholic acid. After development in chloroform–acetone–methanol 70:25:5 (8), the plate was dried and bile acids were visualized as before. This solvent system (TLC-III) separated bile acids into fractions appropriate for GLC analysis. The plate was therefore divided into zones (**Fig. 1**) potentially containing the following: *lower zone*, cholic, hyocholic, ω-MC, and β-MC; *middle zone*, HDC with a small portion of β-MC and minor amounts of 7-keto, 3α, 12α; *upper zone*, all other dihydroxy and keto bile acids and all monohydroxy bile acids. Bile acids were eluted from the zones with acetone, and 0.5 mg of cholestane was added to each fraction as internal standard for GLC.

One-fourth of each of the bile acid fractions was used for scintillation counting to assess recovery. The remaining bile acids were converted to trimethylsilyl ethers as described by Grundy et al (1).

Samples were analyzed using a Hewlett-Packard Model 402, dual-U-column oven with dual flame-ionization detectors; or a Packard Model 420, dual-column instrument with dual flame-ionization detectors. Samples were chromatographed on two 6-ft columns: 3% QF-1 and 1% SE-30, both on 100/120 mesh Gas-Chrom Q (Applied Science, State College, Pa.). Oven temperatures were 230°C and 220°C, respectively, with injector and detector ports 20–30°C higher than oven temperatures. Both instruments were interfaced to an Autolab System IV computing integrator, which calculated retention times and integrated peaks areas.

Determination of conjugated and free bile acids

Older methods have employed liquid–liquid extraction to achieve separation of free and conjugated bile acids (6), a procedure we have found ineffective, or have used a TLC method which did not achieve total separation of these components (3). In the present method we have employed selective methylation and TLC to separate free and conjugated bile acids completely. To establish recovery, ^{3}H- and ^{14}C-labeled standards were added prior to analysis.

Samples were collected in 50% ethanol as described under Sample Preparation and Analysis. Four ml of 15% KOH were added to the usual 20-ml sample and the material was left overnight. Subsequently, neutral sterols were extracted with 4 × 40 ml of hexane. After adding 2.5 ml of conc. HCl to the water phase, fatty acids were extracted with 4 × 40 ml of hexane. Ethanol was removed and replaced with water to a volume of 30 ml. 4 × 40 ml of butanol were used to extract the free and conjugated bile acids, reconstituting the water phase each time. The butanol extract was evaporated to dryness and methylated (see under Bile Acids above). In this step, the free bile acids were methylated, while the amide bond of the conjugated bile acids remained unaffected. The mixture was applied to a TLC plate, which was developed in chloroform–acetone–methanol 70:25:5 (TLC-III). Conjugated bile acids remained at or near the origin, while the methyl esters migrated as in the total bile acids analysis (Fig. 1). If the methyl bile acids separated into fractions as desired (as judged by viewing the plate under ultraviolet light) they were recovered from the gel and used immediately for radioactive recovery and GLC analysis. However, in case of insufficient separation it may be necessary to elute the methyl blie acids and repeat TLC-III. The methyl bile acids constituted the "free" bile acid fraction of the sample.

The origin of the TLC plate, carrying all the conjugated bile acids, was scraped into a centrifuge bottle with 20 ml of

[3] β-Muricholic acid was a gift from Dr. H. Eyssen, Rega Institute, Louvain, Belgium.

TABLE 1. Small intestinal and fecal bile acids of the germfree and conventional Wistar Rat

Bile Acids	Germfree		Conventional	
	MIC[a] (14)[b]	Feces (8)	MIC (12)	Feces (16)
		% of Total[c]		
Lithocholate	tr[d]		0.9 ± 0.4	1.2 ± 0.4
Deoxycholate			2.5 ± 0.4	15.6 ± 2.5
Chenodeoxycholate	0.8 ± 0.1	1.4 ± 0.7	0.5 ± 0.2	
Cholate	51.1 ± 2.1	40.5 ± 3.1	69.8 ± 3.2	3.9 ± 0.4
Hyodeoxycholate			5.4 ± 0.8	34.0 ± 2.4
β-Muricholate	47.9 ± 2.1	56.0 ± 3.3	18.8 ± 1.9	2.2 ± 0.4
ω-Muricholate			1.8 ± 0.3	18.7 ± 1.6
3-Keto				1.6 ± 0.4
"Keto-274"[e]			tr	tr
12-Keto, 3α				8.6 ± 1.1
"Keto-375"[f]				9.6 ± 0.9
Other keto[g]	tr	tr	tr	4.4 ± 0.6
Total[h]	22.2 ± 2.8	2.4 ± 0.2	5.1 ± 1.6	5.5 ± 0.7

[a] Third quarter of small intestine.
[b] Number of samples.
[c] Mean ± SE.
[d] tr, trace: less than 0.5%.
[e] Keto bile acid; retention time (relative to cholic acid = 100): SE-30, 120; QF-1, 274, not identical with any available standard.
[f] Keto bile acid; retention time: SE-30, 120; QF-1, 375.
[g] Others include 7-keto, 3α12α, 12-keto, 3α7α; and at least two (probably di-) keto bile acids (see text).
[h] Total mg in MIC; mg/rat/day in feces.

95% ethanol and 20 ml of water. Alkalinization (4 ml of 10 N NaOH) and autoclaving (3 hr) of the sample served two functions: deconjugating the bile acids, and thereby freeing them from the silica gel. (Conjugated bile acids are difficult to desorb quantitatively from silica gel.) After acidification with 5.0 ml of conc. HCl, 25 ml of methanol were added and bile acids were extracted with 3 × 50 ml of chloroform. The extract was dried, methylated, and analysis was carried out as usual. This constituted the "conjugated" bile acid fraction of the sample.

Calculations

Quantitation of data was done using the cholestane internal standard, radioactive recovery factor, and any dilution factors made during analysis. Final results were expressed in convenient units, such as mg of bile acid/rat, or mg/unit body wt.

Occurrence and identification of ω-muricholic, hyodeoxycholic and hyocholic acids

The material that GLC suggested to be ω-MC was purified by TLC and analyzed by TLC, GLC and nuclear (proton) magnetic resonance as described in detail elsewhere.[4] Proof that HDC was present in our samples was obtained by the use of trifluoroacetate (TFA) derivatives (9) of the bile acids since TFA derivatives of cholate and HDC have different relative retention times on the GLC columns. TLC-III was shown to effect a complete separation of cholate and HDC by the use of standards of HDC and [^{3}H]cholate.

The occurrence of HDC made it necessary to establish the presence or absence of hyocholic acid (HC) in bile from germfree and conventional rats. After developing the bile acid extract in the TLC-III system, the area possibly containing β-MC and HC was removed and bile acids were eluted. Since these two bile acids do not separate on either of the GLC columns in use, it was necessary to do a preliminary separation by TLC. The bile acids were streaked on a fresh plate (TLC-IV) and developed in benzene–isopropanol–acetic acid 30:10:1 which separates these bile acids. The two fractions from TLC-IV were then analyzed by GLC.

RESULTS

Determination of free and conjugated bile acid in cecal contents

As a check on the efficiency of our method for determining conjugated and free bile acids, we analyzed cecal contents of germfree and conventional rats. Bile acids in cecal contents of germfree rats were 96% conjugated; in conventional cecal contents at least 96% of the bile acids were in the deconjugated (free) form. These results were as expected. We presume the presence of a few percent of free bile acid in germfree cecal contents to be an artifact of sample preparation.

[4] Madsen, D., B. Wostmann, and D. Pasto. 1975. Occurrence and possible physiological significance of ω-muricholic acid in the rat. (Manuscript in preparation.)

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Identification of hyodeoxycholic, ω-muricholic, and hyocholic acids

Analysis by GLC of TFA derivatives of the appropriate TLC-III fractions of fecal samples of conventional rats had indicated that materials with relative retention times of both HDC and cholic acid were present. However, since these do not resolve in the currently used GLC system as TMS ethers, it became necessary to determine the efficacy of the TLC-III system to effect a complete separation of cholic acid from HDC. When standards of ^{3}H-labeled cholic acid and HDC were mixed, and developed on TLC-III, cholic acid and HDC were found by GLC in their appropriate zones, with no bile acid detectable in the intervening region. 80–90% of the ^{3}H was found in the cholic acid region, the remainder being distributed unevenly along the plate to the solvent front due to impurity of the [^{3}H]cholic acid stock. Additional proof that, in the TLC procedure, cholic acid does not carry over into the HDC zone is found in the fact that biological samples, to which [^{14}C]cholic had been added to determine procedural losses, seldom showed any label in the HDC zone. Contamination of the cholic acid zone by HDC was excluded by GLC of TFA derivatives of this zone, showing no peak indicative of HDC.

The identity of ω-MC in the conventional rat was established through comparison of putative ω-MC purified from rat feces with a sample of authentic material.[5] Identification was confirmed by TLC, GLC, and proton magnetic resonance spectra.[4] GLC analysis of the trihydroxy bile acid range of TLC-III, prepared as described above, never indicated HC in bile samples of conventional or germfree rats.

Intestinal and fecal patterns in germfree and conventional rats

Comparison and average amounts of bile acids from the third quarter of small intestines and feces of germfree and conventional Wistar rats are given in **Table 1.** Total amounts of bile acids approximated those reported earlier (5), with germfree animals excreting less per day, but retaining much higher levels in the enterohepatic circulation. However, present techniques demonstrate a relatively large amount of HDC, especially in the feces of the conventional rat. Neither HDC nor its supposed derivative, ω-HC[4] (10) are present in germ-free rats. Together with ω-MC, HDC may comprise 50% of total fecal bile acids of the conventional male Wistar rat.

Approximately one quarter of all bile acids found in feces of conventional rats are present in the form of keto acids (Table 1). Tentative identification is based on comparison of GLC retention times on SE-30 and QF-1 with values from the literature, and values obtained with standard preparation from commercial or private sources. "Keto-274" and "keto-375" are putative bile acids eluted from the upper range of TLC-III (retention time on QF-1, relative to cholic acid = 100, 274, and 375, respectively). They always occur in conventional feces, but at this time they cannot be clearly identified by the above means. In general, only traces of keto acids were found in small intestinal contents.

On the average, 92.0% of the GLC peak area could be identified in material originally from conventional rats and 98% in germfree rat samples. α-Muricholic acid is not resolved from cholic by any of the TLC or GLC procedures, but the former bile acid constitutes only a small percentage of the total in this rat strain (2).

DISCUSSION

The present methodology for identification and quantitation of enterohepatic bile acids is an extension of procedures described by Grundy et al. (1). It may seem lengthy in requiring three separate TLC steps. However, the first two of these (which are slightly modified from (1)) are essential to separate the bile acids from the mass of other materials occurring, especially in large intestinal contents and feces. TLC-III then separates bile acids into discrete fractions convenient for GLC analysis; this is especially suitable and necessary for the separation of cholic acid from HDC, the latter an important metabolite in rats and mice. In the hands of an experienced technician, recovery of originally added [^{14}C]-cholic acid carried through the various procedures usually exceeds 90%. In less "dirty" biological samples two TLC steps may be sufficient—the first (TLC-I) and the third (TLC-III).

Bile acids found in the third quarter of the small intestine (Table 1) represent the physiologically functional pattern before major reabsorption in the ileum and alteration by large intestinal microflora. Fecal bile acid patterns reflect those microbially caused changes in bile acids occurring in the cecum and colon of the rat. Of particular interest here is the pathway involving β-MC, HDC, and ω-MC. This pathway appears to be dependent on the occurrence of the primary bile acid, β-MC. Combinations of microbial and hepatic actions convert this bile acid to HDC, thence to ω-MC[4] (10). This sequence may have important implications for the quantitative aspects of bile acid and cholesterol metabolism in the rat.[4]

The recent publication by Cohen et al. (11) describes a methodology based on (1). However, although they report substantial amounts of fecal ω-MC from rats, their method fails to detect HDC. This is a crucial point because of the quantitative importance of HDC, at least in rodents, and because of the probable relationship between HDC and ω-MC[4] (10).

The present methodology could be simplified if a particular column packing were available that separated cholic and HDC, as well as all of the other bile acids found in rodents. Because of the quantitative importance of keto bile acids in rodent feces, we utilized the present GLC system since it allows measurement of these bile acids within a reasonable time, as well as of all hydroxy bile acids. We are not yet aware of a GLC column packing(s) that will separate all of the components listed in Table 1. Samples (e.g., human feces (12)) which do not contain HDC would not require analysis by

[5] ω-Muricholic acid and some keto bile acids were a gift from W. Elliott, Department of Biochemistry, St. Louis University, St. Louis, Missouri.

this methodology. Also, laboratories not requiring analysis of most of the individual bile acids could forego GLC in favor of enzymatic quantification.

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