Biliary bile acids, bile alcohols, and sterols of *Alligator mississippiensis*

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Abstract Bile from *Alligator mississippiensis* was found to contain a mixture of more than twenty bile acids, bile alcohols, and neutral sterols. Bile acids and bile alcohols were purified by reversed-phase high performance liquid chromatography and thin-layer chromatography. Concentrations were measured by gas–liquid chromatography on 1% HiEFF-8BP and identifications were made by mass spectrometry. The major neutral sterols consisted of 98% cholesterol and 0.8% cholestanol. Bile acids recovered from the acidic fraction were 3α,7α,12α-trihydroxy-5β-cholestanoic acid (61%), 3α,7α-dihydroxy-5β-cholestanoic acid (9%), 3α,7α,12α-trihydroxy-5α-cholestanoic acid (8%), and 3-oxo-7α,12α-dihydroxy-5β-cholestanoic acid (10%). Other C_{27} bile acids identified were: 3α,12α-dihydroxy-5β-cholestanoic acid, 7-oxo-3α,12α-dihydroxy-5β-cholestanoic acid, and 3-oxo-7α,12α-dihydroxy-5α-cholestanoic acid. Small quantities of C_{24} cholic, 5α-cholic, chenodeoxycholic, and ursodeoxycholic acids were also detected, as were trace amounts of the C_{27} bile alcohols 5α-cholestane-3α,7α,12α,26-tetrol, and 5β-cholestane-3α,7α,12α,25-tetrol. These results suggest that the alligator is capable of synthesizing both the 5α and 5β-isomers of the C_{27} bile acids. The small amounts of the C_{24} bile acids present might originate either from C_{27} bile acid or bile alcohol precursors or from exogenous sources.—**Tint, G. S., B. Dayal, A. K. Batta, S. Shefer, T. Joanen, L. McNease, and G. Salan.** Biliary bile acids, bile alcohols, and sterols of *Alligator mississippiensis*. *J. Lipid Res.* 1980. **21:** 110–117.

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Early studies of bile acid metabolism in the rat and in man led to general agreement among most investigators (1) that the C_{27} bile acid 3α,7α,12α-trihydroxy-5β-cholestanoic acid was an obligate precursor in the biosynthesis of cholic acid from cholesterol. Several recent investigations (2–9) have provided evidence for the existence of a second pathway and suggest that at least some cholic acid may be synthesized via a 25-hydroxylation pathway with 5β-cholestane-3α,7α,12α,25-tetrol and 24S-5β-cholestane-3α,7α,12α,24,25-pentol as intermediates. Since the major bile acid of the American alligator, *Alligator mississippiensis*, is known to be 3α,7α,12α-trihydroxy-5β-cholestanoic acid (10–12) synthesized from chlesterol (13), this reptile might be interesting as a potential animal model to study side-chain oxidations in the biosynthesis of cholic acid. Although it has been reported previously that bile from *A. mississippiensis* is a highly complex mixture of bile acids, only one other compound, 3α,7α-dihydroxy-5β-cholestanoic acid has, as yet, been identified (14, 15).

It would seem, therefore, that before comparisons can be made between human and alligator bile acid metabolisms, a more complete knowledge of the components of alligator bile must be secured. The following report describes the bile acids, bile alcohols, and neutral sterols found in the bile of *A. mississippiensis*.

METHODS

Bile analysis

Gallbladder bile was collected in the field by needle aspiration from several wild specimens of *A. mississippiensis*. The samples were placed on ice within 2 hours and were frozen 3 days later. The bile was maintained at −20°C for 3 weeks until it was analyzed; a portion was cultured and was found to be sterile. A 2-ml por-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography; TMSi, trimethylsilyl; RRT, relative retention time (to 5α-cholestane); RTR, retention time ratio; cholic acid, 3α,7α,12α-trihydroxy - 5β - cholanoic acid; chenodeoxycholic acid, 3α,7α - dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; cholesterol, cholest-5-ene-3β-ol; cholestanol, 5α-cholestan-3β-ol.

tion of the bile was refluxed with 20 ml of 1N ethanolic NaOH for 1 hr. The non-polar neutral sterols (3.1 mg) were extracted with 3 × 50 ml portions of hexane. Then the ethanol layer was evaporated, 15 ml of 2N aqueous NaOH was added, and the bile acids were deconjugated by heating for 3 hr at 120°C. After acidification to pH 1–2 with 10N HCl and the addition of 20 ml of methanol, the free bile acids and the polar bile alcohols (36 mg recovered) were extracted with 3 × 40 ml portions of chloroform. The mixture was evaporated to dryness with benzene–methanol 86:14 (v/v) and was methylated by overnight treatment with 5 ml of 3N methanolic HCl (Supelco, Inc., Bellefonte, PA). Under these conditions, recoveries of 3α,7α,12α-trihydroxy-5β-cholestanoic acid were 20–30%. With increased alkaline concentration and prolonged heating this can be increased to 50–60%.

The bile components were then identified by a combination of TLC, GLC, HPLC and mass spectrometry and were quantitated by GLC and HPLC.

Thin-layer chromatography (TLC)

The neutral sterols were separated by argentation TLC (16). The plates were made by dipping precoated 0.25 mm silica gel O plates (Analabs, North Haven, CT) in a solution of A_gNO_3, 10% by weight, in methanol–water 1:1 (v/v) and then drying in air prior to activating at 110°C for 30 min.

The plates were developed in chloroform–acetone 97:3 (v/v) at 4°C. In this system, the R_f value for cholesterol was 0.25 while that of cholestanol was 0.32. Sterols were made visible by ultraviolet illumination after spraying the plates with a half saturated aqueous solution of rhodamine 6G and were recovered by elution with ethyl ether.

The bile acid methyl esters were chromatographed on pre-coated 0.25 mm silica gel O plates developed in chloroform–acetone–methanol 70:20:5 (v/v/v) at room temperature. The bands were located by spraying both edges of the plate with 3.5% phosphomolybdic acid in isopropanol and heating only the sprayed regions on a hot plate. The esters on the central unsprayed, unheated, portions were then recovered by elution with methanol.

Gas–liquid chromatography (GLC)

Trimethylsilyl (TMSi) ether derivatives of aliquots of the neutral sterols, bile alcohols, and bile acid methyl esters were formed by treatment with 100 μl of Sil-Prep (Applied Science Laboratories, Inc., State College, PA) at 45°C for 30 min. The TMSi ethers of the neutral sterols were injected onto a 4 mm ID × 120 cm silanized glass column packed with 3% QF-1 on Gas-Chrom Q 80/100 mesh (Applied Science) operated at 225°C with a nitrogen flow of 25 ml/min. The TMSi ether derivatives of the bile alcohols and bile acid methyl esters were chromatographed on a 4 mm ID × 180 cm silanized glass column packed with 1% HiEFF-8BP on Gas-Chrom Q 100/120 mesh (Applied Science) at 240°C and a nitrogen flow of 40 ml/min. The instrument used was a Hewlett-Packard Model 5830A equipped with a flame ionization detector, with the injector temperature set at 250°C and the detector temperature at 275°C.

Quantitation was accomplished by mixing a known quantity of 5α-cholestane with a measured aliquot of the sample and then comparing areas under the GLC curves. All of the retention times listed in the text are given as relative (RRT) values which are calculated with respect to the absolute retention time of 5α-cholestane (5.5 min on 1% HiEFF-8BP, 9.5 min on 3% QF-1).

High performance liquid chromatography (HPLC)

The alligator biliary bile acids were chromatographed as the methyl esters on a Waters Associates ALC 201 system employing a Waters model 401 refractive index detector and a Waters 4 mm ID × 30 cm μBondapack C_{18} column (Waters Associates Inc., Milford, MA). The column was operated in the reversed-phase mode using two aqueous solvent systems; the more polar of the two, MWC 1, consisted of methanol–water–chloroform in the ratio 80:25:3 (v/v/v) while the less polar, MWC 2, was methanol–water–chloroform 85:17:3. The elution volumes at a flow of 1.0 ml/min for several bile acid methyl ester and bile alcohol standards chromatographed with both solvent systems are listed in **Table 1.** At a flow of 1.0 ml/min, solvent MWC 2 yielded 2800 theoretical plates for methyl cholate. The solvents used were reagent grade and were filtered and degassed by passing them through a 0.5 micron Millipore filter. The eluant was collected manually as each peak appeared on the recorder trace, and the resulting HPLC fractions were re-chromatographed on HPLC, TLC and GLC and were subjected to analysis by gas–liquid chromatography–mass spectrometry.

Mass spectrometry

Gas–liquid chromatography–mass spectrometry was carried out on a Varian MAT-111 GLC–mass spectrometer as described previously (7, 8).

Standard compounds

Methyl 3-keto-7α,12α-dihydroxy-5β-cholanoate and methyl (25RS) 3-keto-7α,12α-dihydroxy-5β-cholestanoate were synthesized according to the procedure of Dayal et al. (17). Methyl cholate or methyl

TABLE 1. HPLC Elution volumes for a number of standard bile alcohols and bile acid methyl esters chromatographed on a μBondapack C_{18}, 4 mm × 30 cm, reversed-phase column

Compound	Elution Volume (ml)		Corresponding HPLC Peaks from Alligator Bile[c]
	MWC1[a]	MWC2[b]	
5β-cholestane-3α,7α,12α,24ξ,25-pentol	8.42	5.54	
Methyl ursodeoxycholate	10.13	6.62	2
Methyl cholate	11.12	7.23	3
Methyl 7-keto-3α-hydroxy-5β-cholanoate	12.54	7.74	
Methyl 5α-cholate	12.64	7.77	4
5β-cholestane-3α,7α,12α,25-tetrol	14.13	7.76	5
Methyl 7-keto-3α,12α-dihydroxy-5β-cholestanoate	15.14	7.98	6
Methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate	17.72	9.92	8
Methyl chenodeoxycholate	19.87	11.09	9
Methyl deoxycholate	19.98	11.23	
Methyl 3α,7α,12α-trihydroxy-5β-cholestanoate	23.30	12.19	10
Methyl lithocholate	40.10	20.31	
Methyl 3α,7α-dihydroxy-5β-cholestanoate	46.63	21.11	13
5β-cholestane-3α,7α,12α-triol		23.96	

[a] Solvent; methanol–water–chloroform 80:25:3 (v/v/v) at 1.0 ml/min.
[b] Solvent; methanol–water–chloroform 85:17:3 (v/v/v) at 1.0 ml/min.
[c] See Tables 2 and 3.

(25RS) 3α,7α,12α-trihydroxy-5β-cholestanoate dissolved in benzene, was refluxed for several hours with an oxidizing reagent consisting of silver carbonate adsorbed to Celite, a procedure that selectively oxidizes the C-3 hydroxyl group of the bile acids. (25RS) 3α,7α,12α-trihydroxy-5β-cholestanoic acid and (25RS) 3α,7α-dihydroxy-5β-cholestanoic acid were synthesized by the electrolytic coupling of the half ester of methyl succinic acid with cholic and chenodeoxycholic acid respectively (18). (25RS) 7-keto-3α,12α-dihydroxy-5β-cholestanoic acid was prepared by the oxidation of (25RS) 3α,7α,12α-trihydroxy-5β-cholestanoic acid by N-bromosuccinimide in dioxane–water (19). The resulting compounds were purified by TLC on Silica Gel O plates as described above. No attempt was made to separate the 25R and 25S stereoisomers, nor was any attempt made to determine the absolute symmetry of the bile acids found in the sample of alligator bile.

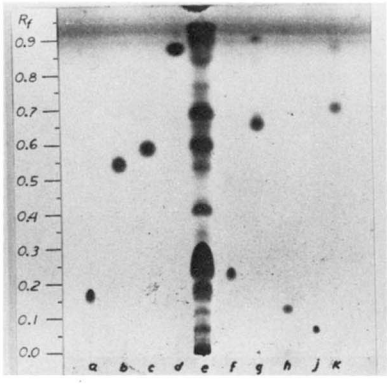

Fig. 1. Thin-layer chromatogram of the methylated acidic fraction of alligator bile together with standard compounds. Silica Gel O plate developed in chloroform–acetone–methanol 70:20:5 (v/v/v). a. methyl cholate; b. methyl chenodeoxycholate; c. methyl deoxycholate; d. methyl lithocholate; e. alligator bile; f. methyl 3α,7α,12α-trihydroxy-5β-cholestanoate; g. methyl ursodeoxycholate; h. methyl 5α-cholate; j. 5β-cholestane-3α,7α,12α,25-tetrol; k. methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate.

Methyl 5α-cholate, methyl 5α-chenodeoxycholate, and methyl 3-keto-7α,12α-dihydroxy-5α-cholanoate were gifts from Professor W. H. Elliott of the Department of Biochemistry, St. Louis University School of Medicine (20, 21). 5α-cholestane-3α,7α,12α,26-tetrol was a gift from Professor N. Hoshita, Department of Biochemistry, Hiroshima School of Medicine (22). 5β-cholestane-3α,7α,12α,25-tetrol was isolated from the bile of a patient with cerebrotendinous xanthomatosis (7).

Identification by GLC retention time ratios (RTR)

The bile acid retention time ratio (RTR) is defined as the quotient of the GLC retention times (or relative retention times) of any two TMSi ether bile acid methyl esters which have the *same* side chain. Observations in our laboratory[1] of consistencies in the GLC retention times of a large number of TMSi ether bile acid methyl esters chromatographed on 1% HiEFF-8BP have led us to propose the following two rules for these ratios:

a) If we let RRT(N_i,S_j) be the relative retention time of any TMSi ether bile acid methyl ester with nuclear configuration N_i and side chain S_j then,

[1] G. S. Tint. Unpublished observations.

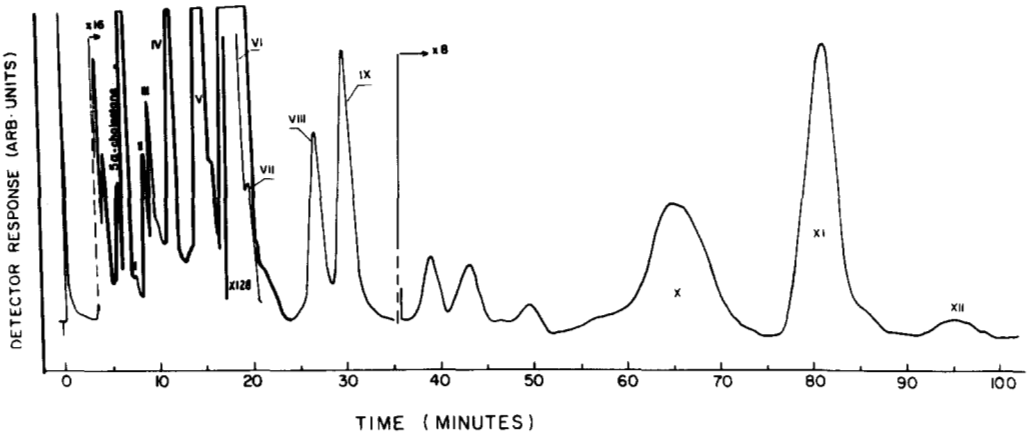

Fig. 2. GLC chromatogram of the TMSi ether derivative of the methylated acidic fraction of alligator bile. Column: 1% HiEFF-8BP (180 cm) at 240°C and N_2 flow 40 ml/min.

$RTR(N_1,N_2) = RRT(N_1,S_1)/RRT(N_2,S_1) = RRT(N_1,S_2)/RRT(N_2,S_2)$.

b) If we let N_i* represent nucleus N_i whose A/B ring junction has been inverted, then, provided there are no C-3β-OTMSi substituents in N_i, $RRT(N_1,S_1)/RRT(N_2,S_1) = RRT(N_1*,S_1)/RRT(N_2*,S_1)$.

Rule a) can be illustrated by noting that the RTR of the TMSi ether derivatives of methyl chenodeoxycholate (retention time relative to 5α-cholestane, 2.76) and methyl cholate (RRT 1.70) is 1.62 while the RTR of the corresponding C_{27} bile acids, methyl 3α,7α-dihydroxy-5β-cholestanoate (RRT 4.21) and methyl 3α,7α,12α-trihydroxy-5β-cholestanoate (RRT 2.54) is 1.66. An example of rule b) is the comparable value (1.63) of the RTR of TMSi methyl 5α-chenodeoxycholate (RRT 2.30) and TMSi methyl 5α-cholate (RRT 1.41) to that of their 5β-analogs.

RESULTS

Neutral sterols

Cholesterol was the major sterol found in the bile of *A. mississippiensis* and constituted 98% of the total neutral sterol fraction. The only other non-polar sterol which could be characterized was cholestanol which was present at a concentration of 0.8%.

Bile acids and bile alcohols

As many as fifteen spots could be detected by TLC (**Fig. 1**), while eighteen chromatographic peaks were seen by GLC (**Fig. 2**). **Fig. 3** is an HPLC chromatogram of the same fraction obtained with solvent system MWC 2 demonstrating that seventeen distinct peaks were observed by this technique. HPLC peaks 8–13 were sufficiently resolved and were collected individually while peaks 1–7 were collected together and were re-chromatographed with the more polar solvent MWC 1 (**Fig. 4**).

Table 2 lists the TLC, GLC and HPLC parameters of ten compounds which were identified by comparing their mass spectra and chromatographic behavior with those of authentic compounds. The HPLC peaks are also listed in Table 1 along with the corresponding standard compounds. **Table 3** notes three additional compounds whose structures were suggested by their mass spectral and chromatographic properties but for which no standards were available.

Only the relative concentrations of the recovered bile acids are indicated in Tables 2 and 3. When two compounds are listed as having the same GLC retention time they were not resolvable by this technique but were separable by HPLC and/or TLC.

The data included in Tables 2 and 3 indicate that the acidic fraction of the bile of *A. mississippiensis* consists predominately of C_{27} bile acids and that 3α,7α,12α-trihydroxy-5β-cholestanoic acid (61%) is the major

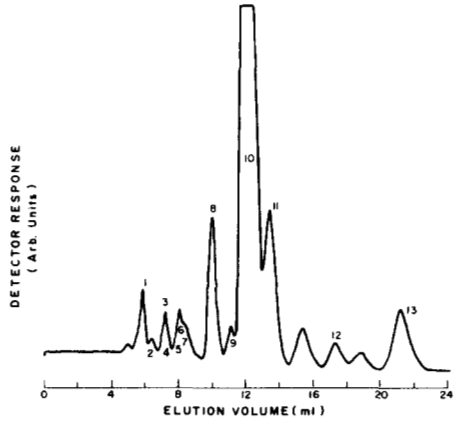

Fig. 3. HPLC chromatogram of the methylated acidic fraction of the bile of *A. mississippiensis*. Column: C_{18} μBondapack; Solvent: MWC 2 methanol–water–chloroform 85:17:3 (v/v/v) at 1.0 ml/min.

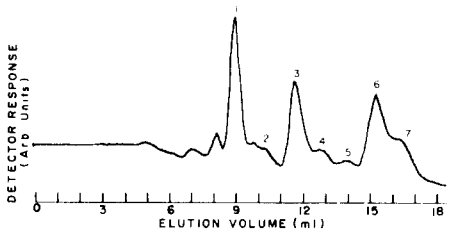

Fig. 4. Peaks 1 through 7 of the chromatogram shown in Fig. 3 re-chromatographed using solvent system MWC 1 methanol–water–chloroform 80:25:3 (v/v/v) at 1.0 ml/min.

compound. Appreciable amounts of 3α,7α-dihydroxy-5β-cholestanoic acid (9%) and 3-keto-7α,12α-dihydroxy-5β-cholestanoic acid (10%) are also present.

Trace quantities of C_{24} bile acids such as cholic acid (1%), 5α-cholic acid (0.2%), and chenodeoxycholic acid (0.4%) were identified, as were two C_{27} bile alcohols (Table 2).

Several other C_{27} bile acids were detected whose mass spectra indicated that they possessed a trihydroxylated nucleus. The most abundant of these compounds chromatographed as peak 11 on HPLC (8%, Table 3, Fig. 3). The mass spectrum of its TMSi ether derivative exhibited a weak molecular ion at m/e 680, and major fragments at m/e 590 (3%), 500 (42%), 410 (13%), 343 (92%), 253 (64%), 226 (9%), and 211 (14%). When this mass spectrum was compared to one obtained from a sample of the TMSi ether derivative of methyl 5α-cholate the relative intensities of "analogous" mass peaks (peaks produced by the identical fragmentation mechanisms in both spectra) were found to be very similar. This consistency of fragmentation pattern together with the observation that the retention time ratios (RRT) of the TMSi ether derivatives of methyl cholate (RRT 1.70) and methyl 5α-cholate (RRT 1.41) were equal to the RTR of the TMSi ethers of methyl 3α,7α,12α-trihydroxy-5β-cholestanoate (RRT 2.54) and HPLC peak 11 (RRT 2.12) suggested that this latter compound was methyl 3α,7α,12α-trihydroxy-5α-cholestanoate.

HPLC peak 13 was separated into two components by TLC. The more polar compound chromatographed with an R_f of 0.60 and corresponded to methyl 3α,7α-dihydroxy-5β-cholestanoate (Table 2) while the less polar one exhibited an R_f of 0.70 (Table 3). The mass spectrum of the TMSi ether derivative of this latter component gave a weak molecular ion at m/e 592 and peaks at m/e 577 (2%), 502 (1%), 487 (1%), 471 (1%), 412 (7%), 397 (3%), 381 (3%), 345 (12%), 255 (100%), 228 (3%), and 213 (8%). The above intensities were in excellent agreement with those obtained from the "analogous" mass peaks of TMSi ether methyl deoxycholate. The conclusion that this compound was methyl 3α,12α-dihydroxy-5β-cholestanoate was supported by the observation that the RTR of the TMSi ethers of methyl deoxycholate (RRT 2.58) and methyl cholate (RRT 1.70) was equal to the RTR of the TMSi ethers of the above compound (RRT 3.87) and methyl 3α,7α,12α-trihydroxy-5β-cholestanoate (RRT 2.54).

The mass spectrum of the TMSi ether derivative of the compound corresponding to HPLC peak 7 was almost identical to the spectrum obtained from the TMSi ether derivative of methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate. A difference between the two was seen, however, in the relative intensities of the mass peaks observed in the region of m/e 356, M-(C-1 to C-4 + 2 × 90), as illustrated in **Fig.**

TABLE 2. Chromatographic properties and relative concentrations of the completely characterized bile acids and bile alcohols found in the bile of *A. mississippiensis*

Compound	HPLC Peak	HPLC Ve (ml)[a]	GLC Peak[b]	TLC R_f[c]	% of Total Recovered Bile Acids
Methyl ursodeoxycholate	2	6.39	IX	0.67	0.5
Methyl cholate	3	7.18	IV	0.16	1
Methyl 5α-cholate	4	7.72	III	0.12	0.2
5β-Cholestane-3α,7α,12α,25-tetrol	5	7.98[d]	II	0.07	0.1
Methyl 7-keto-3α,12α-dihydroxy-5β-cholestanoate	6	7.98[d]	XII	0.61	0.5
Methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate	8	9.97	XI	0.69	10
Methyl chenodeoxycholate	9	11.02	VII	0.54	0.4
Methyl 3α,7α,12α-trihydroxy-5β-cholestanoate	10	12.00	VI	0.23	61
Methyl 3α,7α-dihydroxy-5β-cholestanoate	13	21.53	IX	0.60	9
5α-Cholestane-3α,7α,12α,26-tetrol[e]			I		0.1

[a] Retention volumes; chromatographed on C_{18} μBondapack, solvent MWC2. Fig. 3.
[b] Chromatographed as the TMSi ethers on 1% HiEFF-8BP (180 cm) at 240°C and N_2 flow 40 ml/min. Fig. 2.
[c] Chromatographed on Silica Gel O in chloroform–methanol–acetone 70:20:5 (v/v/v). Fig. 1.
[d] These compounds were separable with solvent MWC1. Fig. 4.
[e] Only seen by GLC.

TABLE 3. Chromatographic properties and relative concentrations of some of the partially characterized bile acids found in the bile of *A. mississippiensis*

Tentatively Assigned Structure	HPLC Peak	HPLC Ve (ml)[a]	GLC Peak[b]	TLC R_f[c]	% of Total Recovered Bile Acids
Methyl 3-keto-7α,12α-dihydroxy-5α-cholestanoate	7	8.38	X	0.71	0.4
Methyl 3α,7α,12α-trihydroxy-5α-cholestanoate	11	13.24	V	0.18	8
Methyl 3α,12α-dihydroxy-5β-cholestanoate	13[d]	21.11[d]	VIII	0.70	0.5

[a] Retention volumes; chromatographed on C_{18} μBondapack, solvent MWC2. Fig. 3.
[b] Chromatographed as the TMSi ethers on 1% HiEFF-8BP (180 cm) at 240°C and N_2 flow 40 ml/min. Fig. 2.
[c] Chromatographed on Silica Gel O in chloroform–methanol–acetone 70:20:5 (v/v/v). Fig. 1.
[d] Separated from methyl 3α,7α-dihydroxy-5β-cholestanoate by TLC. Table 2.

5. This suggested that HPLC peak 7 was the 5α isomer of methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate (20, 21, 23, 24).

Additional evidence for the assignment of methyl 3-keto-7α,12α-dihydroxy-5α-cholestanoate as the structure of this compound was provided by the retention time ratios of the TMSi ether derivatives of the following four pairs of compounds. The RTR of HPLC peak 7 (RRT 9.38) and methyl 3α,7α,12α-trihydroxy-5α-cholestanoate (RRT 2.12) is 4.42 while the RTR of methyl 3-keto-7α,12α-dihydroxy-5α-cholanoate (RRT 6.29) and methyl 5α-cholate (RRT 1.41) is 4.46. The RTRs for the corresponding isomeric 5β compounds are 4.45 for both methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate (RRT 11.30) and methyl 3α,7α,12α-trihydroxy-5β-cholestanoate and for methyl 3-keto-7α,12α-dihydroxy-5β-cholanoate (RRT 7.56) and methyl cholate.

The mass spectra of the TMSi ether derivatives of HPLC peak 1 and HPLC peak 12 (GLC peaks VIII and IX, relative concentrations 1% and 0.8% respectively) indicated that they were the methyl esters of trihydroxy C_{27} bile acids. They could not be characterized further.

DISCUSSION

High performance liquid chromatography has proved to be very useful in resolving the complex mixture of bile acids and bile alcohols obtained by the alkaline hydrolysis of the bile of *A. mississippiensis*. Using a two-stage reversed-phase HPLC separation in combination with TLC, 15 components were isolated and characterized from the acidic fraction of the hydrolysate. This two-step HPLC procedure was found to be more sensitive and more efficient than a single-stage separation with a solvent more polar than system MWC1 which would have resulted in exceedingly long retention times for the less polar compounds.

Eight bile acids and two bile alcohols were identified by direct comparison of their mass spectral and chromatographic properties with those of authentic compounds (Table 2). 3α,7α,12α-trihydroxy-5α-cholestanoic acid and 3α,12α-dihydroxy-5β-cholestanoic acid were tentatively identified by comparison of the mass spectra of their TMSi ether methyl esters with those of analogous C_{24} bile acids (Table 3).

The compound which eluted as peak 7 on HPLC (Table 3) was tentatively identified as methyl 3-keto-7α,12α-dihydroxy-5α-cholestanoate, because of the overall similarity of the mass spectrum of its TMSi ether derivative with that of the TMSi ether of authentic methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate.

The assignment of the 5α configuration of this compound was based on the difference between the relative intensities of the peak seen at m/e 356 in the mass spectra of these two compounds (Fig. 5). This fragment, which is formed by cleavage of ring A leading to the expulsion of carbons 1 to 4 along with two molecules of trimethylsilanol ($HOSi(CH_3)_3$, 90 amu) is considerably less intense in the spectrum of the TMSi either of HPLC peak 7. The more facile cleavage of ring A in 5β compounds as compared to their 5α-isomers was first noted for the 3-keto-steroids (23) and has been used to differentiate between the

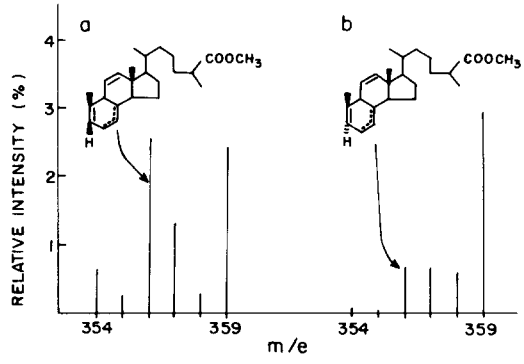

Fig. 5. Partial mass spectra in the vicinity of m/e 356 of the TMSi ether derivatives of methyl 3-keto-7α,12α-dihydroxy-5β-cholestanoate (a) and HPLC peak 7 (b).

5α and 5β isomers of the 3-oxo derivatives of cholic (20), chenodeoxycholic (21), and deoxycholic (24) acids. The GLC retention time ratios (RTR) were consistent with all of the above structures. The RTR proved to be a very valuable parameter in suggesting the most probable structures for several of these compounds.

Until the present study, only 3α,7α,12α-trihydroxy-5β-cholestanoic and 3α,7α-dihydroxy-5β-cholestanoic acids (10, 11, 15, 16) had been identified in alligator bile. The results summarized in Tables 2 and 3 suggest that there are several other families of bile acids present. Small amounts of the C_{24} analogues of these two C_{27} bile acids were found as well as the 5α epimeric forms of both the C_{24} and C_{27} 3α,7α,12α-trihydroxy acids. The concentrations were such that the ratio of the amount of cholic acid to the amount of 5α-cholic acid was about equal to the ratio of the concentrations of the 5β and 5α epimeric C_{27} 3α,7α,12α-trihydroxy acids. The physiological significance of these results is not clear. Also, it is not known whether the C_{24} bile acids are endogenous or are absorbed from the diet.

It has been demonstrated that the alligator makes 3α,7α,12α-trihydroxy-5β-cholestanoic acid from cholesterol (14), however, it has not been established whether a pathway exists in the alligator for the conversion of the C_{27} 3α,7α,12α-trihydroxy acid (or C_{27} 3α,7α-dihydroxy acid) to cholic acid (or chenodeoxycholic acid).

5α-Bile acids have been chemically synthesized from their 5β-analogues via a 3-keto intermediate (25). The simultaneous isolation of both the 5α- and 5β-isomers of 3-keto-7α,12α-dihydroxy cholestanoic acid and 3α,7α,12α-trihydroxy-cholestanoic acid from alligator bile suggests the possibility that the biochemical isomerization of the cis-A/B to the trans-A/B ring junction may proceed by a similar mechanism.

Although it has been established that cholestanol is a precursor of the 5α bile acids in several animal species (26) very little (0.8% of the total sterol fraction) of this compound was found in these samples of bile.

The significance of the bile alcohols, 5α-cholestane-3α,7α,12α,26-tetrol and 5β-cholestane-3α,7α,12α,25-tetrol as intermediates in bile acid synthesis in this species requires further exploration.

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