

Bile Acids of the Teleost *Cottus quadricornis*:* Isolation of 3 α ,12 α -Dihydroxy-5 β -chol-7-enoic Acid

Bile Acids and Steroids 199 **

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Gall bladder bile of *Cottus quadricornis* was found to contain trihydroxycholanoic acids and dihydroxycholanoic acids in a ratio of 4:1. The following bile acids were identified by gas liquid chromatography and combined gas liquid chromatography-mass spectrometry: cholic acid, deoxycholic acid, chenodeoxycholic acid, and 3 α ,12 α -dihydroxy-5 β -chol-7-enoic acid.

The teleost *Cottus quadricornis* is a Baltic and fresh water relict belonging to the order Scleroparei. This fish and other species of the family Cottidae have been extensively used as experimental animals in biochemical,^{1,2} physiological,^{3,4} and histological^{5,6} studies and a thorough ecological study on *Cottus quadricornis* will be published.⁷

Previous studies have indicated that bile from most teleosts contains taurocholic acid as the main trihydroxy bile acid and taurochenodeoxycholic acid as the predominant dihydroxy bile acid.⁸ The present study describes the composition of the bile acids in gall bladder bile of *Cottus quadricornis*.

EXPERIMENTAL

Three batches of animals were caught at various places in the archipelago of Stockholm and in the Bothnian Sea. One of the batches was caught in August, the other two in November. The animals feed differently at various times of the year, the main diet consisting of Crustacea and Mollusca. Different varieties of *Cottus quadricornis* have been

* During the publication of this report the name of the fourhorn sculpin has been changed to *Myoxocephalus quadricornis* (L).

** The following systematic names are given for the compounds referred to in the text by trivial names: cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid; chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholanoic acid; deoxycholic acid, 3 α ,12 α -dihydroxy-5 β -cholanoic acid; and apocholic acid, 3 α ,12 α -dihydroxy-5 β -chol-8(14)-enoic acid.

The following abbreviations are used: GLC, gas-liquid chromatography; TLC, thin layer chromatography; MS, mass spectrum; RRT, relative retention time; TFA, trifluoroacetate.

recognized in the Baltic as well as in the lakes.⁷ The animals used were of both sexes and between 15 and 20 cm long. Their gall bladders usually contained less than 0.5 ml of bile. The animals were killed by a stitch in the head and the entire gall bladder immediately removed and put into ethanol. Gall bladders from about 150 animals were collected, torn by a Waring Blendor and filtered. The filtrate was made alkaline by addition of an equal amount of 2 N KOH and extracted with ether. The aqueous fraction was hydrolyzed in a sealed steel tube at 120° for 6 h and again extracted with ether. These ether fractions were not analyzed. The aqueous phase was acidified with 2 N HCl and acids extracted with ether. After evaporation of the ether, the residue was dissolved in methanol and methylated by the addition of freshly prepared diazomethane.

TLC of the crude bile was performed in a mixture of butanol, acetic acid, and water (10:1:1).⁹ Dihydroxycholanoic acids and derivatives were chromatographed in phase system S11 and trihydroxycholanoic acid derivatives in phase system S6.¹⁰ The chromatoplates were sprayed with concentrated H₂SO₄ and developed by heating to about 130°.

After methylation, the dihydroxycholanoic and trihydroxycholanoic acid fractions were separated by chromatography on a column of aluminium oxide, activity grade IV. The column was eluted with increasing amounts of ethyl acetate in benzene. Ethyl acetate, 10–20 % in benzene, eluted a crude mixture of methyl dihydroxycholanoates and the methyl trihydroxycholanoate fraction was eluted with ethyl acetate and 5 % methanol in ethyl acetate.

GLC analyses of methyl esters, trifluoroacetates, and oxidation products of the methyl esters were performed on a 6 foot × 5 mm column with 3 % QF-1 as stationary phase. TFA's were also analyzed on a similar column with 1 % SE-30 as stationary phase. Column temperature was 215–245° and argon pressure 2.2 kg/cm².¹¹ TFA's and oxidation products of the bile acids were prepared as described by Eneoth *et al.*¹¹

Mass spectra were recorded with a combined GLC-MS instrument (LKB 9000) using an SE-30 coated column.

3 α ,12 α -Dihydroxy-5 β -chol-8(14)-enoic acid (apocholic acid) was prepared as described by Devor and Marlow,¹² and had m.p. 166–167°, reported m.p. 166–168°. This compound showed a negative Liebermann-Burchard reaction. *3 α ,12 α -Dihydroxy-5 β -chol-7-enoic acid* was prepared by the method described by Berner *et al.*,¹³ and had m.p. 208°, reported m.p. 210–212°. This compound was fast reacting with Liebermann-Burchard reagent giving an absorption maximum at 355 m μ . Methyl esters were prepared by dissolving the acids in methanol and adding diazomethane.

Methyl *3 α ,12 α -dihydroxy-5 β -chol-7-enoate*, 5 mg, was isomerized to methyl apocholate by shaking with Adams catalyst under hydrogen for 4 h.¹⁴ As estimated from the decrease in the absorption at 355 m μ isomerization had occurred to more than 80 %. No methyl deoxycholate was found by GLC of the trifluoroacetylated reaction mixture. This method of analysis would have revealed the presence of less than 1 % of methyl deoxycholate.

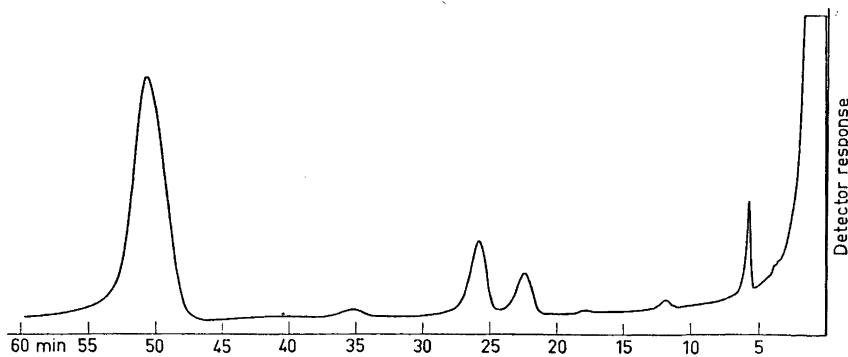


Fig. 1. GLC of methyl esters of crude bile hydrolysate on a QF-1 column. Conditions as described in the text.

Table 1. RRT's of isolated bile acid fractions and suitable reference compounds. The retention time of methyl deoxycholate has arbitrarily been put to 1.000.

Compound	QF-1 column			SE-30 column
	Methyl ester	TFA	Oxidation product	TFA
Dihydroxy fraction	0.987	0.562	2.55	0.230
	1.14	0.662	2.76	0.260
		0.839		0.316
Dihydroxy fraction after treatment with Pt/H ₂ and acetic acid			2.59	0.231
			2.80	0.260
Trihydroxy fraction	2.21	1.36	5.85	0.314
Cholic acid	2.22	1.33	5.90	
Deoxycholic acid	1.000	0.662	2.78	0.262
Chenodeoxycholic acid	1.14	0.838	2.78	0.314
3 α ,12 α -Dihydroxy-5 β -chol-7-enoic acid	0.993	0.566	2.55	0.232
Apocholeic acid			2.60	0.228

RESULTS

TLC of the crude bile showed the presence of materials with the same mobilities as simultaneously run taurine conjugates of trihydroxycholanoic acids and dihydroxycholanoic acids. The solvent system used does not separate taurodeoxycholic acid and taurochenodeoxycholic acid. No material was present at the place characteristic of glycine-conjugated bile acids.

A GLC-recording of the total bile acid fraction — after hydrolysis and methylation — is shown in Fig. 1. Peaks at 23, 26, and 50 min have the same RRT's as methyl deoxycholate, methyl chenodeoxycholate, and methyl

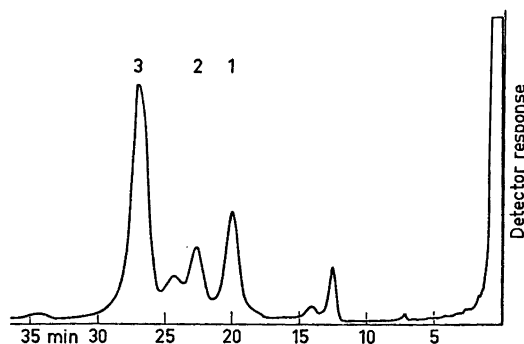


Fig. 2. GLC of TFA of methyl esters of dihydroxycholanoic acid fraction. Numbers are referred to in the text.

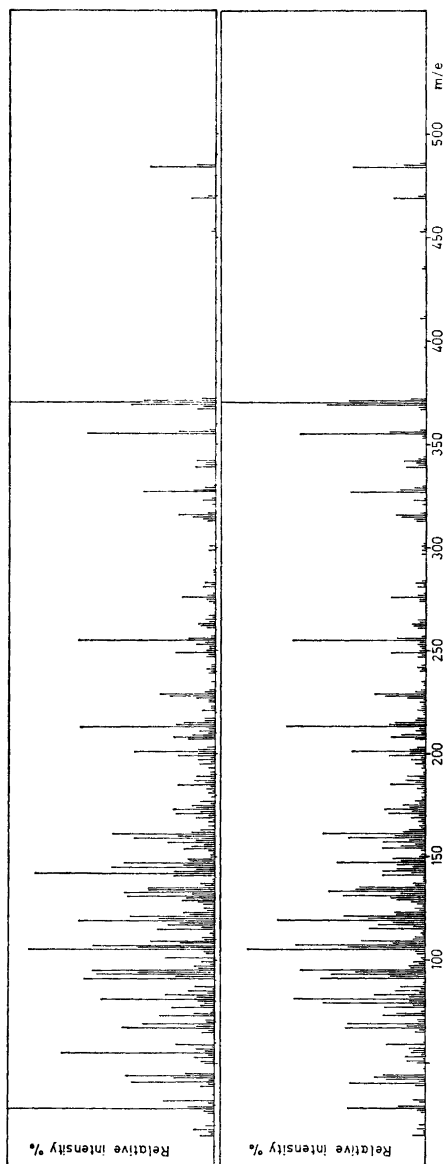


Fig. 3. MS of TFA of compound 3 (left) and TFA of methyl chenodeoxycholate (right).

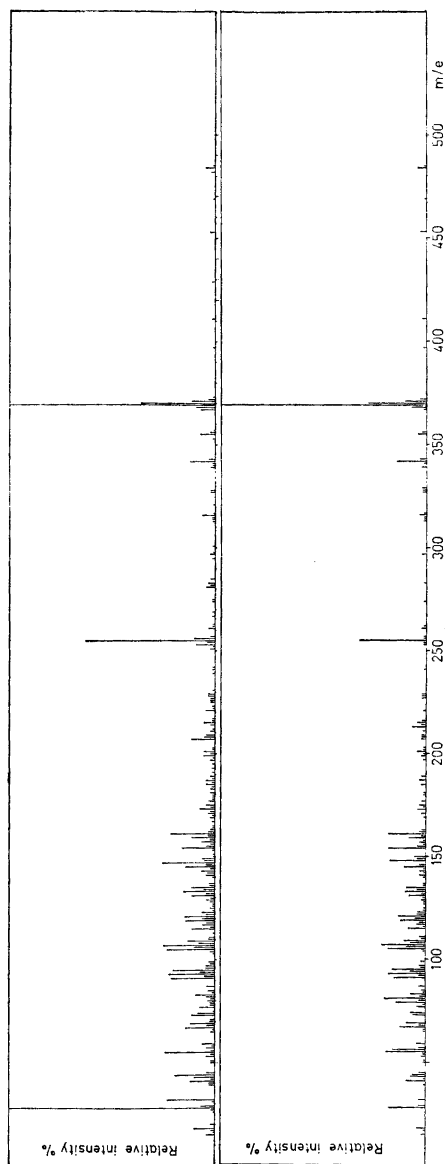


Fig. 4. MS of TFA of compound 2 (left) and TFA of methyl deoxycholate (right).

cholate, respectively. By weight, the ratio between the trihydroxycholanoic acids and dihydroxycholanoic acids was about 4:1. This ratio was about the same in the three different bile samples.

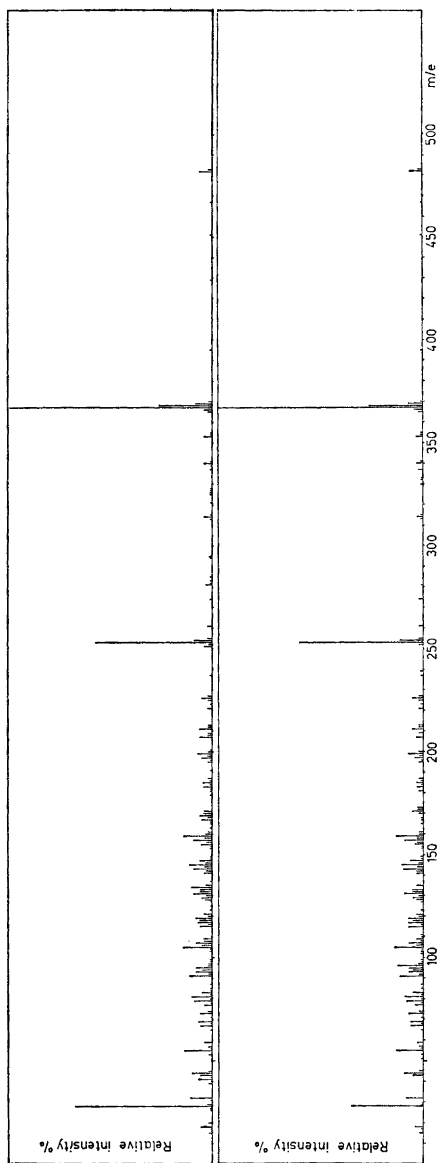


Fig. 5. MS of TFA of compound 1 (left) and TFA of methyl ester of $3\alpha,12\alpha$ -dihydroxy- 5β -chol-7-enoate (right).

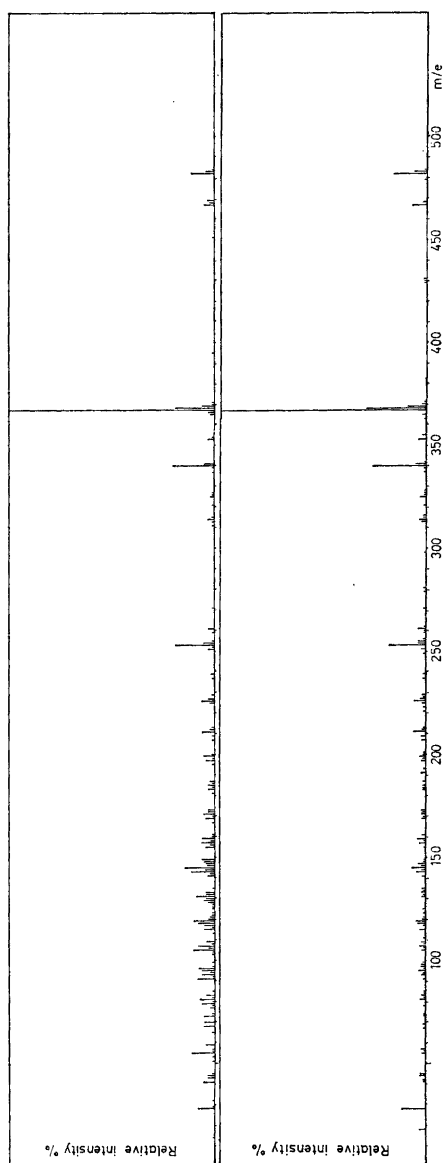


Fig. 6. MS of TFA of the reduction product with the same GLC properties as the TFA of compound 1 (right) and of TFA of methyl apocholate (left).

The methyl trihydroxycholanoate fraction isolated from the aluminium oxide column was further analyzed by TLC and by GLC. GLC was also performed on the TFA and after oxidation with Djerassi's reagent. By these methods this fraction proved homogeneous and showed the same properties as the corresponding derivatives of cholic acid (Table 1). The MS of the trifluoroacetylated methyl ester of this compound was identical with that of methyl cholate-TFA. After crystallization from methanol, the methyl ester of the isolated material had a m.p. of 156°. No depression of the melting point of this compound was observed on admixture with methyl cholate.

GLC of the TFA of the methyl dihydroxycholanoate fraction was shown to contain several compounds, three of which were invariably present in about the same relative amounts in different bile samples (Fig. 2). The MS of these compounds are shown in Figs. 3, 4, and 5. The MS of compounds 2 and 3 nicely corresponded to the TFA of methyl deoxycholate and methyl chenodeoxycholate, respectively, and the identity of these compounds was further established by determination of the RRT's for the TFA's and the oxidation products on a QF-1 column (Table 1).

The MS of compound 1 (Fig. 5) showed a poor fragmentation at lower mass numbers, a feature characteristic of 3,12-dihydroxycholanoic acid derivatives. The base peak occurred at m/e 367, two units lower than in the spectrum of the TFA of methyl deoxycholate where the peak at m/e 369 (M-229) is ascribed to the fragment M-(CF₃COOH+the elements of the side chain).¹⁵ The occurrence of the base peak two mass units lower than in the MS of methyl deoxycholate-TFA indicates the presence of a double bond in the steroid nucleus of compound 1. A large peak is also seen at m/e 367 in the MS of methyl cholate-TFA where it is attributed to the fragment M-(2CF₃COOH+the elements of the side chain). Thus, the spectrum and the chromatographic properties of compound 1 imply that this compound is an unsaturated 3,12-dihydroxycholanoic acid with the double bond in the steroid nucleus.

A small sample of the methyl dihydroxycholanoate fraction was treated with H₂/Pt in acetic acid. GLC of the TFA of the reaction mixture showed a chromatogram that was identical to that obtained before this treatment (Table 1). The MS of the peak which corresponded to compound 1 (Fig. 6) also showed a base peak at m/e 367 (cf. Fig. 5), but an increase was observed of the peak at m/e 340 and a decrease of the peak at m/e 253 compared with the spectrum of compound 1. The fragment m/e 253 is recognized as an ion consisting of the steroidal A, B, C, and D rings including 3 double bonds. The fragment m/e 340 is interpreted as an ion formed by the loss from the fragment m/e 367 of two carbon atoms (16 and 17) and three hydrogen atoms, equal to m/e 27. The increase in the intensity of the peak at m/e 340 in the "reduced compound 1" could be explained as an effect of the migration of a 7(8) double bond to the 8(14) position during the reducing conditions. Thus, carbon atom 15 would be in an allylic position to the latter double bond and a fission of the bond between carbon atoms 15 and 16 would be enhanced.¹⁶ In comparing the MS of the TFA's of the reference compounds, methyl 3 α ,12 α -dihydroxy-5 β -chol-7-enoate and methyl apocholate, the same characteristic differences were observed (Figs. 5 and 6). The identity of compound 1 with

3 α ,12 α -dihydroxy-5 β -chol-7-enoic acid was further established by determination of the RRT for the TFA and oxidation product on a QF-1 column (Table 1).

DISCUSSION

Chemical. The dihydroxycholanoic acid fraction of *Cottus quadricornis* invariably contained three different bile acids which could be separated by GLC of the TFAs of the compounds. It was not possible, however, to separate these compounds on a preparative scale and the components therefore had to be identified in the mixture. The identity of the compounds was established by comparing the RRT's of different derivatives on various columns with those of the reference compounds. An indication of the position of the hydroxyl groups is obtained by oxidation and this treatment may also reveal configurational differences in the carbon skeleton, e.g. the ratio between the RRT's of methyl 3,12-diketo-5 α -cholanoate and methyl 3,12-diketo-5 β -cholanoate is 1.18.¹⁷ It should be pointed out, however, that it is not known if such a difference would be found between the RRT of the 5 α - and the 5 β -epimers of the 7(8) or 8(14) unsaturated homologues of deoxycholic acid. Therefore the 5 β -structure of compound 1 is assigned on the basis of chromatographic and mass spectrographic identity with a reference compound.

Biological. The main bile acids in teleosts are cholic acid and chenodeoxycholic acid, whereas the occurrence of deoxycholic acid has been uncertain.⁸ The present study has revealed that the main bile acid present in *Cottus quadricornis* is taurocholate and that taurine conjugates of three different dihydroxycholanoic acids, chenodeoxycholic, deoxycholic, and 3 α ,12 α -dihydroxy-5 β -chol-7-enoic acids, occur. The latter acid is a bile acid which has not been previously isolated from biological sources. In fact, no sterol with the cholic acid nucleus and a double bond in the nucleus has previously been isolated from bile.¹⁸

In mammals, cholic acid and chenodeoxycholic acid are primary bile acids produced in the liver by oxidation of cholesterol. During the enterohepatic circulation of bile these acids are modified by the action of intestinal microorganisms and secondary bile acids are formed. The main secondary bile acid is deoxycholic acid, formed by the dehydroxylation of cholic acid. In some animals, e.g. the rat, the deoxycholic acid absorbed from the intestine is again hydroxylated in the liver and excreted as cholic acid conjugates in the bile. In other animals, e.g. the rabbit, no hydroxylation occurs during the enterohepatic circulation and deoxycholic acid is one of the main bile acids in bile.

The occurrence of deoxycholic acid in the bile of teleosts has been previously denied¹⁹ or uncertain,⁸ which could be interpreted to be due to either absence of 7-dehydroxylation in fish intestine, or efficient 7 α -hydroxylation during the enterohepatic circulation. The presence of deoxycholic acid in gall bladder bile of *Cottus quadricornis* raises the question whether deoxycholic acid is a primary or secondary bile acid in this species. If deoxycholic acid is a secondary bile acid, the question also arises whether or not the mechanism of the dehydroxylation is the same as in mammals.

The dehydroxylation of cholic acid in the intestine of mammals proceeds by means of the intermediary formation of $3\alpha,12\alpha$ -dihydroxy- 5β -chol-6-enoic acid.²⁰ $3\alpha,12\alpha$ -Dihydroxy- 5β -chol-7-enoic acid is apparently not formed and cannot be metabolized by the rat.²⁰ The presence of $3\alpha,12\alpha$ -dihydroxy- 5β -chol-7-enoic acid in gall bladder bile of *Cottus quadricornis* is not understood at present and work is in progress to establish the origin of this acid. It should be borne in mind that $3\alpha,12\alpha$ -dihydroxy- 5β -chol-7-enoic acid might be of dietary origin — molluscs for instance are known to be rich in 3β -hydroxy- 5α - Δ^7 -sterols.²¹ On the other hand, the batches of fish were caught at various places and under various dietary habits and $3\alpha,12\alpha$ -dihydroxy- 5β -chol-7-enoic acid was invariably present in bile.

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