Analyses of bile from gallbladders of fish *(Arius platystomus, Arius tenuispinis, Pomadasys commersonni* and *Kishinoella tonggol)*

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Abstract: Bile from gallbladders of *Arius platystomus* (Singhara), *Arius tenuispinis* (Khagga), *Pomadasys commersonni* (Holoola) and *Kishinoella tonggol* (Dawan) were derivatised and analysed by GC-MS for identification of bile acids and bile alcohols. Cholic acid and Chenodeoxycholic acid were found as major bile acids in *Arius platystomus*, *Arius tenuispinis* and *Pomadasys commersonni*. Other bile acids identified in *Arius platystomus* were *allo*chenodeoxycholic acid, *allo*deoxycholic acid, 3α,7α,12α-trihydroxy-24-methyl-5β-cholestane-26-oic acid, and 3α,7α,12α, 24-tetrahydroxy-5α-cholestane-26-oic acid. Cholesterol was found as major bile alcohol in *Arius platystomus*, *Arius tenuispinis* and *Pomadasys commersonni*. Cholic acid was the major bile acid identified in the bile of *Kishinoella tonggol* while other bile acids included 3α,7α,12α-tridydroxy-5α-cholestanoic acid and 3α,7α,12α-tridydroxy-5β-cholestanoic acid. Bile alcohol 5β-cyprinol was present in significant amounts with 5β-cholestane-3α,7α,12α,24ξ-tetrol being the other contributors in the bile of *Kishinoella tonggol*.

Keywords: Bile alcohols, bile acids, GC-MS, Marine fish.

INTRODUCTION

Cholesterol catabolism yields either bile alcohols or bile acids (fig. 1). Bile alcohols are usually esterified with sulfate groups whereas bile acids conjugate with taurine or glycine (Hofmann and Hagey, 2008).

Scientists and clinicians take interest in bile acids for numerous reasons. Firstly, they are quantitatively important as about half of the cholesterol elimination occurs through its conversion to bile acids. Secondly, bile acids derivatives the bile salts play central role in digestion and absorption of dietary fats and exert potent antimicrobial activity in the small intestine (Hofmann and Hagey, 2008). Thirdly, their secretion in bile maintains normal bile flow and prevents cholestasis, a primary factor for build-up of cholelithiasis. Most importantly, certain bile acids have proven therapeutic applications (Thistle and Hofmann, 1973). Finally, recent studies have revealed that bile acids play fundamental role as cellular regulatory molecules sometimes inducing apoptosis (Qiao *et. al.*, 2002) while at other occasions inhibiting the same (Rodrigues *et. al.*, 1998, Rodrigues *et. al.*, 2003).

Structural diversity in bile salts and its relation with biodiversity leads to significant facts indicating the molecular evolution in the basic C_{27} skeleton of cholesterol. Most of the compounds isolated from the bile are found to contain C_{24} to C_{27} cholestane skeleton. C_{26} alcohols and acids are also produced by the oxidation of the side chain of Cholesterol (Hofmann and Hagey, 2008; Hoshita, 1985).

The earliest evolving fish are believed to be the jawless fish (Agnatha), which are currently available as hagfish and lampreys (Forey and Janvier, 2000, Nelson, 2006). Jawless fish were found to have C_{27} bile alcohols. Sea lamprey is the only fish with C_{24} bile alcohol (5α-petromyzonol) (Haslewood, 1969) while 5α-myxinol (as disulfate) is the major bile alcohol in hagfish (Haslewood, 1966).The major bile salt of all species of Elasmobranchii (sharks, skates, and ray; subgroup of Chondrichthyes or cartilaginous fish) is 5β-scymnol (Bridgwater *et. al.*, 1962) while the major bile alcohol of chimaerae is 5β-chimaerol (Bridgwater *et. al.*, 1963). Ray-finned fish (Actinopterygii), consisting of a greater number of species (Nelson, 2006), bears common C_{24} bile acids, cholic acid and chenodeoxycholic acid as major bile salts. The most primitive living member of bony fish is the *Latimeria chalumnae*, with atimerol as the main constituent of the bile salt (Anderson and Haslewood, 1964). The principal component in *Protopterus aethiopicus* and *Lepidosiren paradoxa* is the C_{26} (or C_{27})-sulfate of 5α-cholestane-3α, 7α,12α,26, 27-pentol (5α-cyprinol). *Neoceratodus forsteri* bile salts were found to contain an appreciable proportion of the sulfate of 5α-cholestane-3α, 7α, 12α, 25, 26-pentol (5α-bufol) (Tammer, 1974). Bile acids of channel catfish (*Ictaturus puctatus*) and blue catfish (*Ictaturus furcatus*) contain taurocholic acid, taurochenodeoxycholic acid and

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taurodeoxycholic acid (von Kellogg, 1975). The bile salts, haemulcholate conjugated with taurine, isolated from *Parapristipoma trilineatum* is unique, with a hydroxyl group at C_{22} (Hoshita *et. al.*, 1967).

In the present study four different species of marine fish (ray-finned fish) were selected to determine the composition of bile salts isolated from their gallbladder bile. In analyzing the bile salts variation patterns, the direct pathway (evolutionary transition from C_{27} bile alcohols to C_{24} bile acids) was found to be common in ray-finned fish, as both C_{27} bile alcohols and C_{24} bile acids were identified in their bile, but C_{27} bile acids were not found in appreciable amount. In contrast to fish, amphibians and reptiles both follow indirect pathway and C_{27} bile acids are common in their bile.

MATERIALS AND METHODS

Samples collection

Various fish were purchased from fish Harbor West Wharf and identified by marine Fisheries Department, Fish Harbor West Wharf, Karachi, Pakistan in 2000. The gallbladder bile from 4-8 fish of each category were removed and dropped into ethanol. Evaporation of the filtered solution left crude bile salts.

Extraction of unconjugated bile acids and bile alcohols from fish bile

The crude bile salts (2.0g) were dissolved in 100 ml water and extracted with two 50 ml portions of petroleum ether to de-fat the content. The aqueous layer was acidified with 2N HCl and then extracted with three 50 ml portions

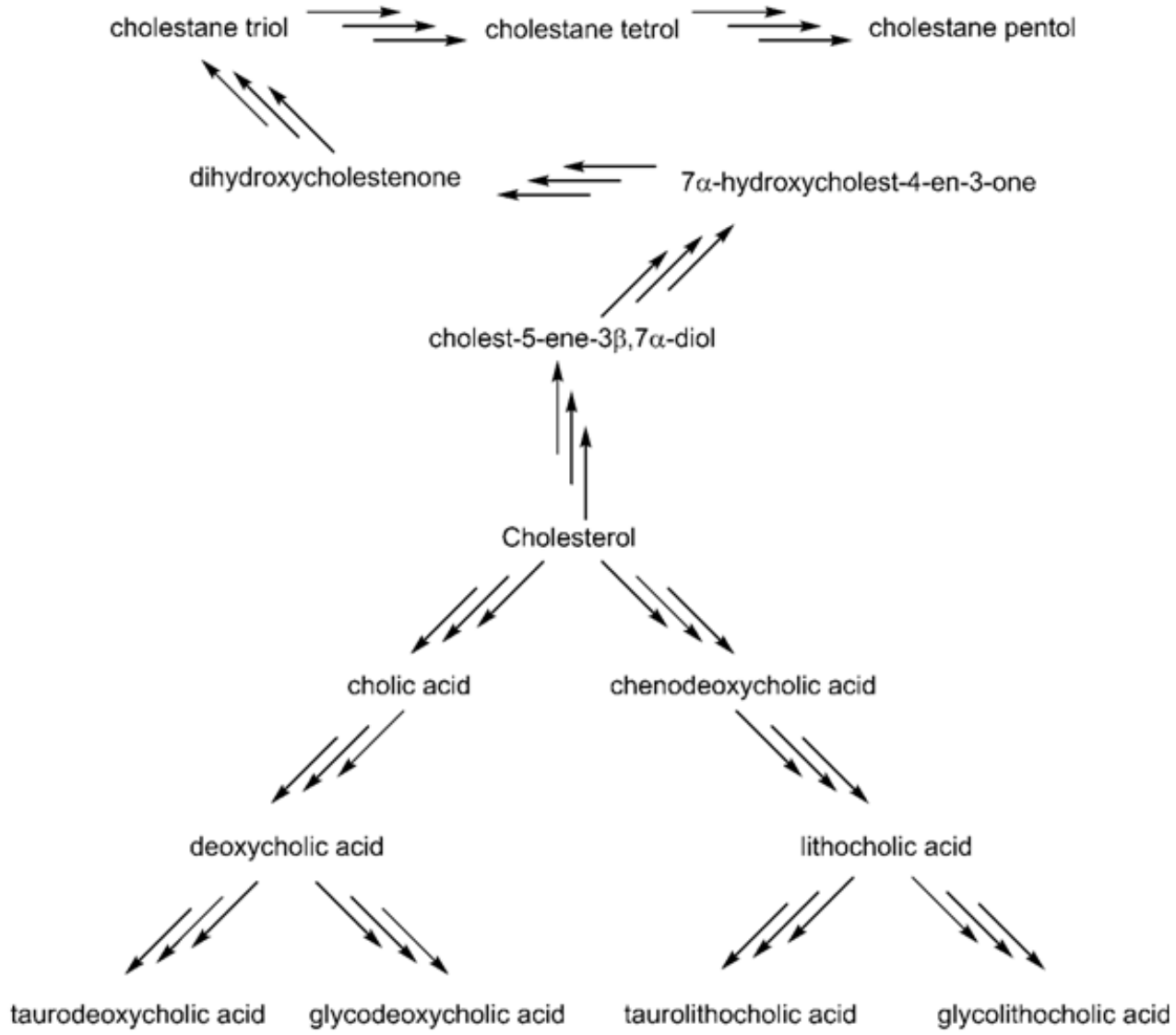


Fig. 1: Major bile acids and bile alcohols from cholesterol metabolism

Table 1: Relative retention times of bile acids on GC-MS

Identified Compound	R.R.T*	R.T.T**	R.T.T***	Fish
Cholic acid **(1)******	1.00	0.90	-	I, II, III, IV
Chenodeoxycholic acid **(2)******	1.11	1.00	-	I, II, III
Allochenodeoxycholic acid **(3)**	1.02	0.92	-	I
Allodeoxycholic acid **(4)**	1.36	1.22	-	I
3α, 7α, 12α-trihydroxy-24 methyl-5β-cholestan-26-oic acid **(5)**	1.59	-	-	I
3α, 7α, 12α, 24-tetrahydroxy-5α-cholestan-26-oic acid **(6)**	1.94	-	-	I
Cholesterol **(7)******	-	-	1.00	I, II, III
5β-Cholestane-3α, 7α, 12α, 24-tetrol **(8)******	-	-	1.23	IV
5β-Cyprinol **(9)******	-	-	2.22	IV

*Relative to methyl cholate; ** Relative to methyl chenodeoxycholate;*** Relative to cholesterol. Bile acids and Bile alcohols were chromatographed as their methyl ester-TMS ethers and TMS respectively;**** identified using GC-MS data. fish: *Arius platystomus(I)*, *Arius tenuispinis(II)*, *Pomadasys commersonni(III)* and *Kishinoella tonggol(IV)*

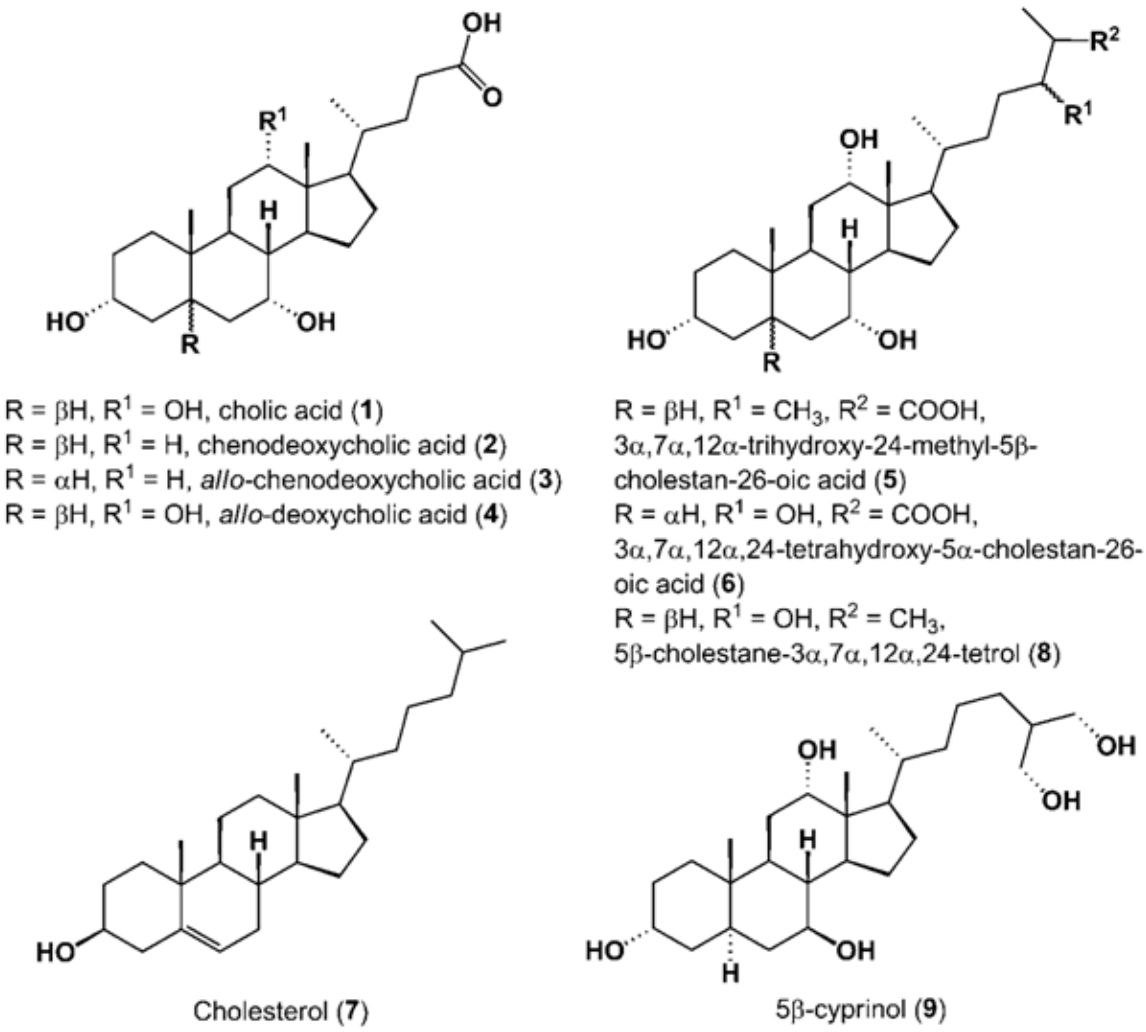
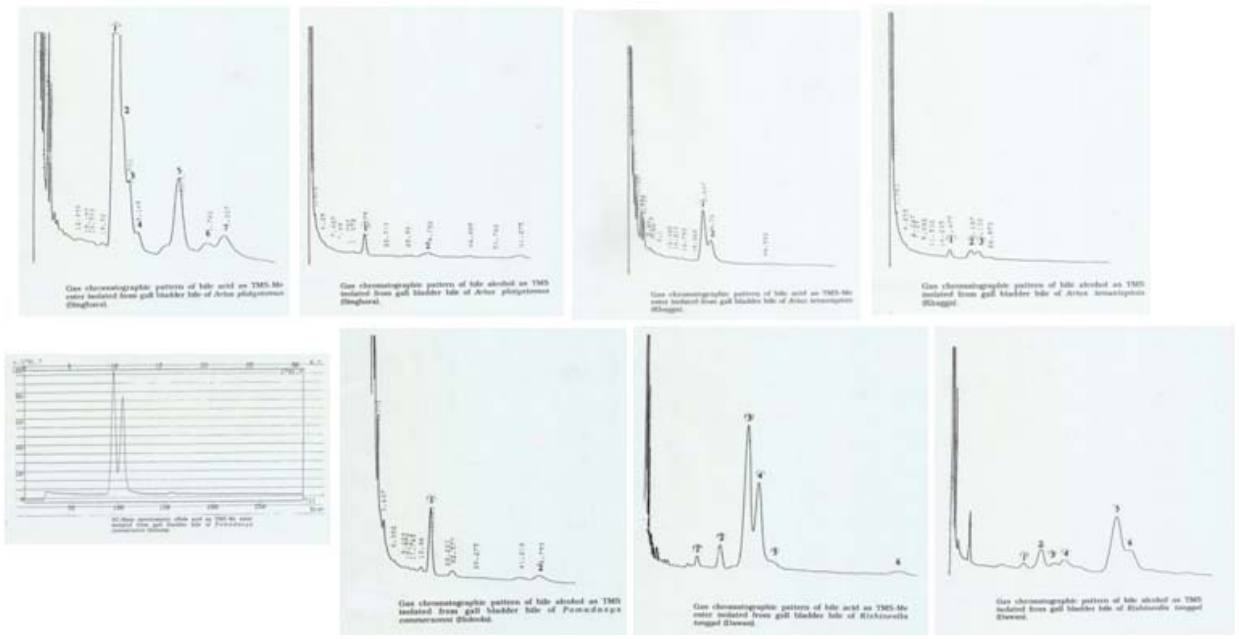


Fig. 2: Bile acids and bile alcohols from marine fish

of ether. The ether extracts were combined and washed with three portions of 5 % Na_2CO_3 solution to extract acidic materials. The ether layer was washed with water until neutral, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness, leaving a residue consisting of unconjugated bile alcohols. The Na_2CO_3 washing were combined, acidified with 2N HCl, and extracted with three 50 ml portions of ether. Evaporation of the solvent from the washed (H_2O) and dried (Na_2SO_4) extracts left a residue consisting of unconjugated bile acids.



Chromatograms

Hydrolysis of conjugated bile acid

Conjugated bile acid was taken in 50ml conical flask with 15ml of 2.5N NaOH and was allowed to hydrolyse in an autoclave at 121°C and 15psi for 8 hours. The hydrolysate was cooled to room temperature, diluted with distilled water, acidified with 2.5N HCl to litmus paper and extracted with three portions of diethyl ether. Ether layers were pooled, washed with distilled water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness under a slow stream of nitrogen gas. The residue (free bile acids) was stored refrigerated till further analyses.

Preparation of methyl esters and TMS ethers was carried out as described previously (Ali *et. al.*, 1976).

Gas-liquid chromatography (GLC)

GLC was done on a Shimadzu CG-14A gas chromatograph using glass column (2m x 3mm) packed with 3% OV-17 on 100/120 chromosorb® WAW (SUPELCO, U.S.A.). All retention times are given relative to the TMS ether of methyl cholate.

Gas-chromatography- mass spectrometry (GC-MS)

GC-MS was carried out on a GC-model HP5890 and MS-model JMS HX110. Capillary column (3% OV-17, 1m x 1.5mm I.D.) with above-mentioned operating conditions was used; EIMS was operated at standard temperature, 270°C and ionization voltage, 70 eV.

Reference compounds

Cholic acid, Chenodeoxycholic acid, and Cholesterol were arranged from Sigma-Aldrich, U.S.A.

RESULTS

The Gas chromatographic pattern of bile acid as TMS-Me ester isolated from gallbladder bile of *Arius platystomus* showed 6 peaks with RRT (relative to methyl cholate) 1.00, 1.11, 1.02, 1.36, 1.59 and 1.94 respectively and the GC-MS data of first two peaks was found as follow: peak-1(Cholic acid): m/z 638 [M, $C_{34}H_{66}O_5Si_3]^+$, 0.8%; 623 [M-$CH_3]^+$, 24%; 548 [M-$C_3H_{10}OSi]^+$, 5%; 533 [M-($C_3H_{10}OSi$ + CH_3)]$^+$, 4 %; 458 [M-2$C_3H_{10}OSi]^+$, 68%; 443 [M-(2$C_3H_{10}OSi$ + CH_3)]$^+$, 7%; 423 [M-($C_3H_{10}OSi$ + $C_6H_{11}O_2$)]$^+$, 6%; 368 [M-3$C_3H_{10}OSi]^+$, 85 %; 353 [M-(3$C_3H_{10}OSi$ + CH_3)]$^+$, 10 %; 343 [M-(2$C_3H_{10}OSi$ + $C_6H_{11}O_2$)]$^+$, 32%; 313, [M-(2$C_3H_{10}OSi$+ $C_6H_{11}O_2$+ 2CH_3)]$^+$ 6%; 253 [M-(3$C_3H_{10}OSi$ + $C_6H_{11}O_2$)]$^+$, 95 %; 226, 13 %; 147, 19 %; 95, [C_7H_{11}, ring A]$^+$, 18% and 73, [$C_3H_9Si]^+$ 100%. Peak-2(Chenodeoxycholic acid): m/z 550 [M, $C_{31}H_{58}O_4Si_2]^+$ (not observed); 535 [M - $CH_3]^+$, 56%; 460 [M-$C_3H_{10}OSi]^+$, 11%; 445 [M-($C_3H_{10}OSi$ + CH_3)]$^+$, 4%; 429, [M-($C_3H_{10}OSi$ + OCH_3)]$^+$, 11%; 370 [M-2$C_3H_{10}OSi]^+$, 68 %; 345 [M-($C_3H_{10}OSi$ + $C_6H_{11}O_2$)]$^+$, 62 %; 316, 8%; 283, [M-(2$C_3H_{10}OSi$ + $C_4H_7O_2$)]$^+$, 8%; 255 [M-(2$C_3H_{10}OSi$ + $C_6H_{11}O_2$)]$^+$, 100 %; 243, 6%; 208, 67%; 161, 20%; 147, 35%; 119, 23% and 107, 47%.

The Gas chromatographic pattern of bile alcohol as TMS showed two peaks and GC-MS data of the major peak-1 (Cholesterol)was found as follow: m/z 458 [M, $C_{30}H_{54}OSi]^+$, 35 %; 443 [M-$CH_3]^+$, 15%; 368 [M-$C_3H_{10}OSi]^+$, 60%; 353 [M-($C_3H_{10}OSi$ + CH_3)]$^+$, 37%; 329 [M-$C_6H_{13}OSi]^+$, 100 % ; 247 [M-$C_{12}H_{22}OSi$, ring B rDA]$^+$, 20%; 129 [CH_2=CH-CH=O-TMS or $C_6H_{13}OSi]^+$, 87 %; 95, [C_7H_{11}, ring A]$^+$, 40% and 73 [$C_3H_9Si]^+$, 57%.

The GC-MS profiles of bile acids from *Arius tenuispinis* showed two peaks (constituting 98% of the total bile acid) and their GC-MS data were found similar to the data mentioned above. The GC-MS pattern of bile alcohol showed three peaks with RRT 1.00, 1.34 and 1.46 and GC-MS data of the first peak was found similar to the data mentioned above. Bile acids and bile alcohols GC-MS data of *Pomadasys commersonni* were found similar as mentioned previously.

The GC-MS profile of bile acids from *Kishinoella tonggol* showed six peaks. GC-MS data of one of the peak was found similar to the data mentioned above. The GC-MS data of bile alcohols was found as follow: peak-2 (5β-Cholestane-3α,7α,12α,24-tetrol): m/z724 [M, $C_{39}H_{80}O_4Si_4]^+$, 4%; 681 $[M\text{-}(CH_3)_3CH]^+$, 19%; 634 $[M - C_3H_{10}OSi]^+$, 40%; 591 $[M\text{-}(C_3H_{10}OSi + (CH_3)_3CH)]^+$, 18%; 544 $[M\text{-}2C_3H_{10}OSi]^+$, 15%; 501 $[M\text{-}(2C_3H_{10}OSi + (CH_3)_3CH)]^+$, 60%; 454 $[M\text{-}3C_3H_{10}OSi]^+$, 6%; 411 $[M\text{-}(3C_3H_{10}OSi + (CH_3)_3CH)]^+$, 47%; 321 $[M\text{-}(4C_3H_{10}OSi + (CH_3)_3CH)]^+$, 29-%; 253 $[M\text{-}(4C_3H_{10}OSi + C_8H_{15})]^+$, 17%; 233, 44%; 213 $[M\text{-}(3C_3H_{10}OSi + C_6H_{13}OSi + C_8H_{15}\text{-}H)]^+$, 15%; 129 [$CH_2$=CH-CH=O-TMS or $C_6H_{13}OSi]^+$, 62 %; 103, 64% and 73 $[C_3H_9Si]^+$, 100 %.

Peak-5(5β-Cyprinol)**:** m/z 812 [M, $C_{42}H_{88}O_5Si_5]^+$, (not observed); 722 $[M\text{-}C_3H_{10}OSi]^+$, 6 %; 632 $[M\text{-}2C_3H_{10}OSi]^+$, 77%; 617 $[M\text{-}(2C_3H_{10}OSi + CH_3]^+$, 8 %; 542 $[M\text{-}3C_3H_{10}OSi]^+$, 62%; 527 $[M\text{-}3C_3H_{10}OSi + CH_3]^+$, 12 %; 452 $[M\text{-}4C_3H_{10}OSi]^+$, 10 %; 437 $[M\text{-}4C_3H_{10}OSi + CH_3]^+$, 6%; 407, 13%; 343 $[M\text{-}(2C_3H_{10}OSi + C_{14}H_{33}O_2Si_2)]^+$, 52%; 315, 7%; 281, 14 %; 253 $[M\text{-}(3C_3H_{10}OSi + C_{14}H_{33}O_2Si_2)]^+$, 87%; 226, 19%; 197 $[C_{14}H_{33}O_2Si_2\text{-}C_3H_{10}Osi\text{-}2H]^+$, 100 %.

DISCUSSION

The composition of bile salts isolated from the gallbladders bile of four marine fish was studied using GC-MS (Chromatograms). The main objective of the present study was to determine the types of bile acids and bile alcohols and to relate differences in their structures with the evolutionary transition across the fish, from C_{27} bile alcohols to C_{24} bile acids (Haslewood, 1967). Isolation and identification of newer bile acids and bile alcohols also prompted the need to assess the bile salts in Pakistani marine fish. The extracted fractions from four different fish bile were subjected to GC-MS after derivatising bile acid enriched fraction as their methyl ester TMS derivatives and bile alcohol enriched fraction as TMS derivative (Tammer, 1974). In order to identify the individual components both bile acid and bile alcohol fractions were analyzed by GC-MS. In addition standard cholic acid, chenodeoxycholic acid and cholesterol were also subjected to GC-MS as reference standard for their mass spectrum and for calculating RRT.

The GC-MS of bile acids from gallbladder of *Arius platystomus* showed 6 peaks. Peaks1, 2 were identified as cholic acid **(1)** and chenodeoxycholic acid **(2)** (fig. 2) due to their comparable RRT and mass spectra to the standard cholic acid TMS-Me ester and chenodeoxycholic acid TMS-Me ester respectively. Other peaks were identified on comparison of their RRT with literature. These included allochenodeoxycholic acid **(3)**, allodeoxycholic acid **(4)**, 3α, 7α, 12α-tridydroxy-24-methyl-5β-cholestan-26-oic acid **(5)** and 3α, 7α, 12α, 24-tetrahydroxy-5α-cholestan-26-oic acid **(6)** respectively (table-1) (Cronholm and Johansson, 1970, Noma *et. al.*, 1980). When bile alcohol was analyzed, the major constituent was found to be cholesterol **(7)**.

The GC-MS profiles of bile acids from *Arius tenuispinis* showed two peaks (constituting 98% of the total bile acid) and were confirmed as **1** and **2**. From bile alcohol fraction three peaks were identified. One of these was **7.** However, the smaller peaks appearing at R.R.T, 1.34 and 1.46 were tentatively assigned as some polar bile alcohol having trihydroxy- and tetrahydroxy-cholestane skeleton. These mass spectra did not match with any of the spectra present in the electronic library (Mallard WG, NIST 2005) or literature.

1 and **2** constituted 100% of the total bile acid in *Pomadasys commersonni* while **7** was the only bile alcohol present in its gallbladder bile.

Bile acid obtained from *Kishinoella tonggol* showed the presence of **1** as major constituent. Mass spectra from another major peak at scan 253 with m/z 682 (expected molecular ion $[M]^+$), with consecutive losses of m/z 90 ($C_3H_{10}OSi$) in mass spectrum, indicated the presence of three hydroxyl groups, interpreted on the basis of m/z 682 $[M]^+$, 592 $[M\text{-}C_3H_{10}OSi]^+$, 502 $[M\text{-}2C_3H_{10}OSi]^+$, 343 $[M\text{-}(2C_3H_{10}OSi+157)]^+$ and 253 $[M\text{-}(3C_3H_{10}OSi+157)]^+$. Further GC-MS showed another peak at scan no. 288, which have mass spectra similar to the one discussed above (scan no. 253). Slight differences in intensities and fragmentation pattern in mass spectrum indicated that both of the discussed compounds could be 5α and 5β isomers of one another (Cronholm and Johansson, 1970). The constituents belonging to these spectra were not identified as these were not present in the available electronic library (Mallard WG, NIST 2005) or literature. The mass spectra of the bile alcohol extracted from *Kishinoella tonggol* corresponded to 5β-Cholestane-3α,7α,12α,24-tetrol **(8)** and 5β-Cyprinol **(9)** (Kuwabara *et.al.*, 1984). Since the literature (Cronholm and Johansson, 1970) reported the mass spectrum of 5β-Cholestane-3α,7α,12α,24-tetrol as diastereomeric mixture without mentioning the contribution of α/β isomers. 5β-Cholestane-3α,7α,12α,24-tetrol **(8)** identified in this study is expected to be a mixture with different contributions of 24-α/β-diastereomers as the intensities of different peaks in mass spectrum was found varying.

CONCLUSION

Present study was conducted to evaluate the structures of bile acids and bile alcohols present in gallbladder bile of different fish species. The cholic acid and chenodeoxycholic acid were found as major bile acids and cholesterol was found as major bile alcohol in all the species. Other bile acids and bile alcohols were result of different enzymatic reactions involved in the metabolism of cholesterol and vary from species to species. The bile acids and bile alcohols (**1** to **9**) of gallbladders bile of these species were not analysed by GC-MS and had not been reported previously. These findings can help to assess the molecular evolution of bile salts in Pakistan marine fish.

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