Bile acids and bile alcohols from *Muraenesox bagio*, *Pomadasys argenteus* and *Lobeo rohita*

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Abstract: Gallbladders bile of three well known commercial fish of South Asia region named *Muraenesox bagio* (locally called bam), *Pomadasys argenteus* (dother) and *Lobeo rohita* (rohu) were analysed on GC-MS, after derivatising the bile alcohols and bile acids as trimethylsilyl ether and trimethylsilyl-methyl ester, respectively. Cholic acid (1) and chenodeoxycholic acid (2) were found as major bile acids in all three species. Major bile alcohol in these fish was cholesterol (4), which was not detected in freshwater specie (*L. rohita*). *M. bagio* was also found to contain 3α,7α,12α-trihydroxy-23-cholesten-26-oic acid (3). Other bile acids and bile alcohols identified in *L. rohita* were *allo* deoxycholic acid (5), 12-oxo-3α-hydroxycholanic acid (6), 3α,7α,12α-trihydroxy-24-cholesten-26-oic acid (7), 5α- and 5β-anhydrocyprinol (8 and 9, respectively) and 5β-homocholane-3α,7α,12α-25-tetrol (10). Besides acting as emulsifying agent in the digestion process, in non-mammalian vertebrates, *e.g.*, fish, reptiles, *etc.* the analytical and elucidative studies on the bile contents disclose the diversity in metabolic pathways of cholesterol and indicate the existence of molecular evolution in the basic C_{27} skeleton of cholesterol.

Keywords: GC-MS, marine and fresh water fish.

INTRODUCTION

According to the studies, in comparison to invertebrates, vertebrates use cholesterol to a much greater extent and evolution of vertebrates is found to have strong relations with cholesterol metabolism (Nes and Nes, 1980). Vertebrates take advantage by utilizing almost all properties of cholesterol for their cell membrane fluidity regulation, nerve fiber insulation and using it as a precursor molecule for synthesizing numerous endogenous compounds. The vast use of cholesterol undoubtedly requires a strictly regulated system for its synthesis and elimination from the body. Nature has fulfilled this requirement by establishing different pathways for its biosynthesis and for the conjugation of bile acids and alcohols, which are basically the catabolic end products of cholesterol (Haslewood, 1967). Bile acids act as powerful detergent or emulsifying agents in the intestine to aid digestion process. Various bile acids and alcohols occur in nature primarily because multiple biochemical pathways are involved in converting cholesterol into highly water soluble molecules.

In non-mammalian vertebrates, such as fish and reptiles, bile alcohols are formed whereas invertebrates do not produce bile acids and bile alcohols. Bile salts are produced by almost every class of vertebrate animals and remarkable diversity in their structure has been revealed across species (Moschetta, Xu *et al*., 2005, Une and Hoshita, 1994). Study of bile salts and acids has led to certain significant facts indicating the existence of molecular evolution in the basic C_{27} skeleton of cholesterol (Nes and Nes, 1980). Most of the compounds isolated from the bile are found to contain C_{24} to C_{27} skeletons (Hofmann and Hagey, 2008) C_{26} alcohols and acids are also produced by the oxidation of the side chain of cholesterol (Danielsson, 1985). It is also reported that majority of the lower animals are unable to oxidize the cholesterol molecule to the extent of (C_{24}) cholic acid and chenodeoxycholic acid (Danielsson, 1985). Conjugation of bile salts varied accordingly. In lower animals, sulfate conjugates of C_{26} and C_{27} alcohols are formed. With biological evolution, a progressive change in the pattern of conjugation is observed. Taurine conjugates are mostly observed in primitive animals containing C_{24} steroids whereas glycine conjugates are found in higher animals (Danielsson, 1985). Presence of bile alcohol in fish was first noted by Hammarsten in the bile of northern Shark *Scymnus borealis* (Hammarsten, 1898). The principal bile alcohol of the hag fish *Myxine glutinosa* and *Eptatretus stoutii* is myxinol, which occurs in bile as C-3,27-disulfate ester (Haslewood, 1966). Haslewood reported that principal bile salt of *Petromyzon marinus* is a monosulfate ester at C-24 of a bile alcohol havin 3α,7α,12α-trihydroxy pattern in a 5α-steroid nucleus (Haslewood, 1969). The chief bile salt in *Chimaera*

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monstrosa is a monosulfate alcohol that has been called chimaerol (Bridgwater, Haslewood *et al*., 1963).Scymnol,

a bile alcohol from Shark *Scymnus borealis* has also been reported (Bridgwater, Briggs *et al*., 1962). The most primitive living member of bony fish is *Latimeria chalumnae*. Its bile contains latimerol as the main constituent (Anderson and Haslewood, 1964). The principal components in *Protopterus acthiopicus* and *Lepidosiren paradoxa* is 5α-cyprinol. *Neoceratodus forsteri* bile salts contain an appreciable proportion of the sulfate of 5α-bufol (Tammar, 1974). Bony fish of fresh water are somewhat different, species of eels ***i.e.***, *Conger myriaster* contains 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (Yukawa, 1965). Biliary acids of channel catfish (*Ictalurus punctats*) and blue catfish (*Ictalurus furcatus*) consists of taurocholic acid, taurochenodeoxy cholic acid and taurodeoxy cholic acid (Kellogg, 1975). Stehly and Hayton reported the presence of glucuronide and sulfate conjugates of pentachlorophenol that were present in the bile of gold fish (*Carassius auratus*) (Stehly and Hayton, 1988). A sulfate-conjugated bile alcohol, 3,12-diketo-4,6-petromyzonene-24-sulfate, was identified using bioassay-guided fractionation from water conditioned with sexually mature male sea lamprey *(Petromyzon marinus)*(Li, Brant *et al*., 2013) . *N*-Methyltaurine, *N*-acyl amidated bile acids and deoxycholic acid were reported in the bile of angelfish *(Pomacanthidae)* (Satoh, Saito *et al*., 2014).

A close study of the patterns of bile acids and bile alcohols present in most of the fish reveals the evolutionary pattern in the formation of bile salts from cholesterol. This information is useful in determining the primitive nature of sterol oxidation in lower animals. However, in few fish the enzymatic systems are seen to be evolved, providing the capability to oxidize the side chain of cholesterol to the C_{24} level (Hofmann, Hagey *et al*., 2010). In current study the patterns of structural variability in bile acids and bile alcohols among selected fish have been analyzed and discussed.

Some fish species used as favorite sea food in our region, has been selected and analyzed. Two of these were from marine (*Muraenesox bagio*, *Pomadasys argenteus*) while *Labeo Rohita* was from fresh waters. To the best of our knowledge bile content of these fish species were not analysed by GC-MS, except a report on bile content of *Labeo Rhita* using TLC, which reported cholic acid, chenodeoxycholic acid and cholesterol (De, Deb *et al*., 2012). Current report presents the biosynthetic diversity in the constituents of bile of these species, which will help in phylogenetic studies.

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals used in general were purchased from Merck. Diazald® (art. no. D28000) and chenodeoxycholic acid (art. no. 859109) was purchased from Aldrich Chemical Corporation. Cholic acid (art. no. 27010) and cholesterol (art. no. C8667), were obtained from Fluka chemicals and Sigma Chemical Co. Saint Louis, MO, USA, respectively.

Extraction of bile acids and bile alcohols

Fish (n=7-9), each weighing ~1-2kg, purchased from Fish Harbor, West Wharf, Karachi, and were identified by Marine Fisheries Department, Fish Harbor, West Wharf, Karachi, Pakistan.

The gallbladder bile from fish of each species was collected and dropped in a conical flask containing 50ml of absolute ethanol. The material was kept for 24 hours in order to facilitate complete dissolution of bile salts in alcohol. The alcoholic extract was filtered over glass wool and was evaporated to dryness in slow stream of nitrogen gas. The dried material containing mainly the bile salts was stored in refrigerator till further analysis.

Extracted bile salts (~2.0g) were dissolved in 100 ml water and extracted with 50ml (x 3) of petroleum ether to remove neutral lipids and cholesterol. The aqueous layer was acidified with 2.0M HCl and then extracted with diethyl ether (x3). The aqueous layer containing bile acid conjugates is evaporated to dryness. In order to remove any free bile acid associated with the ether extract, the ether extracts were combined and washed thrice with 5% Na_2CO_3 solution and washed with water till neutral. These washings were combined, acidified to litmus paper with 2.0M HCl, and finally extracted with diethyl ether to give free bile acid. Extract was filtered over glass wool, evaporated to dryness in slow stream of nitrogen gas, and stored in refrigerator till further analyses.

Conjugated bile acid (1.0g) was taken in 50 ml conical flask with 2.5M NaOH (15 ml) and was hydrolyzed in an autoclave at 121°C and 15(psi) for 8 hours. The hydro lysate was cooled to room temperature, diluted with distilled water, acidified to litmus paper with 2.5M HCl and extracted with diethyl ether (x 3). Ether layers were pooled, washed with distilled water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness in a slow stream of nitrogen. The residue as free bile acids was stored refrigerated till further analyses.

Derivatization of bile acids and bile alcohols

Bile acids, dissolved in a few drops of anhydrous methanol, were allowed to react with excess of freshly distilled ice cold diethyl ether solution of diazomethane. The diazomethane was generated *in-situ* from Diazald®. The reaction mixture was allowed to stand at room temperature for 30 minutes before the excess diazomethane and solvent were removed under nitrogen stream. Bile acid methyl ester, so produced, were dissolved in a few drops of dry pyridine (0.2ml) and allowed to react with hexamethyldisilazane (0.5ml) at

Table1: Gas chromatographic data of bile constituents identified in gallbladder bile of *Muraenesox bagio* (Bam) and *Labeo rohita* (Rohu)

Identified compounds	*M. bagio* (Bam)	*L. rohita* (Rohu)		
Bile acids	RT (%)[a]		RRT[b]	RRT[c]
cholic acid (1)[d,e]	22.643 (80.5)	22.395 (37.9)	1.00	0.90
chenodeoxycholic acid (2)[e,f]	25.240 (12.1)	24.852 (26.0)	1.11	1.00
allo-deoxycholic acid (5)	-	28.902 (7.2)	1.29	1.16
Unidentified	29.657 (1.0)	-	1.31	1.18
3α,7α,12α-trihydroxy-23-cholesten-26-oic acid (3)	40.687 (2.6)	-	1.80	1.61
12-oxo-3α-hydroxy-cholanic acid (6)	-	46.140 (22.9)	2.06	1.85
3α,7α,12α-trihydroxy-24-cholesten-26-oic acid (7)	-	53.105 (6.0)	-	2.12
Unidentified	53.665 (3.8)	-	2.37	-
Bile alcohols	RT (%)[a]		RRT[g]	RRT[h]
cholesterol (4)[e,i]	16.877 (79.7)	17.503 (10.0)	1.00	1.00
5α-anhydrocyprinol (8)[e]	-	26.412 (45.4)	-	1.51
5β-anhydrocyprinol (9)[e]	-	30.263 (33.1)	-	1.73
5β-homocholane-3α,7α,12α,25-tetrol (10)[e]	-	48.427 (11.5)	-	2.76
Unidentified	49.630 (20.30)		2.94	

[a]Concentrations calculated from area normalization method using TIC of GC-MS; Relative retention times with reference to [b]methyl cholic acid-TMS and [c]methyl chenodeoxycholic acid-TMS; [d,f,i]1,2, and 4, also present in bile constituents of *P. argenteus,* are also confirmed by co-injection; [e]Supported by GC-MS; Relative retention times with reference to cholesterol in [g]*M. bagio* and [h]*L. rohita*.

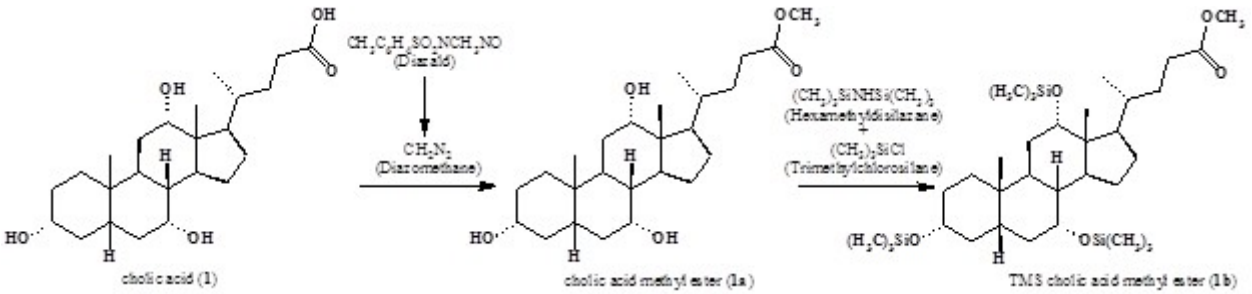

Fig. 1a: Scheme for derivatization taking cholic acid (1) as an example

room temperature for 30 to 60 minutes with few drops of trimethylchlorosilane, added as catalyst. Using similar protocol TMS derivatives of alcohols were also prepared (fig. 1a and 1b).

Identification of bile acids and alcohols using gas chromatography (GC) and gas chromatography-mass spectrometric (GC-MS) analyses

Gas chromatography (GC) was performed on a Shimadzu GC-14A gas chromatograph (Japan) equipped with a flame ionization detector. The glass column (2.1m x 3mm (*ID*)), packed with 3% OV-17 on 100/120 chromosorb (R) WAW (Supelco Co, USA) and the column temperature was kept at 235°C (isothermal).The GC operating conditions for both bile acids and bile alcohols were same. Nitrogen was used as carrier gas at a flow rate of $2.5cm^3.min^{-1}$.

GC-MS profiles of TMS-ME derivatives of bile acids and TMS derivatives of bile alcohols were obtained on GC-model HP-5890 (USA) coupled with MS-model Jeol JMS HX-110 (Japan). The following operation conditions were employed; column 3% OV-17 (100/120 chromosorb® WAW), (1m x 1.5mm) (Supelco Co, USA); column temperature, 270°C (isothermal) with standard operating conditions for MS (fig. 2-4).

RESULTS

Gas chromatographic pattern of bile acids as TMS-Me ester isolated from gallbladder bile of *Muraenesox bagio* showed 5 peaks with RRT (relative to methyl cholate) 1.00, 1.11, 1.31, 1.80 and 2.37 respectively. GC- MS data of first two peaks were found as follow:

Peak-1 (cholic acid (1)): m/z 638 [M^+], 0.8%; 623 [M^+ -15], 24%; 548 [M^+ - 90], 5%; 533 [M^+ - (90 +15)], 4%;458 [M^+ - (2 x 90)], 68%; 443 [M^+ - (2 x 90 +15)], 7%;368 [M^+ - (3 x 90)], 85%; 343 [M^+ - (2 x 90 + side chain, $C_6H_{11}O_2$)], 32%; 253 [M^+ - (3 x 90 + side chain, $C_6H_{11}O_2$)], 95%; 226, 13%; 147, 19%; 95, 18%; and 73 [$C_3H_9Si]^+$, 100% (base peak).

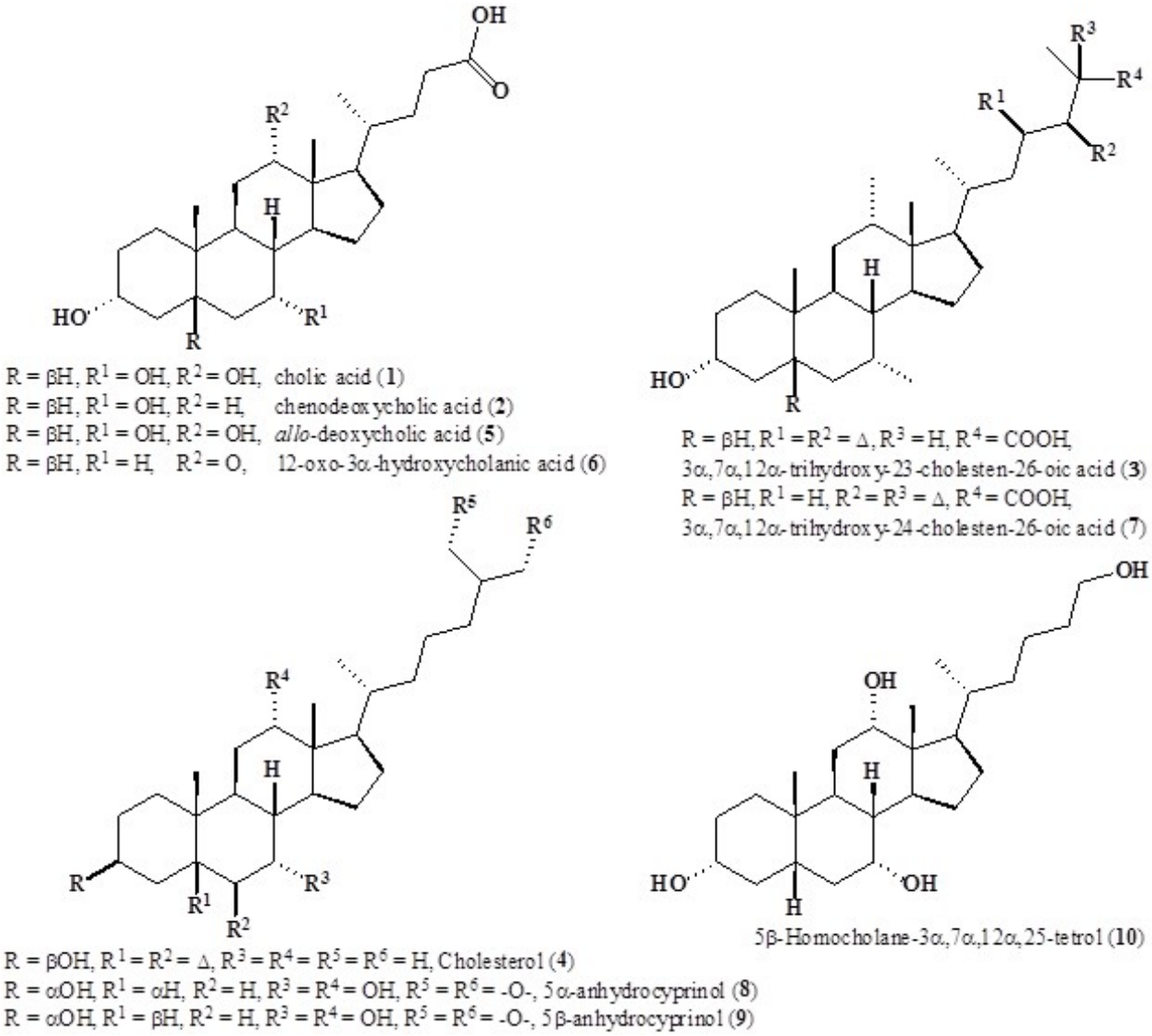

Fig. 1b: Identified bile constituents

Peak-2 (chenodeoxycholic acid (2)): m/z 550 [M^+] (not observed); 535 [M^+ - 15], 56%; 460 [M^+ - 90], 11%; 445 [M^+ - (90 + 15)], 4%; 429 [M^+ - (90 + 31(OCH_3)], 11%; 370 [M^+ - (2 x 90)], 68%; 345 [M^+ - (90 + side chain, $C_6H_{11}O_2$)], 62%; 316, 8%; 283 [M^+ - (2 x 90+ side chain, $C_4H_7O_2$], 8%; 255 [M^+ - (2 x 90 + side chain, $C_6H_{11}O_2$)], 100% (base peak); 243, 6%; 208, 67%; 161, 20%; 147, 35%; 119, 23% and 107, 47%.

Peak-4 (3α,7α,12α-trihydroxy-23-cholesten-26-oic acid (3) was identified from RRT. Peak-3 and 5 remained unidentified, data not shown. When the bile alcohol fraction was analyzed as TMS derivative two peaks were observed (constituting about 100% of the total bile alcohol) with RRT 1.00 and 2.94 respectively. GC- MS data of the peaks were found as follow:

Peak-1 (cholesterol (4)): m/z 458 [M^+], 35%; 443 [M^+ - 15], 15%; 368 [M^+ - 90], 60%; 353 [M^+ - (90 + 15)], 37%; 329 [M^+ - (CH_2=CH-CH=O-TMS or $C_6H_{13}OSi$)], 100% (base peak); 247, 20%; 129[CH_2=CH-CH=O-TMS or $C_6H_{13}OSi]^+$, 87%; 95, 40%; and 73, 57%. Peak-2 remained unidentified, data not shown (fig. 1b and 2).

The GC-MS profiles of bile acids from *Pomadasys argenteus* showed five peaks, two of these (peak-2 and 3) constituting 98.54% of the total bile acid in their GC-MS data were identified as 1 and 2. The minor peaks (peak-1, 4 and 5) remained unidentified, data not shown. The GC-MS pattern of bile alcohol showed only one peak (~100%) and GC-MS data of the peak was found similar to 4 (fig. 1b and 3).

Gas chromatographic pattern of bile acids as TMS-Me ester isolated from gallbladder bile of *Lobeo rohita* showed 5 peaks with RRT (relative to methyl chenodeoxycholate) 0.90, 1.00, 1.16, 1.85 and 2.12 respectively. GC-MS data of first two peaks were found similar to the data of 1 and 2. While remaining three peaks were identified as *allo*deoxycholic acid (5), 12-oxo-

3α-hydroxycholanic acid (6) and 3α,7α,12α-trihydroxy-24-cholesten-26-oic acid (7) on the basis of RRT comparable to literature (Ali, Farhat *et al*., 1976; Ali, Stephenson *et al*., 1982). The bile alcohol fractions showed four peaks with RRT (relative to cholesterol) 1.00, 1.51, 1.73 and 2.76 respectively and the GC-MS data of first peak was found similar to 4. GC-MS data of the remaining three peaks were found as follow:

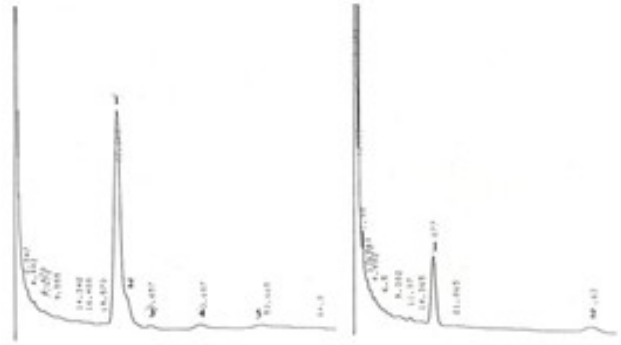

Fig. 2: GC-MS chromatogram of derivatized bile acids (left) and alcohols extracted from *Muraenesox bagio*

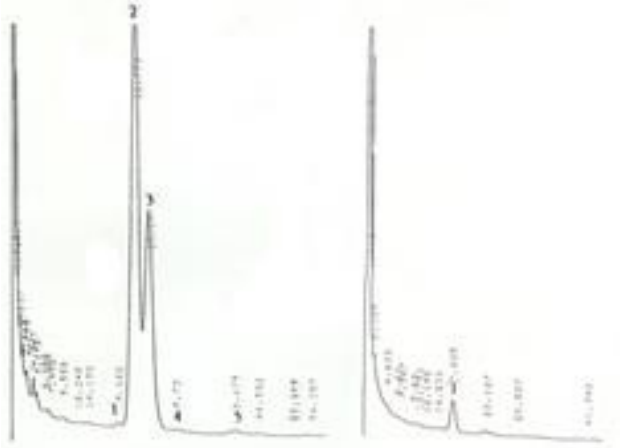

Fig. 3: GC-MS chromatogram of derivatized bile acids (left) and alcohols extracted from *Pomadasys argenteus*

Peak-2 (5α-anhydrocyprinol (8)): m/z 650 [M^+], 1.5%; 635 [M^+ - 15], 1.5%; 560 [M^+ - 90], 7%; 545 [M^+ - (90 + 15)], 1%; 530 [M^+ - (90 + CH_2O)], 8%; 470 [M^+ - (2x 90)], 100% (base peak); 455 [M^+ - (2 x 90+15)], 12%; 440 [M^+ - (2x 90 +CH_2O)], 66%; 433 [M^+ - (90 + $CH(CH_3)$-$(CH_2)_3CH(CH_2)_2O$)], 4%;380 [M^+ - (3 x 90)], 8%; 343 [M^+ - (2 x 90 + $CH(CH_3)$-$(CH_2)_3CH(CH_2)_2O$)], 98%; 253 $CH(CH_3)$-$(CH_2)_3CH(CH_2)_2O$)], 98%; 73 [C_3H_9Si]$^+$,100% (base peak).

Peak-4: (5β-homocholane-3α,7α,12α,25-tetrol (**10**)): m/z [M^+ - (3 x 90 + $CH(CH_3)$-$(CH_2)_3CH(CH_2)_2O$)], 35%. Peak-3 (5β-anhydrocyprinol (9)): m/z 650 [M^+], 1.5%; 635 [M^+ - 15], 1.5%; 560 [M^+ - 90], 7%; 545 [M^+ - (90 + 15)], 3%; 530 [M^+ - (90 + CH_2O)], 8%; 470 [M^+- (2x 90)], 80%; 455 [M^+ - (2 x 90+15)], 12%; 440 [M^+ - (2x 90 +CH_2O)], 38%; 433 [M^+-(90+$CH(CH_3)$-$(CH_2)_3CH(CH_2)_2O$)], 4%; 380 [M^+ - (3 x 90)], 40%; 343 [M^+-(2 x 90 + $CH(CH_3)$-$(CH_2)_3CH(CH_2)_2O$)], 57%;253 [M^+ - (3 x 90 + 696 [M], (not observed); m/z 681 [M -15], 3%; 578 [M - (CH_2-CH_2O-TMS)], 100% (base peak), 501 [M^+ - (2 x 90 +15)], 9%; 343 [M+ - (2x 90 + $CH(CH_3)$-$(CH_2)_3$-CH_2-OTMS)], 62%; 321 [M^+- (4 x 90 +15)], 5%; 253 [M+ - (3x 90 + $CH(CH_3)$-$(CH_2)_3$-CH_2-OTMS)], 23% (fig. 1b and 4).

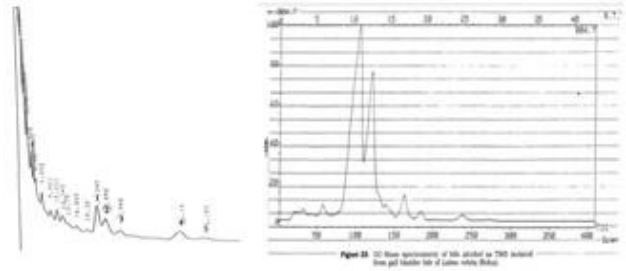

Fig. 4: GC-MS chromatogram of derivatized bile acids (left) and alcohols extracted from *Lobeo rohita*

DISCUSSION

The composition of bile acids and bile alcohols isolated from the gall bladder bile of two marine fish and a fresh water fish was studied using GC and GC-MS as their trimethylsilyl-methyl ester (TMS-Me) and trimethylsilyl ether (TMS) derivatives, respectively (fig. 1a and 1b).

Bile acids and bile alcohols were assessed qualitatively and quantitatively and the findings were made available for the phylogenic studies with reference to the molecular evolution of acidic and neutral steroids within the vertebrate series. In connection to the significance of bile acids and salts, the identification of these moieties in Pakistani fish requires further studies.

The GC-MS of bile acids from gallbladder bile of *Muraenesox bagio* showed 5 peaks. Peaks-1 & 2 identified by co-injecting standards, RRT and GC-MS were cholic acid (1) and chenodeoxycholic acid (2). Peaks-3 & 5 remained unidentified but peak-4 was found 3α,7α,12α-trihydroxy-23-cholesten-26-oic acid (3) (table-1 and fig. 2) by comparing its RRT with reported data (Ali, Stephenson *et al*., 1982).

When the bile alcohol fraction was analyzed as TMS derivative peak-1 (RRT 1.00) was identified as cholesterol (4) (table-1, fig. 2) on the basis of RRT (Annan, Lequesne *et al*., 1993), co-injection and GC-MS data. GC-MS data of peak-2 (RRT 2.94) was found very close to the 5β-anhydocyprinol but due to greater difference with the RRT of 5β-anhydocyprinol (table-1 and fig. 2) it is declared unidentified.

GC-MS pattern of bile acid as TMS-Me ester of gallbladder bile of *Pomadasys argenteus* showed two

major peaks constituting 98.54% of the total bile acid and were confirmed as 1 and 2. The bile alcohol fraction gave only one peak (~100%) on GC and GC-MS and identified as 4 (table-1, fig. 3).

GC-MS pattern of bile acid as TMS-Me ester of gallbladder bile of *Lobeo rohita* showed five peaks. First two were identified as 1 and 2 whereas remaining three were identified on the basis of RRT as *allo*deoxycholic acid (5) (Ali, Farhat *et al*., 1976), 12-oxo-3α-hydroxycholanic acid (6) and 3α,7α,12α-trihydroxy-24-cholesten-26-oic acid (7) (Ali, Stephenson *et al*., 1982)) respectively (table-1, fig. 4).

The bile alcohol fractions showed four major peaks. First peak (peak-1) is identified as 4 and peak-2 and 3 appearing at scan no. 105 and 121, were found to provide fragmentation pattern specific to 5α- and 5β-anhydrocyprinol (8 and 9 respectively) (fig. 1) (Ali, 1992). Fragmentation pattern of the peak scanned at 105 showed that the relative abundance of m/z 343 is greater than that of m/z 253. Such pattern is indicated by most of the structurally related 5α-derivatives of bile acids and alcohols. The reverse (scanned at 121) is true for those having 5β-configuration (Ali, 1992). Thus the two peaks are finalized as 5α- and 5β-anhydrocyprinol. Another peak of bile alcohol was identified as 5β-homocholane-3α,7α,12α,25-tetrol (10) (fig. 1). The base peak at m/z 578 represents the elimination of the terminal -CH_2-CH_2O-TMS fragment, such elimination is not usual but possibility of such cleavage may not be ignored (Ali, 1992). However, a peak appearing at m/z represents the loss of methyl from the M^+.

CONCLUSION

In current study, cholic acid and chenodeoxycholic acid are identified as the major bile acids in both marine and fresh water fish. Cholesterol is identified as the major bile alcohol in both marine and fresh water fish. Trihydroxycholestenoic acid and anhydrocyprinol were also common in both marine and fresh water species but homocholane tetrol was the only bile alcohol that was found in fresh water specie. The bile acids and bile alcohols (1 to 10) of gallbladder 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 and fresh water fish.

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