Isolation of Bile from Fish and Identification by Thin Layer Chromatography

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The paper deals with the collection of gall bladders, isolation of bile and identification of the constituents of the bile salts from different fishes. The yield of bile contents from fresh water fishes rohu, mrigal and catla were compared with that from marine fishes seer, tuna, shark and sardine. Considerable variation in yield was showed between marine and fresh water fish as well as between the species in both groups. It ranged from 0.04 to 0.06% of the body weight of fish in catla, mrigal and rohu. The bile constituents from rohu and mrigal were analysed by thin layer chomatography. The result showed that bile of rohu and mrigal contains mainly taurine derivative of lithocholic acid.

Bile is produced in the liver of animals and stored in the concentrated form in the gall bladders. These bile salts secreted in the intestine emulsify fat, aiding digestion and absorption. These are usually a mixture of salts of cholic acid and/or its different amino acid derivatives (Hawk, 1965). The nature and composition of bile salts vary widely depending on the type of animal. The principal bile acids are cholic acid, deoxycholic acid, anthropodeoxycholic acid, dehydrocholic acid, lithocholic acid, taurocholic acid and chenodeoxycholic acid (Hawk, 1965).

Human bile contains the first three of the above acids and ox bile contains cholic acid and deoxycholic acid whereas goose and chicken bile contains mainly anthropocholic acid (Hawk *et al.*, 1954). Cholic acids are closely related to cholesterol in structure. Sex hormones are synthesised from bile salts obtained from mammalian gall bladder (Brody, 1965). Bile salts are, therefore, very important for many pharmaceutical preparations. Dugal & Lafromboise (1956) reported that fish bile contains cholic acid and deoxycholic acid combined with taurine and sodium. Stansby (1953) reported that bile salts from fishery waste have been utilized in medicine and in the manufacture of organic chemicals.

The gall bladders from fish being very small the collection of the galls and isolation of bile are laborious and hence limits its use as a commercial source for bile acids. But considering the annual availability of 2.4 million tons of fish in the country an attempt has been made on the collection of galls, isolation of bile and identification of constituent bile acids from certain species of fish and the results are reported here.

Materials and Methods

Marine fishes used were tuna *(Euthynnus affinis)*, seer *(Scrombromorus commorson)*, shark *(Scoliodon sorrakowah)* mackerel *(Rastrelliger kanagurta)*, oil sardine *(Sardinella longiceps)* and jew fish *(Johnius argentatus)* weighing on an average 1.5 kg, 2 kg, 3 kg, 40 kg, 150 g, 100 g, 600 g respectively. Freshwater fishes used were catla *(Catla catla)* mrigal *(Carprinus mrigala)* and rohu *(Labeo rohita)* size ranging from 29 to 35 cm and weighing on an average of 500 g, cultured in a fish farm at Alleppy. They were brought live to the laboratory and length and weight were noted (Table 1).

The gall bladders were collected by opening the beelly with a knife and separating the gall bladder located near the liver without causing any mechanical damage

Table 1. *Yield of gall bladder from freshwater fish in relation to size*

Mrigal

Wt. of fish g	Length in cm	Wt. of gall g	Yield of gal (% of body wt)
335	28.5	1.14	0.340
495	33.0	1.46	0.295
307	22.5	1.50	0.488
372	31.5	1.27	0.340
420	33.5	1.48	0.352
460	34.0	1.52	0.330
388	32.7	1.22	0.314
400	31.3	1.21	0.300
398	32.5	1.07	0.360
450	34.0	1.506	0 340
458	33.3	1.505	0.309
505	33.4	1.561	0.309
435	33.0	1.520	0.340
375	31.3	1.350	0.350
375	31.5	1.350	0.350

Rohu

205	23.2	1.53	0.746
298	27.0	3.09	1.036
272	26.5	3.73	1.370
382	28.5	4.09	1.500
260	25.0	2.66	1.070
325	27.5	4.14	1.020
309	28.5	3.29	1.060
285	26.3	2.84	0.996
445	31.5	2.90	0.762
350	28.0	2.67	1.050
315	28.0	3.38	1.050

to the bile sack. They were plucked out by hand and dipped in water to remove blood and other contaminants. The bile was collected by piercing the gall bladder with a needle and squeezing it. The weight of the content was noted (Table 2A) and the residual adhering bile was also collected through a filter paper by washing with minimum quantity of water. The bile was collected separately from each species and dried in sun as well as under vacuum at a temperature of 40–50°C. The yield of dry matter and physical characteristics like colour, appearance and odour were noted. The samples were also analysed for moisture nitrogen, fat and ash contents (A.O.A.C., 1975) and presented in Table 2B.

Table 2a. *Yield of bile from freshwater and marine fish*

Name of fish	Bile content-wet wt (percentage of body wt)
Mrigal	0.34
Rohu	1.05
Catla	0.6
Tuna	0.065
Seer	0.033
Mackerel	0.075
Sardines	0.038
Tuna (blue fin)	0.137
Shark	0.065
Jew fish	0.020

Table 2b. *Yield, physical and chemical characteristics of dried bile salts from freshwater fish*

	Catla	Mrigal	Rohu
Yield %	0.04	0.05	0.06
Moisture %	6.712	5.8	4.7
Nitrogen (dry basis)	0.144	0.128	0.089
Ether extractable (dry basis)	2.28	1.56	2.1
Ash (dry basis)	17.69	14.8	15.5
Colour	Yellowish green	Yellowish green	Yellowish green
Odour	odourless	odourless	odourless
Appearance	crystalline	crystalline	crystalline

Separation and identification of the bile constituents were done by thin layer chromatography (TLC) following the guidelines of Waldi (1965). This was performed on glass plates of size 20 × 20 cm coated with silica gel G (0.5 mm thickness) heated to 110°C for 1 h. The solvent system used was toluene: acetic acid: water (50:50:10) equilibrated for 1 h in 24 × 10 × 26 cm chamber.

The dried bile salts were dissolved in A.R. methanol to a concentration of 1 mg/ml and centrifuged to remove undissolved impurities. Standard bile salts (Sigma)

were also run along with the samples. The standards and samples were spotted (10 ul) on the plate about 2 cm from the bottom. The solvent was allowed to run 16 cm from the surface of the solvent. The plates were then taken out dried in air and colour was developed by spraying 0.5% solution of phosphomolybdic acid in ethanol and heating at 80–90°C for 5 min (Waldi, 1965).

Bile acids and derivatives are located as blue spots in visible light (Plate 1) and Rf values were determined (Table 3).

Table 3. *Rf values of standard and purified bile in toluene:acetic acid:water (50:50:10)*

Name of bile acid	Rf × 100
Chenodeoxycholic acid	77.05
Lithocholic acid	82.35
Deoxycholic acid	67.70
Cholic acid	42.35
Dehydrocholic acid	67.70
Taurocholic acid	5 88
Purified bile (Mrigal)	7 05
Purified bile (Rohu)	7.05

Dried bile salts (100 mg) were hydrolysed using 6 N hydrochloric acid by heating at 110°C for 24 h taken in sealed test tube. The bile acids formed by hydrolysis were separated, concentrated and dried over warm water bath (50–60°C). The residue was dissolved in methanol (1 mg/ml) and TLC was performed alongwith standards (Plate 2) as described above (Table 4). The aqueous layer of the hydrolysate was freed from

Table 4. *Rf values of standard and solvent extract of hydrolysed bile salt in toluene:acetic acid:water (50:50:10)*

Chenodeoxycholic acid	70.66
Lithocholic acid	89.33
Deoxycholic acid	70.66
Cholic acid	34.66
Dehydrocholic acid	86.66
Taurocholic acid	0
Chloroform extract (hydrolysed) Rohu bile	89.33
Chloroform extract (hydrolysed) Mrigal bile	89.33

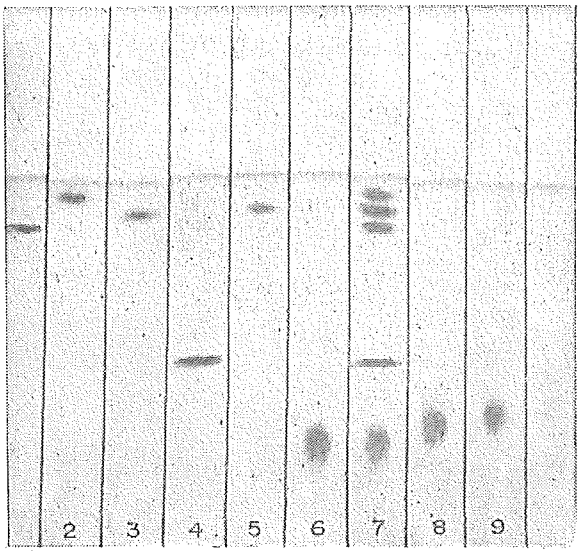

Plate 1. Thin layer chromatogram of standard bile acids and purified bile from mrigal and rohu.
(1) Cheno deoxy cholic acid, (2) Lithocholic acid, (3) Deoxy cholic acid, (4) Cholic acid, (5) Dehydrocholic acid, (6) Taurocholic acid, (7) Mixture of acids, (8) Purified bile (Mrigal) (9) Purified bile (Rohu)

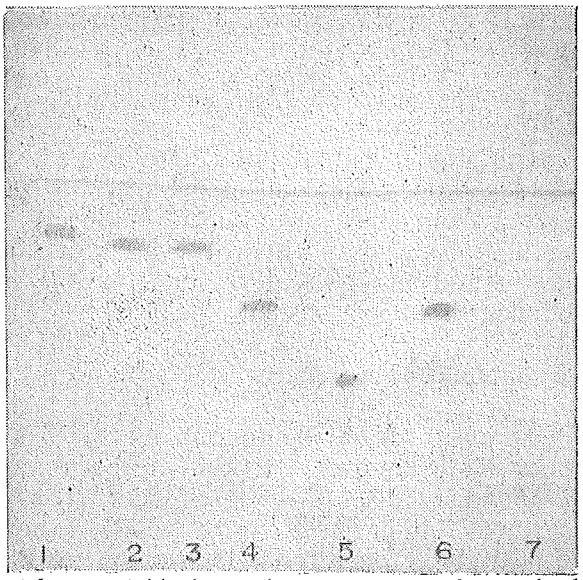

Plate 2. Thin layer chromatogram of standard bile acids and chloroform extract of hydrolysed bile from mrigal and rohu.
(1) Lithocholic acid, (2) Chloroform extract (hydrolysed rohu bile), (3) Chloroform extract (hydrolysed mrigal bile), (4) Chenodeoxycholic acid, (5) Cholic acid (6) Dehydrocholic acid (7) Taurocholic acid

hydrochloric acid by evaporating under vacuum and dried at 60°C. The amino acids present were extracted by dissolving in butanol: acetic acid:water (80:20:20) and by centrifuging. TLC was developed on

silica gel G in a solvent mixture of butanol: acetic acid : water (80:20:20) (Brenner *et al.* 1965) and Rf values are given in Table 5. The amino acid was identified (Plate 3) by the pink colour developed by spraying a solution of 300 mg ninhydrin in 100 ml n-butanol containing 3 ml glacial acetic acid and heating at 110°C for 10 min (Waldi, 1965).

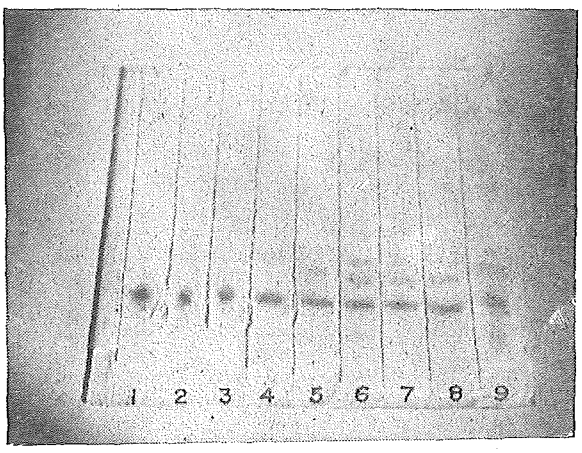

Plate 3. Thin layer chromatogram of water extract of hydrolysed bile from Mrigal, Rohu and Taurine.
(1–4) Taurine, (5, 6, 7) Rohu, (8, 9) Mrigal.

The single visible spot (Plate 1) portions were scrapped and collected. The bile was eluted from silica gel G using chloroform-methanol, dried and hydrolysed as described above and TLC was developed for bile salt concentrate.

Results and Discussions

The size of gall bladder in marine fish is very small compared to that in fresh water fishes (Table 1). The gall bladders from oil sardines and jew fish were found to be so tiny that the isolation of it from the viscera is commercially not viable. Eventhough gall contents (volume) of the different fresh water fishes studied showed appreciable difference, they do not differ considerably in the dried form. Comparatively higher yield was obtained from rohu. The dry yield ranged from 0.04 to 0.06% of the body weight. The gall bladders being very small, the chances of contamination with blood and other proteinaceous matter is high during isolation of galls and collection of bile. The presence of blood, protein and such other extraneous matter affects both physical and chemical characteristics of the dried bile salts. The more the impurities, the darker is the colour. The dried bile salt collected is odourless and yellowish green in colour. Freshness of fish was found to affect the content and quality of the bile. Even slight spoilage of the fish or careless handling breaks the bladders causing loss of bile and bile contents. Size and weight of bladders collected from different fishes are presented in Table 2A. The drying in sun and drying in vacuum did not show any significant difference in physical or chemical characteristics.

Table 3 shows the Rf values with solvent system toluene acetic: acid: water (50:50:10) of the standard bile acids and bile of mrigal and rohu. Rf values of the purified bile salt did not correspond to any of the standard bile acids studied. Plate 1 shows spots of the experimental samples along with standard acids. Plate I and Table 3 shows that experimental sample did not contain any of the standard acids studied. Assuming that the sample may be an amino acid derivative with peptide bond, it was hydrolysed with 6 N HCl and the aqueous and chloroform extracts were analysed separately by TLC. Plate 2 shows the spots of standard bile acids and the chloroform, methanol extract of the hydrolysed bile salts of the freshwater fish with a modified solvent system, toluene-aceticacid:water (50:50:10). The corresponding Rf values are given in Table 4. The Rf values and the only spots obtained correspond to lithocholic acid, showing that the bile acid from rohu and mrigal were derivatives of lithocholic acid.

The Rf values of the spots obtained from the aqueous fraction of the hydrolysed bile and that of taurine are presented in Table 5 and Plate 3. The dominant spot corresponds to that of taurine showing that the bile from rohu and mrigal may be taurine derivative of lithocholic acid. Since there were four more insignificant spots seen in the TLC (Plate 3) of the aqueous fraction of the hydrolysed bile, it can be inferred that there are other amino acids as well in combination with lithocholic acid. There was no appreciable difference between the amino acid pattern of bile salts from rohu and mrigal.

Table 5. *Rf values of taurine and aqueous portion of hydrolysed bile in butanol : acetic acid: water (80:20:20)*

	Rf x 100
Taurine	33.3
Aqueons portion of hydrolysed bill from rohu	33.3; 28.57; 40.0; 45.0
——,,——from mrigal	33.3; 28.57; 40.0; 48.0

Conclusion

The gall bladders from catla, rohu and mrigal are comparatively bigger in size than those of marine fishes and therefore can be collected for commercial exploitation. The bile of rohu and mrigal contain mainly taurine derivative of lithocholic acid.

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