AN EXTENDED CHEMICAL ANALYSIS OF GALLSTONE

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ABSTRACT

Chemical composition of gall stones is essential for aetiopathogenesis of gallstone disease. We have reported quantitative chemical analysis of total cholesterol bilirubin, calcium, iron and inorganic phosphate in 120 gallstones from Haryana. To extend this chemical analysis of gall stones by studying more cases and by analyzing more chemical constituents. A quantitative chemical analysis of total cholesterol, total bilirubin, fatty acids, triglycerides, phospholipids, bile acids, soluble proteins, sodium potassium, magnesium, copper, oxalate and chlorides of biliary calculi (52 cholesterol, 76 mixed and 72 pigment) retrieved from surgical operation of 200 patients from Haryana state was carried out. Total cholesterol as the major component and total bilirubin, phospholipids, triglycerides, bile acids, fatty acids (esterified), soluble protein, calcium, magnesium, iron, copper, sodium, potassium, inorganic phosphate, oxalate and chloride as minor components were found in all types of calculi. The cholesterol stones had higher content of total cholesterol, phospholipids, fatty acids (esterified), inorganic phosphate and copper compared to mixed and pigment stones. The mixed stones had higher content of iron and triglycerides than to cholesterol and pigment stones. The pigment stones were richer in total bilirubin, bile acids, calcium, oxalate, magnesium, sodium, potassium, chloride and soluble protein compared to cholesterol and mixed stones. Although total cholesterol was a major component of cholesterol, mixed and pigment gall stone in Haryana, the content of most of the other lipids, cations and anions was different in different gall stones indicating their different mechanism of formation.

KEY WORDS

Gallstone, Biliary calculi, Serum composition, Triglyceride, HDL, Metal, Enzymes

INTRODUCTION

The chemical composition of gallstones is essential to study aetiopathogenesis of gallstone disease, which is common in northern part of India. Reports on chemical analysis of gallstones are available from different endemic regions of India like Kolkata (1), Varanasi, Aligarh, Kanpur (2-4), Mumbai (5), Patiala, Chandigarh (6-7). Recently, we have reported the quantitative chemical analysis of two metabolites viz total cholesterol and total bilirubin, two cations calcium, iron and one anion inorganic phosphate in gallstones collected from patients of Haryana state, a nuclear region of northern India (8). The present study describes an extensive quantitative

analysis of gallstones from Haryana which includes metabolites like fatty acids, triglycerides, phospholipids, bile acids, soluble protein and cations and anions like sodium, potassium, magnesium, copper, oxalate and chloride besides the above five constituents.

MATERIALS AND METHODS

Gallstones from 200 patients of cholelithiasis were collected after cholecystectomy at local hospital (Pt.B. D. Sharma PGIMS, Rohtak hospital). In all, 200 gallstones were collected from 62 males and 138 females. The stones were divided into 3 groups depending upon their colour: pale yellow and whitish stones as cholesterol calculi, black and blackish brown as pigment calculi and brownish yellow or greenish with laminated features as mixed calculi. The other relevant information about the patients such as age, sex and number of calculi were obtained from hospital records. The various physical parameters of stones such as number, shape, size, texture and cross-section were noted. The stones were powdered in

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a pestle and mortar and dissolved in different solvents depending upon the type of chemical constituent to be analyzed. To determine total cholesterol and total bilirubin, 30mg stone powder was dissolved in 3 ml chloroform in a test tube. The tube was kept in boiling water bath for 2 min. The stone solution thus obtained was used for determination of total cholesterol and total bilirubin. To determine calcium, oxalate, inorganic phosphate, magnesium, chloride, soluble protein, triglycerides, iron, copper, sodium and potassium, 30 mg stone powder was dissolved in 3 ml 1N HCl in graduated 10 ml tube and its final volume was made up to 10 ml with distilled water. The tube was kept in boiling water bath for 1 hr. To analyze phospholipids, stone powder (20 mg) was dissolved in 15 ml $\text{CHCl}_3 + \text{CH}_3\text{OH}$ in 2:1 ratio, containing 1N HCl. To measure bile acids and fatty acids, the stones were dissolved in chloroform-methanol (2:1) mixture and ethyl alcohol-solvent ether in (3:1 mixture) respectively.

Total cholesterol by enzymic colorimetric method of Bayer Diagnostic India Ltd (9), total bilirubin by colorimetric method of Accurex Biomedical Pvt. Ltd (10), triglycerides by enzymic colorimetric method of Bayer Diagnostics India Ltd (11), soluble protein by colorimetric method of Lowry *et al* (12), oxalate as described by Satyapal and Pundir based on enzymic colorimetric method (13), calcium by OCPC kit method of Miles India Ltd. (14), phospholipid & inorganic phosphate by colorimetric method of Fiske and Subba Row (15), magnesium by colorimetric method of Neill and Neely (16), chloride by colorimetric method of Bayer Diagnostics India Ltd. (17), iron & copper by Atomic Absorption Spectrophotometer (Make Hitachi, Japan), sodium & potassium by Flame photometer (Systronics 'Mediflame' 127, Hyderabad), fatty acids by colorimetric method of Stern and Shapiro (18) and bile acids by colorimetric method of Carey (19) were estimated. The dissolved stone solutions were stored at 2-8°C, when not in use.

RESULTS AND DISCUSSION

The biliary calculi collected from 200 gallstone patients were divided into 3 groups based on their colour: cholesterol calculi, mixed calculi and pigment calculi. Out of the 200 stones collected, 76 were mixed calculi, 72 were pigment calculi and 52 were cholesterol calculi indicating the incidence of gallstones in the studied population from Haryana state as follows: Mixed calculi (38%) > pigment calculi (36%) > cholesterol calculi (26%). These observations were in confirmation of our earlier report (8) and also similar to other reports from Aligarh (3) and Kanpur (4) region but different from Guwahati (20) and Japan (21). The differences may be

attributed to different dietary conditions and habitats and different socio-economic status of the people in these areas. Out of the total 200 stones collected, 74 were solitary, while rest were multiple. Cholesterol stones were bigger in size as compared to mixed stones and pigment stones similar to earlier reports from Calcutta (1) and Haryana (8). Out of the total number of stones collected, the incidence of gallstone was higher in age group 41-50 yrs (Table 1). These observations are in conformity with our earlier study from Haryana (8) and also reports from Assam (20) and Aligarh (3). The size (B and L) of cholesterol calculi was in the range 0.2-2.4 cm and 0.4-3.2 cm with an average of 0.97 cm and 1.50 cm, while for mixed calculi it was in the range 0.1-2.5 cm and 0.1-3.0 cm with an average of 0.6 cm and 0.93 cm and for pigment calculi it was 0.1-1.9 cm and 0.2-2.5 cm with an average of 0.69 cm and 1.01 cm, similar to our earlier report from Haryana (8) and from Calcutta (1). Cholesterol calculi were soft with soft surface, while pigment calculi were soft with rough surface. Mixed calculi had both rough and smooth surface. Cholesterol calculi showed radiations like spokes of wheel with a nuclear cross-section. Pigment calculi were amorphous, while mixed had laminated features. These results are comparable to reports from Singapore (22). Out of 200 gallstone patients, 138 were females and 62 were males and thus the female to male ratio was 2:1 which is comparable to earlier reports. Rains (23) advocated that concentration of bile salts in bile is reduced by oestrogen and thereby making it lithogenic. Horn (24) postulated that under the influence of female sex hormone, the muscle may relax, biliary passage dilates and duodenal content of pancreatic secretion regurgitates into gallbladder and promote conditions which favour the formation of gallstones. Females had a greater risk of gallstone disease, especially if they had used oral contraception and/or had four or more children (25).

Table I : Incidence of different types of gallstones in relation to age

Age group (yrs)	Type of gallstone			Total gallstones
	Cholesterol	Mixed	Pigment	
1-10	-	-	-	-
11-20	4	2	2	8
21-30	2	18	6	26
31-40	12	8	22	42
41-50	20	20	16	56
51-60	8	14	16	38
> 60	6	4	10	20
Total	52	76	72	200

Table II : Quantitative analysis of metabolites in different types of biliary calculi expressed as mg/gm dry stone powder (Data are mean±SE)

Group	Type of stone	Total Cholesterol	Total Bilirubin	Bile acids	Fatty acids	Triglycerides	Phospholipids	Soluble Protein
1.	Cholesterol (52)	597.98±33.22	2.10±0.32	19.84±2.48	20.65±1.08	64.31±5.35	8.28±0.62	25.58±5.45
2.	Mixed (76)	594.78±24.91	1.72±0.19	19.11±2.16	13.73±1.67	74.43±5.92	4.49±0.44	9.97±2.36
3.	Pigment (72)	489.85±28.68	4.87±0.49	21.55±2.37	14.63±1.71	31.53±1.89	6.44±0.48	97.43±12.39
		gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ <0.05	gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ <0.001	gp ₁ vs gp ₂ <0.01
		gp ₂ vs gp ₃ <0.01	gp ₂ vs gp ₃ <0.001	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ <0.001	gp ₂ vs gp ₃ <0.01	gp ₁ vs gp ₂ <0.001
		gp ₁ vs gp ₃ <0.05	gp ₁ vs gp ₃ <0.001	gp ₁ vs gp ₃ >0.05	gp ₁ vs gp ₃ <0.01	gp ₁ vs gp ₃ <0.001	gp ₁ vs gp ₃ <0.05	gp ₁ vs gp ₂ <0.001

Chemical analysis of biliary stones The cholesterol, bilirubin, bile acids, fatty acids, triglycerides, phospholipids, soluble protein, sodium, potassium, calcium, magnesium, copper, iron, inorganic phosphate, oxalate and chloride were found in all 200 gallstones from Haryana. The results of quantitative analysis of the constituents of 200 gallstones are expressed as mg/gm dry wt of stone powder in Table II-III.

Determination of metabolites

Total cholesterol: The total cholesterol was significantly higher in cholesterol calculi, compared to pigment stones ($p < 0.05$) and in mixed stones as compared to pigment stones ($p < 0.01$). However, there was an insignificant difference between total cholesterol content of cholesterol stones and mixed stones ($p > 0.05$). These results were in confirmation with our earlier report (8). Gallstones are believed to form, when the concentration of cholesterol exceeded that which can be held in mixed micellar solution with bile acids and phospholipids. Supersaturation of cholesterol is believed to be due to abnormal production of bile from liver. The concept of cholesterol supersaturation as a basis for gallstone formation has been emphasized for cholesterol stones, which are composed of mainly cholesterol. The high level of cholesterol in cholesterol calculi has been related to high carbohydrate diet (26).

Total bilirubin: The total bilirubin concentration was highest in pigment calculi and lowest in mixed calculi. It was significantly higher in pigment calculi compared to mixed calculi and cholesterol calculi ($p < 0.001$) and insignificantly higher in cholesterol calculi as compared to mixed calculi ($p > 0.05$). These observations are in agreement with earlier reports from Varanasi (2), Kanpur (4), Delhi (27) and Haryana (8). The colour of the pigment stones could be attributed to colour of bilirubin, which form salt with calcium to form calcium bilirubinate. It is known that β -glucuronidase of bacterial origin hydrolyses conjugated bilirubin into free bilirubin, which form salt with calcium as calcium bilirubinate (28-29).

Fatty acid (esterified): The esterified fatty acid content was highest in cholesterol calculi and lowest in mixed calculi. The high content of esterified fatty acids in cholesterol stones might be due to interaction between excessive cholesterol and fatty acids. The difference of fatty acid content was significant between cholesterol calculi and mixed calculi ($p < 0.05$) and between cholesterol calculi and pigment calculi ($p < 0.01$) but insignificant between mixed and pigment calculi ($p > 0.05$).

Triglycerides: The triglyceride content was highest in mixed calculi and lowest in pigment calculi. The triglyceride content was significantly higher ($p < 0.001$) in cholesterol calculi and

Table III: Quantitative analysis of cations and anions in different types of biliary calculi expressed as mg/gm dry stone powder (Data are mean±SE)

Group	Type of stone	Sodium	Potassium	Calcium	Magnesium	Copper	Iron	Inorganic Phosphate	Oxalate	Chloride
1.	Cholesterol (52)	1.05±0.19	0.229±0.02	10.66±1.85	8.48±1.01	0.70±0.10	0.64±0.05	18.43±2.71	6.54±0.29	19.93±1.19
2.	Mixed (76)	1.197±0.21	0.296±0.02	26.52±2.39	8.26±0.98	0.354±0.03	1.09±0.08	11.74±1.45	6.44±0.29	23.66±1.62
3.	Pigment (72)	3.12±0.38	0.645±0.06	27.60±3.13	10.84±1.06	0.348±0.05	0.78±0.05	8.49±1.49	7.02±0.25	32.82±1.67
		gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ <0.001	gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ <0.001	gp ₁ vs gp ₂ <0.001	gp ₁ vs gp ₂ <0.05	gp ₁ vs gp ₂ >0.05	gp ₁ vs gp ₂ >0.05
		gp ₂ vs gp ₃ <0.001	gp ₂ vs gp ₃ <0.001	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ <0.05	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ >0.05	gp ₂ vs gp ₃ <0.001
		gp ₁ vs gp ₃ <0.001	gp ₁ vs gp ₃ <0.001	gp ₁ vs gp ₃ <0.001	gp ₁ vs gp ₃ >0.05	gp ₁ vs gp ₃ <0.01	gp ₁ vs gp ₃ >0.05	gp ₁ vs gp ₃ <0.001	gp ₁ vs gp ₃ >0.05	gp ₁ vs gp ₃ <0.001

mixed calculi as compared to pigment calculi. However, the difference was insignificant between cholesterol calculi and mixed calculi ($p > 0.05$). The triglycerides get accumulated along with cholesterol salts to form gallstones. The higher content of triglyceride in mixed stone or cholesterol stone compared to pigment stone might be due to more deposition of calcium salts of cholesterol and esters of fatty acids in mixed and cholesterol stone as compared to pigment stone, where calcium bilirubinate is the major salt.

Phospholipids: The phospholipids content was highest in cholesterol stones and lowest in mixed stones. There was a significant difference between phospholipid content of cholesterol calculi and mixed calculi ($p < 0.01$), mixed calculi and pigment calculi ($p < 0.01$) and between cholesterol calculi and pigment calculi ($p < 0.05$). This might be due to accumulation of phospholipids alongwith cholesterol during cholesterol stone formation. The accumulation of phospholipids could either be due to their enhanced biosynthesis or decreased utilization.

Bile acids: The bile acid content in pigment stones was comparatively higher than that in cholesterol calculi and mixed calculi but the difference was insignificant ($p > 0.05$). This was five times higher than that reported from Japan (19). The difference might be due to the different dietary habits of people of these two countries.

Soluble protein: The soluble protein content was highest in pigment calculi and lowest in mixed calculi. The protein content was significantly higher in pigment calculi ($p < 0.001$) as compared to mixed calculi and cholesterol calculi and in cholesterol calculi as compared to mixed calculi ($p < 0.01$). Binette *et al* (30) suggested that the proteins to be the candidates either to facilitate or hinder the formation of stones. Maki (31) proposed the ability of bacterial β -glucuronidase enzymes (protein) to hydrolyse the bilirubin glucuronide complex thus releasing a poorly soluble bilirubin to explain the formation of pigment stones as well as cholesterol stones.

Determination of cations and anions

Sodium: The mean sodium content was highest in pigment calculi and lowest in cholesterol calculi. The difference in sodium content was highly significant ($p < 0.001$) in pigment calculi as compared to cholesterol calculi and mixed calculi but insignificant ($p > 0.05$) between cholesterol calculi and mixed calculi. Sodium forms salt with bile acid which gets accumulated during cholelithiasis. The more the content of

bile acid, the more content of sodium as was observed in pigment calculi compared to other calculi.

Potassium: The mean potassium content was highest in pigment calculi and lowest in cholesterol calculi. The potassium content was significantly higher ($p < 0.001$) in pigment calculi as compared to mixed and cholesterol calculi. The difference of potassium content of cholesterol calculi and mixed calculi ($p > 0.05$) was however insignificant. A certain ratio of sodium and potassium is maintained in bile of gall bladder. Higher the sodium content, higher the potassium content. This might be the reason for higher potassium content in pigment stones compared to other stones.

Calcium: The mean calcium content was highest in pigment calculi and lowest in cholesterol calculi. It was significantly higher in pigment calculi and mixed calculi as compared to cholesterol calculi ($p < 0.001$). However, there was an insignificant difference between calcium content of pigment calculi as compared to mixed calculi ($p > 0.05$). The calcium content in various gallstones was in the following order. Pigment calculi > Mixed calculi > Cholesterol calculi. It is in agreement with our earlier report (8) and reports from Varanasi (2), Singapore (22), Kanpur (4) and Chandigarh (7). Earlier calcium carbonate was identified as the most frequently occurring compound in pigment stones (32). This could be attributed to the suggestion that the copper and iron may act as chelating agents for calcium bilirubinate. The central aggregates of calcium salts constitute hard foreign bodies which may lead to ulceration of gallbladder mucosa and microscopic haemorrhage. The iron released by this process may be another source of its deposition in gallstones. Injury to gallbladder mucosa also provides an opportunity for release of epithelium β -glucuronidase, an additional enzyme contributing towards precipitation of calcium bilirubinate (7). It has been postulated that calcium precipitation in bile is a critical event in cholelithiasis (25).

Magnesium: The magnesium content was highest in pigment calculi and lowest in mixed calculi. However, there was no significant difference ($p > 0.05$) between magnesium content of three calculi. The oxalate content was also higher in pigment stone compared to cholesterol and mixed stone. Both magnesium and oxalate form insoluble salt which might be crystallized to strengthen the texture of pigment stones.

Copper: The copper content was highest in cholesterol calculi and lowest in pigment calculi. The content of copper in cholesterol calculi was significantly higher as compared to mixed calculi ($p < 0.001$) and pigment calculi ($p < 0.01$). However,

the difference in copper content of mixed calculi and pigment calculi ($p > 0.05$) was insignificant. The role of copper in pathogenesis of calcium bilirubinate in gallstones is suggested (33).

Iron: The iron content was highest in mixed calculi and lowest in cholesterol calculi. The content of iron was significantly higher in mixed calculi as compared to cholesterol calculi ($p < 0.001$) and pigment calculi ($p < 0.05$). However, there was an insignificant difference ($p > 0.05$) between iron content of cholesterol calculi and pigment calculi. These observations are in agreement with our earlier report (8) and report from Chandigarh (7). Been *et al* (33) reported the presence of small amount of iron in a thick black shell around the central dark inclusion of gallstones. The high iron content in mixed gallstones might be promoting aggregation of calcium bilirubinate particles which are the major constituents especially due to the polyelectrolytic nature of iron. Verma *et al* (7) suggested that central aggregates of calcium salts may lead to ulceration of gall bladder mucosa and microscopic haemorrhage. The iron released by this process might be another source of its deposition in gallstones.

Inorganic Phosphate: The inorganic phosphate content was highest in cholesterol calculi and lowest in pigment calculi. There was no significant difference of inorganic phosphorous content between mixed calculi and pigment calculi ($p > 0.05$). However, there was a significant difference between cholesterol calculi and mixed calculi ($p < 0.05$) and cholesterol calculi and pigment calculi ($p < 0.001$) but no significant difference between mixed and cholesterol calculi ($p > 0.05$). This is in agreement with report from Varanasi (2) but differed from our earlier report (8) where mixed calculi had highest inorganic phosphate content, however, there was no significant difference between mixed and cholesterol calculi. Inorganic phosphate might be playing an important role in the formation of cholesterol gallstones by forming salt with calcium.

Oxalate: The oxalate content in gallstones was in the following order: Pigment calculi > Mixed calculi > Cholesterol calculi. However, there was no significant difference ($p > 0.05$) between oxalate content of the three calculi. The higher content of oxalate in pigment stone compared to that in cholesterol stones could be explained on the basis of its harder texture due to more deposition of calcium oxalate crystals in these stones. Raha *et al* (1) found calcium oxalate to be one of the major component of mixed stones. Calcium oxalate was also a major component in the 2nd layer from within, in pigment

stones. Calcium oxalate was also detected in outer layer of stones.

Chloride: The mean chloride content was highest in pigment calculi and lowest in cholesterol calculi, which might be due to their easy deposition with calcium bilirubinate. The chloride content was significantly higher ($p < 0.001$) in pigment calculi as compared to cholesterol and mixed calculi but insignificant between cholesterol calculi and mixed calculi ($p > 0.05$). Chloride ions are always present in biological fluids in human beings including bile, which might get deposited in the form of sodium chloride salt along with major salts of the gallstones.

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