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Original Article

Quantitative Estimation of Biochemical and Inorganic Constituents Present in the Gallstones

Swati Jain*1 and M.C. Kanchan2

1Research Student, Department of Botany, Bipin Bihari P.G. College, Jhansi, India
2Ex. Professor, Department of Botany, Bipin Bihari P.G. College, Jhansi, India

***Corresponding Author**
Dr. Swati Jain
Research Student,
Department of Botany,
Bipin Bihari P.G. College, Jhansi, India
E-mail: swatijain12001@gmail.com

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Abstract

Carcinoma of gallbladder has an unusual geographic distribution. Gallbladder cancer is the most common type of biliary tract cancer which is the fifth most common gastrointestinal tract cancer and sixth most common digestive tract malignancy. Thus, the study was carried out based on quantitative analysis of biochemical constituents (cholesterol, bilirubin, bile acids, fatty acids, phospholipids and soluble protein) and inorganic constituents (sodium, potassium, calcium, magnesium, inorganic phosphate, oxalate and chloride) in all the three types of gallstones and also compare the present findings with other researchers findings. The results indicated that out of 179 gallstones, 41 were of cholesterol type, 77 mixed and 61 of pigment type. Cholesterol content was found to be the major component in all the three types of stones. Also, cholesterol stones had higher content of total cholesterol, fatty acids, phospholipids and inorganic phosphates as compared to mixed stones and pigment stones. While in case of pigment stones bilirubin, bile acids, soluble protein, sodium, potassium, calcium were found to be higher as compared to cholesterol and mixed stones. The overall findings with respect to biochemical constituents resulted that the cholesterol saturation is mostly responsible for crystallization sequences in human gallbladder.

1. Introduction

Gallstones, complex biomineralized deposits formed in the gallbladder, are still a major health problem all over the world. Carcinoma of gallbladder has an unusual geographic distribution. The chemical composition of gallstones is essential to study aetiopathogenesis of gallstone disease, which is especially of high prevalence in the northern region than in the southern part of India. Reports on chemical analysis of gallstones are available from different endemic regions of India like Kolkata, Varanasi, Aligarh, Kanpur, Mumbai, Patiala and Chandigarh. According to ICMR report (1996)[1] on gallbladder cancer at Delhi, Mumbai, Chennai, Bangalore etc. revealed that there is a definite preponderance of disease in females as compared to males. Various workers have worked on the chemical analysis of gallstone. Chandran *et al*(2007)[2] during chemical analysis of gallstone found total cholesterol as the major component and total bilirubin, phospholipids, bile acids, fatty acid, soluble proteins, calcium, magnesium, sodium, potassium, inorganic phosphates, oxalates and chlorides as minor components have been reported. The pigment stones were reported to be rich in total bilirubin, bile acids, calcium, oxalates, magnesium, sodium, potassium, chloride and soluble proteins compared to cholesterol and mixed stones. Verma *et al*(2002)[3] found calcium and trace elements in chronic cholelithiasis. Studies on gallstone composition carried out in different parts of the world indicate a close link with dietary habits and ethnicity[2][3][4]. Gallstone formation is relatively increased with consumption of dietary fats rich in saturated fatty acids [5][6]. This study describes an extensive quantitative biochemical and inorganic constituents of gallstones, including cholesterol, bilirubin, bile acids, fatty acids, phospholipids, soluble protein, sodium, potassium, calcium, magnesium, inorganic phosphate, oxalate and chloride and their association to induce neoplastic changes. Hence, the objective of this study was to determine the chemical composition of gallstones and their association to induce neoplastic changes.

When enough cholesterol is deposited from saturated fat they crystallize with the bile to form gallstone in the gallbladder or common duct of gallbladder commonly called cholelithiasis. In people when there is a rapid destruction of red blood cells (hemolytic anemia) generally develop gallstone composed of bilirubin, a bile pigments. Mostly gallstones are a mixture of cholesterol, acids, lipids and soluble protein. The quantity and the size of the stone may vary from one large stone to hundreds or thousands very small ones. Often persons with gallstones have no symptoms and require no treatment. These stones are known as silent gallstones and freely pass into the small intestine with no problem. The problem occurs when gallstones lodge into the cystic duct blocking the flow of bile from the gallbladder into the duodenum. Thus, the aim of the current study is to determine the quantitative estimation of biochemical and inorganic constituents present in the Gallstones.

2. Materials and Methods

2.1 Collection of Gallbladder samples

Total 179 Gallbladder samples were collected from seven different hospitals of Jhansi, India which were engaged in performing cholecystectomy operations.

2.2 Preparation of samples

Stones were collected from the gallbladder and were immediately processed under sterilized conditions. The stones were divided into three groups namely cholesterol, pigment and mixed stones depending upon their colour. The pale yellow or light brown and whitish stones as cholesterol, black and blackish brown are pigment stones and finally brownish yellow or greenish with laminated features are represented as mixed stones. The stones in the gallbladder were then separately powdered in pestle and mortar and dissolved in different solvents depending upon the type of chemical constituents to be analyzed. To determine the total cholesterol and total bilirubin; 30 mg stone powder was dissolved in 3 ml chloroform in a test tube.

The tubes were kept in boiling water bath for 2 minutes. The stone solution thus obtained was used for determination of total cholesterol and bilirubin. To determine calcium, oxalate, magnesium, chloride, inorganic phosphate, soluble protein, sodium and potassium; 30 mg stone powder was dissolved in 3 ml 1N HCl in graduated tube and its final volume was made up to 10 ml with distilled water. The tubes were kept in boiling water bath for 1 hour. To analyze phospholipids stone powder; 20 mg was dissolved in 15 ml Chloroform + Methanol in 2:1 ratio containing 1N HCl. To measure bile acids and fatty acids; the stone powder was dissolved in chloroform + methanol (2:1) mixture and ethyl alcohol + solvent ether in 3:1 mixture respectively.

2.3 Methods used for analytical measurements of various parameters

Total cholesterol was determined by enzymatic colorimetric method of Bayer diagnostic[7], Total bilirubin by colorimetric method of POINTE Scientific, INC.[8], Soluble protein by colorimetric method of Bayer's diagnostic[9], Oxalate by enzymatic colorimetric method, calcium by ACCUREX Biomedical Pvt. Ltd., Harold Varley, phospholipids and inorganic phosphate by colorimetric method of Fiske and Subbarow[10], Magnesium by colorimetric method by Neill and Neely[11], chloride by colourimetric method Bayer diagnostic by Schoenfied[12]; sodium and potassium from standard book (APHA)[13]; fatty acids by titration method by Stern[14], and bile acids by colorimetric method of Carey[15], The dissolved stone solution was stored at 2 – 8 ^{0}C when not in use.

3. Results and Discussion

Gallstones remain a serious health concern affecting millions of people throughout the world. It exhibits prevalence rates of about 25% in industrialized societies but are uncommon in underdeveloped or developing societies. The biochemical constituents of gallstones samples for cholesterol, mixed type and pigment type are presented in **Table 1.** Total 179 gallstones were recovered. Out of which 41 were of cholesterol type, 77 mixed type and 61 pigment type. However, it was found that mixed stones were in largest number followed by pigment stones and cholesterol stones Pundir *et al*[4] *and* Chandran *et al*[2] also reported similar type of results. But these results are in contrast to the observation made by Masao *et al*[16], who found the rapid increase of cholesterol stone in Japan and decrease in bilirubin stones.

The biochemical constituent's in different types of stone samples i.e. cholesterol, bilirubin, bile acids, fatty acids, phospholipids and soluble protein are summarized in **Table 1.** The quantitative estimation of 179 gallstones presented in **Table 1** reveals that the mean value and standard error of cholesterol contents found in the 3 types of stones namely cholesterol, mixed type and pigment type showed slight variation having its highest values in cholesterol stones (582.29 ±3.93) followed by mixed (576.34±4.63) and pigment stones (485.94±5.64) respectively. These results are quite insignificant as observed during the study period. The bilirubin content was significantly high in pigment stone (4.97±0.10) as compared to cholesterol (2.01±0.05) and mixed stones (1.74 ±0.02) respectively. The colour of the pigment stones could be attributed due 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. The content of bile acid was high in the pigment stone (22.28±0.25) followed by mixed stone (19.87±0.05) and least in cholesterol stone (19.23±0.10) respectively. Similar results were reported by Jaraari *et al*[6]and Chandran *et al*[2]. Super saturation of bile with calcium bilirubinate is inhibited by bile salts, which bind calcium, reducing the activity of free calcium ions. When supersaturation occurs, usually due to increased concentrations of bilirubinate anion, nuclea-tion may be initiated by binding of calcium bilirubinate to mucin glycoproteins in bile[17]. The fatty acids content was highest in cholesterol stone (20.77±0.20) followed by mixed stone (14.41±0.17) and least value in the pigment stone (14.35±0.16) respectively. The high content of fatty acids in cholesterol stones might be due to interaction between excessive cholesterol and fatty acids.

The phospholipids content was high in cholesterol stone (8.36±0.08) followed by mixed stone (6.37±0.05) and least in pigment stone (4.59±0.04) respectively. As the mean phospholipids content was high in cholesterol, it has to be in relative proportion with the bile salts and phosphatidyl choline to remain soluble in the bile and thereby stone formation[18]. About 5% of cholesterol is barely soluble in 20% phosphatidyl choline and this should require approx-imately 60% of bile salts. Any concentration above this level would cause precipitation of cholesterol, as shown by the presence of the highest amount of cholesterol in cholesterol stone is an indicator of this fact[19]. Pigment stone contained less cholesterol than the other two types of stones. Cholesterol in pigment stone is mostly because of co-precipitation with bilirubin and other compounds but not due to its super saturation in the bile[20]. In contrast to this, mixed stone have less cholesterol than cholesterol stone but more than pigment stone. This might be due to the presence of both cholesterol and bile pigment in these stone. The soluble protein content was high in pigment stone (33.36±3.93) followed by cholesterol stone (24.72±0.68) and least in mixed stone (10.03±0.05) respectively. Binette *et al*[21] suggested that the proteins to be the candidates either to facilitate or hinder the formation of stones. Maki[22] 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 stone as well as cholesterol stone. These results indicated that cholesterol plays significant role in the cholesterol stone formation which is evident from comparatively high content of cholesterol in cholesterol stones than that in pigment stones and mixed ones.

The inorganic constituent's in different types of stone samples i.e. sodium, potassium, calcium, magnesium, inorganic phosphate oxalate and chloride are summarized in **Table 2.** The sodium content was highest in the pigment stone (4.822±0.23) followed by mixed stone (2.35±0.14) and least in cholesterol stone (1.40±0.14) respectively. Similarly, the potassium content was also high in pigment stone (1.608±0.10) followed by mixed stone (0.449±0.02) and cholesterol stone (0.252±0.02) respectively. This indicated that an increase in sodium content facilitates excessive formation of bile salts. Potassium was also higher in pigment stone. Similar results were observed by Jaraari *et al*[6]. It is presumed that the sodium to potassium ratio will be maintained in the bile. Hence, higher sodium content is associated with higher potassium content, al-though the increase in the latter was not as much as that of the sodium content.

The calcium content was high in pigment stone (32.705±0.19) followed by mixed stone (29.61±0.31) and least in cholesterol stone (12.04±0.12) respectively. This is in agrees with studies by Jaraari *et al*[6] and Pundir *et al*[4]. It is known that bilirubin combines with calcium to form a precipitate of calcium bilirubinate[23]. Since pigment stone has excess bilirubin, calcium forms calcium bilirubinate[24]. These results indicated that the inorganic constituents were significantly higher in pigment stone as compared to mixed and cholesterol stones as depicted in **Table 2.** The magnesium content was highest in pigment stone (12.55±0.13) as compared to cholesterol (10.87±0.07) and mixed stone (9.80±0.08) respectively. This is in conformity with observations made by others [2][6].

The inorganic phosphate was high in cholesterol stone (20.89±0.04) followed by the mixed stone (12.63±0.04) and lowest in pigment stone (8.57±0.06) whereas oxalate content was significantly high in cholesterol stone (8.44±0.09) as compared to pigment stone

(8.22±0.13) and mixed stone (7.75±0.05) respectively. Insignificant difference was found between cholesterol stones (8.44±0.09) and pigment stones (8.22±0.13). The chloride content was high in pigment stone (40.88±0.05) followed by mixed stone (37.93±0.04) and least in the cholesterol stone (25.31±0.16) respectively. Whereas, Raha *et al*(1966) were found calcium oxalate to be one of the major component of mixed stone. Both magnesium and oxalate form insoluble salt which might be crystallized to strengthen the texture of pigment stone[25]. The mean chloride content was highest in pigment stone and lowest in cholesterol stone. Similar result also obtained by others in their series[2][6]. Same authors proposed that the 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. These results indicated that increase in inorganic constituents such as inorganic phosphate, calcium etc. may be due to increase in sera of cholesterol stone patients. This is evident from the presence of inorganic phosphate and calcium salts as a major components of cholesterol stones.

Table 1: Quantitative estimation of biochemical constituents in stone sample (in mg/gm stone powder)

S. No	Types of stone	Cholesterol	Mixed	Pigment
1.	Cholesterol	582.29 ± 3.93	576.34 ± 4.63	485.94 ± 5.64
2.	Bilirubin	2.01 ± 0.05	1.74 ± 0.02	4.97 ± 0.10
3.	Bile acids	19.23±0.10	19.87±0.05	22.28±0.25
4.	Fatty acids	20.77±0.20	14.41±0.17	14.35±0.16
5.	Phospholipids	8.36±0.08	6.37±0.05	4.59±0.04
6.	Soluble protein	24.72±0.68	10.03±0.05	33.36±3.93

(Data are of Mean Value ± Standard Error)

Table 2: Quantitative estimation of inorganic constituents in different stone samples (in mg/gm stone powder)

S. No	Types of stone	Cholesterol	Mixed	Pigment
1.	Sodium	1.40±0.14	2.35±0.14	4.822±0.23
2.	Potassium	0.252±0.02	0.449±0.02	1.608±0.10
3.	Calcium	12.04±0.12	29.61 ± 0.31	32.705 ± 0.19
4.	Magnesium	10.87 ± 0.07	9.80 ± 0.08	12.55 ± 0.13
5.	Inorganic Phosphate	20.89 ± 0.04	12.63 ± 0.04	8.57 ± 0.06
6.	Oxalate	8.44± 0.09	7.75 ± 0.05	8.22 ± 0.13
7.	Chloride	25.31 ± 0.16	37.93 ± 0.04	40.88 ± 0.05

(Data are of Mean Value ± Standard Error)

4. Conclusion

The overall results indicated that the cholesterol stone showed significantly higher cholesterol content than pigment stones though not significantly higher than mixed stones. These findings resulted that the cholesterol saturation is mostly responsible for crystallization sequences in human gallbladder.

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