FTIR SPECTROSCOPIC ANALYSIS OF HUMAN GALLSTONES

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Abstract. Fourier transform infrared spectroscopy (FTIR) has been carried out to analyze the organic and inorganic constituent in human gallstones (n = 34) from Rajah Muthiah Medical College and Hospital. Statistical correlations between stone weight, age, stone type, number of pregnancy, food habit and chemical composition of gallstones were performed. The quantitative estimation was calculated for the particular peaks of bilirubinate salt (3421 cm^{-1}), cholesterol (2932 cm^{-1}), calcium palmitate (2849 cm^{-1}) and calcium carbonate (1448 cm^{-1}). The study reveals that mixed and brown type gallstones were predominant whereas black stones were less frequent in the selected region. Calcium carbonate is found in all the stones, and it is in a higher amount in mixed stones.

Key words: Gallstones, FTIR, quantitative analysis.

INTRODUCTION

Gallstones, complex biomineralized deposits formed in the gallbladder [3], is still a major health problem all over the world. 20–30% of western and around 10% of non-western population have been affected by gallstones [7, 11]. Gallstones are frequently composed of more than one crystalline compound, although the exact mechanism of formation of multicomponent stones is not clearly understood. They are generally classified into three major types: pure cholesterol, pigment and mixed gallstone. Pigment gallstones are further subdivided into laminated brown stones and amorphous black stones [2]. Cholesterol stones contain more than 70% of cholesterol whereas pigment stones contain mainly of various bilirubinate salts with less than 20% cholesterol by weight. Pigment stones are predominant in India [10]. Brown and black stones are chemically, morphologically and clinically distinct. Black stones are formed in the gallbladder and are associated with hemolysis, cirrhosis and old age [15, 25]. Classification of pigment stones was made on the basis of the Proceedings of the first National Institute of Health – International Workshops on pigment gallstone diseases [26]. The major chemical constituent of brown stones is calcium palmitate (up to 25%), whereas calcium

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phosphate and calcium carbonate are both usually found in black stones. Chemical composition of gallstones is essential for aetiopathogensis of gallstone disease [5].

Non-spectroscopic methods, such as enzymatic and calorimetric methods, have been used to determine chemical composition of gallstones. The lack of specificity, sensitivity and inaccurate values are common in these methods. Infrared spectroscopy is the most widely used method because of its advantages over chemical analysis or other techniques. This technique requires minimal sample volume, specificity of all components and also provides quantitative results with greater reproducibility [29]. FTIR spectroscopy is a valid method for gallstones composition evaluation [9]. The present study was carried out to analyze the chemical composition of human gallstone using FTIR and quantitative analysis was performed using the baseline method.

MATERIALS AND METHOD

The fresh stones were collected from Rajah Muthiah Medical College and Hospital (RMMCH), Annamalai University, Annamalainagar since December 2006 till April 2008 and the stones were kept at 4 ºC until further analysis. The age of patients ranged from 25 to 83 years, including 13 men (57.77 ± 10.33 years) and 21 women (37.00 ± 8.21 years). All the patients belong to the same socio economic status. The stones were washed with running deionised water for an hour to remove debris and bile and dried at 60 ºC for four hours. During analysis the stones were cut into two halves by using a razor blade and one quarter was ground with a pestle and mortar, 2 mg of sample was mixed with 40 mg of spectral grade KBr crystals, and ground up as fine as possible to make the 13 mm diameter disc in silver dies at 5 ton pressure. The FTIR measurements were performed using a Perkin Elmer Spectrum RX1 Model FTIR spectrometer in the frequency range 4000–400 cm^{-1} at 4 cm^{-1} resolution. To obtain a high signal/noise ratio 100 scans were accumulated for each sample. Graphs were plotted by spectrum ver. 5.0.1 software baseline correction performed before drawing the spectra. The extinction coefficient (K) can be calculated using the relation

$$K = \frac{D \cdot A}{m} \text{ cm}^2/\text{mg} \text{ and } D = \log\left(\frac{I_0}{I}\right) \quad (1)$$

where D is the optical density of the particular absorption peak, A is area of the pellet and m is the mass of the pellet (in mg).

The quantitative method was adopted by using the baseline method. This method automatically corrects the cell absorbance, reflection losses and eliminates the possible errors.

STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical package for the social sciences (SPSS) version 12.0 (SPSS, Inc, Chicago, IL, USA) for windows, continuous variables are expressed as mean ± standard deviation (SD). One sample run test with ($p \leq 0.001$) was used to check the random mixture of stones in age group. The correlation studies were performed between age and stone weight, extinction coefficients and stone weight. One sample run test shows the stone type ($p < 0.001$) randomly mixed with age group. There is no significant relation between age and stone weight, age and extinction coefficients. Positive correlations between the peaks 2932 cm^{-1} and 3421 cm^{-1}, as well as between 2932 cm^{-1} and 2849 cm^{-1} have been observed.

RESULTS AND DISCUSSION

From the FTIR spectra, the collected gallstones were grouped into mixed ($n = 16$), brown ($n = 14$) and black ($n = 4$) type gallstones. This analysis showed that cholesterol crystal is the predominant composition in cholesterol and mixed gallstones. Presence of Cholesterol in the gallstone was characterized by a large O–H stretching absorption band at 3398 cm^{-1}, a C–H stretching vibration band at 2934 cm^{-1}, C–H deformation bands at 1466 cm^{-1} and a sharp absorption peak at 1056 cm^{-1} can be attributable to ring deformation of cholesterol [3, 12]. The characteristic strong peak at 2901 cm^{-1} is due to CH_2 symmetric stretching vibration. The doublets at 1378 and 1365 cm^{-1} are attributed to the (CH_2 and CH_3) bending vibration of cholesterol in the mixed gallstone [3, 13, 14, 19]. In the entire pigment gallstones weak band at 3398 cm^{-1} is due to the presence of cholesterol.

Various bilirubinate salts were observed by FTIR spectroscopy. The calcium bilirubinate has characteristic bands at 1254 cm^{-1} which are assigned to (C–O) stretching or C–N stretching coupled with NH deformation ν(C–N) + δ(NH) [19]. The reference peak for the amount of unconjugated bilirubin is 1614 cm^{-1}, but this shifts to 1624cm^{-1}. In this study, it confirms the presence of calcium bilirubinate in the pigmented stones [15, 19]. In black brown stone the unconjugated bilirubin peak at 3421 cm^{-1} is shifted to 3398 cm^{-1}, this is due to complex formation with some metals like calcium, copper. Vinyl polymer in the mixed stone and pigmented stone confirmed in by the peak of 986 and 991 cm^{-1}. Methyvinylamaleimide, which is a monopyrrole derived from the A and D ring of bilirubin and has a pendant vinyl group, demonstrated a prominent peak at 986 cm^{-1}. On the other hand, haematinic acid methyl ester, which is derived from the B or C ring of bilirubin and lacks pendant vinyl groups but has a pendant propionic acid group, shows little absorbance in the 990 cm^{-1} region [9].

Two crystalline forms of calcium carbonate have been detected in human gallstone: calcite and aragonite [6]. Calcite is characterized by two weak bands 1435 cm^{-1} (C–O stretching) Aragonite form of the $CaCO_3$ is found at weak intensity of 1085 and 699 cm^{-1}. Only the third crystalline form of calcium carbonate (vaterite), detected by X-ray diffraction method [24], was seldom observed by the weak band at 741 cm^{-1}. All the samples showed the vaterite peak in the present study. Frequency may be somewhat different in the different isomers.

Apatite and whitlockite are the two crystalline forms of calcium phosphate. They are rare constituents of gallstones [22]. This can be identified by the characteristic frequency of low intensity around 605 cm^{-1}. It can be easily detected as small white spheroids described as "beads" [24]. Calcium palimitate is the fourth most abundant component of human gallstone [23]. In the FTIR the identification of calcium palmitate could be based on the presence of a specific peak at 1412 and 2849 cm^{-1}, which is present in all mixed stones, but in black pigmented stone it is found at 1577 cm^{-1} [28] and a sharp peak at 1700 cm^{-1} for brown pigmented stone. The phosphate peaks are found in all the stone specimens with different frequencies, calcium phosphate is found to medium peak 957 cm^{-1} of mixed stone and a weak peak in black type of gallstone at 606 cm^{-1}. The peak at 1247 cm^{-1} may be due to P=O symmetric stretching vibration of PO_2 phosphodieters. Magnesium is the metal which has been detected next in frequency to calcium [1]. Longitudinal optic mode frequency of MgO is observed with weak peaks at 1236 and 799 cm^{-1} for black and mixed type stones [19]. The very broad bands in the spectra of pigment gallstones are said to be indicative of multiple metal ions (Mg, Ca, Fe, Cu) present in high concentration.

The peak at 1448 cm^{-1} (C–O stretching vibration) indicates that the gallstone had a high amount of calcium carbonate [1, 3, 21]. The very small peak around 1236 cm^{-1} in mixed stone indicates the presence of apatite. Interaction of calcium bilirubinate and hydroxy apatite peak was found at 617 cm^{-1}, which peak is very weak and rarely found in limited samples of mixed and black stones [27]. The bending and rocking vibrations characteristic of a long methylene chain are observed at 720 cm^{-1}[9]. Existence of amides II and I was confirmed in 1650 and 1543 cm^{-1} [28]. A less important spectral characteristic of calcium oxalate monohydrate (COM) found in mixed stones at 882 cm^{-1}, which can be distinguished from calcium oxalate dihydrate (COD) by the peak of 518 cm^{-1} [16]. Figure 1 and Table 1 show the spectrum and corresponding tentative assignments of human gallstones.

The present investigation was undertaken to assess the constituents of the pure and mixed stones and their inter relationship was also quantitatively estimated in the bilirubin content, cholesterol, calcium palmitate and calcium carbonate in all the gallstone samples. For this purpose spectral extinction coefficients (K) were calculated for the absorption bands of bilirubinate salt (3421 cm^{-1}), cholesterol (2932 cm^{-1}), calcium palmitate (2849 cm^{-1}) and calcium carbonate (1448 cm^{-1}).

The age of women patients lies in less than fifty, and men having the age of above forty, i.e. female patients having the gallstone disease at less than 50 years and male patients having the disease above 50 years. This is similar to previous studies [10, 17]. In this study area 61.76% female and 38.24% male patients had gallstone diseases. Within the female patients 4.76% had no pregnancy, 9.52% had one pregnancy, 52.38% had two pregnancies, 33.33% had more than 3 pregnancies till this study.

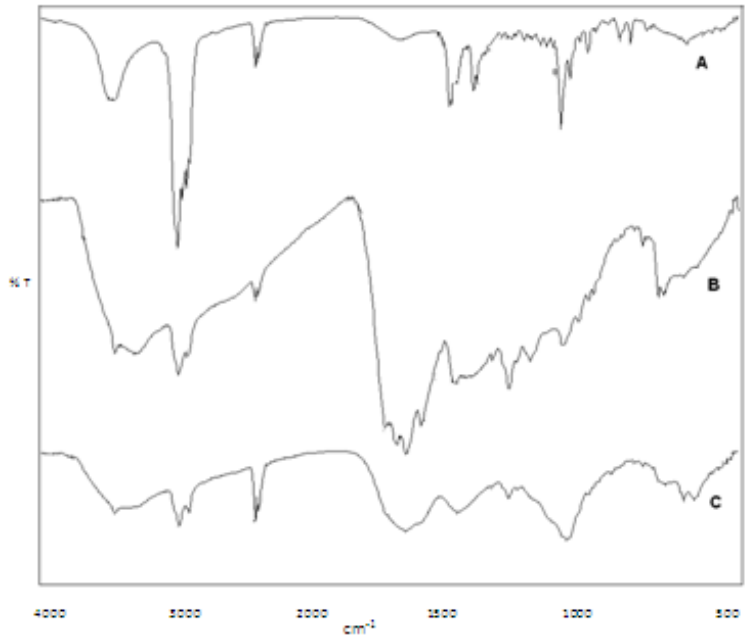

Fig. 1. FTIR spectrum of gallstone varieties A. mixed, B. brown, C. black.

Several recent studies showed the chemical composition of gallstones using FTIR [29, 18]. The present work, FTIR spectral extinction coefficients of the gallstones were studied for the particular peaks of 3421, 2932, 2849, and 1448 cm^{-1} which is the predominant peak for bilirubinate salts, cholesterol, calcium palmitate and calcium carbonate. The averages over the extinction coefficients are shown in Table 2.

Male patients comprise of 61.54% vegetarian and 38.46% non-vegetarian, similarly 23.81% and 76.19% of female patients are vegetarian and non-vegetarian, respectively. Many of the stones weight were lying in the range of less than one gram (70.59%). Chemical composition does not depend upon the stone weight. More than 95% of the pigment stones were multiple stones. 12.5% of mixed stones contain more than one stone in gallbladder.

Table 1

FTIR absorption frequencies and corresponding tentative assignment for human gallstones

Composition	**Absorption frequencies for different types of gallstone (cm^{-1})**		
	Mixed	**Black**	**Brown**
Cholesterol	3402, 2933, 2901, 2867,1466, 1459, 1376, 1365,1333 and 1056	3393, 2932, 1052	2929, 2866, 1052
bilirubin and its compound	3421, 3032, 1272, 1169, 986	3421, 992	3398, 1629, 991
calcium palmitate	2849, 1412	2850, 1577	2849, 1830, 1700
calcium oxalate	3446	–	–
amide I	1649	1654	1647
amide II	1543	1247	-
calcium carbonate	1448, 840	1446	1447
calcite $CaCO_3$	1438	–	–
aragonite $CaCO_3$	1082, 699	875, 698	698
vaterite $CaCO_3$	741	–	740
calcium oxalate monohydrate (COM)	882	–	–
Apatite	1236	–	–
Phosphate	957, 605, 593	1105, 606	1248

Table 2

Extinction coefficients for particular peaks in human gallstones

Type of stone	**Extinction coefficients (cm^2/mg)**			
	K_{3421}	K_{2849}	K_{2932}	K_{1448}
Mixed	3.232 ± 0.395	1.912 ± 0.490	1.066 ± 0.342	3.663 ± 0.748
Black	5.131 ± 1.184	6.399 ± 1.459	5.493 ± 1.225	6.576 ± 1.003
Brown	2.556 ± 0.217	4.015 ± 0.205	3.404 ± 0.195	4.148 ± 0.099

The present study shows that the extinction coefficient for mixed stones at 2949, 2932 and 1448 cm^{-1} peak is decreased. This represents the negative relationship with the amount of cholesterol, calcium palmitate, calcium carbonate present in the sample. The increase in value of extinction coefficient represents the decrease in actual quantity; this is in good agreement with quantitative reports of other methods such as calorimetric [4] and chemical analysis [8].

CONCLUSION

Identification of the constituents of gallstone is the first step in medical diagnosis. Spectroscopy is one of the major analytical tools for analyzing the chemical composition of gallstones qualitatively as well as quantitatively. An FTIR spectrum is a very sensitive, reliable and less time consuming technique for classifying the human gallstones. The quantitative analysis was performed for four consecutive peaks for cholesterol, calcium bilirubinate, calcium palmitate, calcium carbonate. The present study shows that no pure cholesterol stone was found in the selected patients and mixed gallstones are high in the selected area, brown stones being seldom found. Mixed varieties of gallstones contained more cholesterol, calcium carbonates, calcium palmitate and fewer amounts of bilirubin compounds. Black pigmented stones contained a lesser amount of cholesterol, calcium carbonate, calcium palmitate and bilirubinate compounds whereas brown stones contained a higher amount of bilirubinate and mixed stones contained an intermediate amount of these four compounds.

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