Gallstone Analysis

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ABSTRACT

Gallstone is a crystal deposit which is formed in the gallbladder or bile duct. Gallstone is classified into cholesterol stone, pigment stone (black and brown), and mixed stone. Mechanism which underlies the formation of cholesterol or pigment gallstone is different. Information on chemical component of the stone will assist the management and prevention of its recurrence. Analysis of gallstone component can be performed by colorimetry method or even gas liquid chromatography (GLC). Chemical component analysis of gallstone by colorimetry includes examination of cholesterol, bilirubin, and calcium. Stone is classified as cholesterol stone if the cholesterol content is > 80%, pigment stone if cholesterol content is < 20%, and mixed stone if cholesterol content is 25-80%. Gallstone analysis by GLC method is conducted by separation of fatty acid chain and evaluation of fatty acid quantity in the methylester derivatives form, which is fatty acid methyl estered. Fatty acid content in cholesterol stone (310.09 ± 49.7 mg/gram) is higher compared to pigment stone (55.59 ±7.71 mg/gram). Saturated to unsaturated fatty acid (S/U) ration in cholesterol stone (8.6 ± 3.1) is higher compared to pigment stone (4.8 ± 1.5).

Keywords: *gallstone, colorimetry, gas liquid chromatography*

ABSTRAK

Batu empedu adalah deposit kristal yang terbentuk dalam kandung empedu atau saluran empedu. Batu empedu diklasifikasikan menjadi batu kolesterol, batu pigmen (hitam dan coklat) dan batu campuran. Mekanisme yang mendasari pembentukan batu empedu kolesterol maupun pigmen berbeda. Informasi tentang komponen kimia batu akan membantu dalam penatalaksanaan dan pencegahan kekambuhannya. Analisis komponen batu empedu dapat dilakukan dengan metode kolorimetri ataupun gas liquid chromatography (GLC). Analisis komponen kimia batu empedu dengan metode kolorimetri meliputi pemeriksaan kolesterol, bilirubin dan kalsium. Batu diklasifikasikan sebagai batu kolesterol bila kandungan kolesterol > 80%, batu pigmen bila kandungan kolesterol < 20% dan batu campuran bila kandungan kolesterol 25-80%. Analisa batu empedu dengan metode GLC dilakukan dengan pemisahan rantai asam lemak dan menilai kuantitas asam lemak dalam bentuk derivat methylester yaitu fatty acid methyl estered. Kandungan asam lemak pada batu kolesterol (310.09 ± 49.7 mg/gram) lebih tinggi dibandingkan batu pigmen (55.59 ±7.71 mg/gram). Ratio saturated to unsaturated fatty acid (S/U) pada batu kolesterol (8.6 ± 3.1) lebih tinggi dibandingkan batu pigmen (4.8 ± 1.5).

Kata kunci: *batu empedu, kolorimetri, gas liquid chromatography*

INTRODUCTION

Gallstone is a crystal deposit which is formed in the gallbladder or bile duct. Gallstone is a disease with multifactorial causes which are associated with dietary habits, loss of body weight, sex, and family history. Its chemical composition can be associated with nutritional, social economical, and genetic factors.[1,2] Incidence of gallstone disease is 10-15% in European population and 3-5% in Asian and African population. Incidence of gallstone disease in American Indian population reaches up to 73% in female adults. The mortality rate is approximately 0.6%. Incidence of gallstone disease in Indonesia is thought to be of no difference with Asian.[3-5]

Gallstone disease is frequently found inadvertently through abdominal ultrasonography (USG) examination for other indication. Epidemiology study states that 70-80% of gallstone disease remains asymptomatic and approximately 20% cause symptoms with complications that emerge in 5-20 years after diagnosis. Gallstone that passes through gallbladder to the intestine is potential to cause ileus and require surgical procedure.[6,7]

Laboratory examination in gallbladder disease is not specific, consisting of haematology examination (complete blood count), liver function test, amylase, and lipase.[8] Other supporting examination for gallbladder and bile duct evaluation can be performed by ultrasound, plain abdominal x-ray, radioisotope, computed tomography, magnetic resonance cholangiopancreatography, endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiogram.[9] Gallstone analysis can give information regarding chemical composition of the stone. Information regarding chemical composition of stone will help in the management and recurrence prevention.[10,11]

PATHOGENESIS OF GALLSTONE

Gallstone is formed due to abnormal composition of bile acid. This stone can be located in gallbladder or even bile duct as shown in Figure 1. Gallstone is classified into cholesterol stone, pigment stone (black and brown), and mixed stone. Cholesterol stone contains > 50% monohydrate cholesterol with other components including calcium salt, bile pigment, protein, and fatty acid. Pigment stone particularly consists of bilirubinate calcium with cholesterol content of < 20%. Mixed stone has cholesterol content between cholesterol and pigment stone.[9,12] Most gallstone cases (70%) in Western countries are cholesterol gallstone, while in East Asia mostly are brown pigment stone.[13,14]

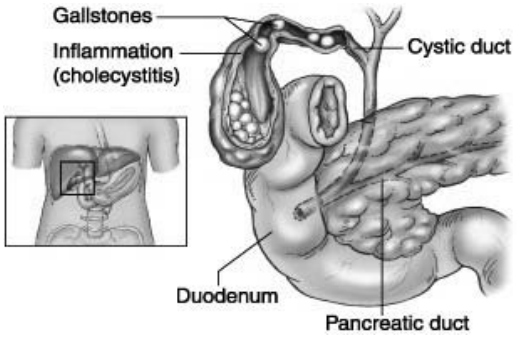

Figure 1. Gallstone in biliary system.[14]

CHOLESTEROL GALLSTONE

Formation of cholesterol gallstone involve several mechanisms, including supersaturation of bile acid with cholesterol, monohydrate cholesterol nucleation, and gallbladder hypomotility.[9] Excessive cholesterol level compared to phospholipid and bile acid causes vesicle rich in cholesterol to aggregate and cholesterol crystal to precipitate. Hypersecretion of cholesterol in obesity condition, high calorie diet, high cholesterol diet may cause bile supersaturation with cholesterol. Hyposecretion of bile acid or phospholipid also may contribute to this condition. Second mechanism is nucleation of monohydrate cholesterol in bile acid which may lead to the excess of pronucleation factors,

Table 1. Predisposition factors of cholesterol gallstone.[9,15-17]

Predisposition factors	Mechanism
Genetic	Mutation in Cyp7A1 gene causing 7-α-OHase deficiency, thus later decrease synthesis of bile acid
	MDR3 mutation which cause defect in phospholipid secretion
Age	Increased secretion of biliary cholesterol
	Decreased secretion of bile salt
Obesity, metabolic syndrome	Increased biliary cholesterol
	Decreased cholecystokinin response
Body weight loss	Breakdown of fat during not eating period and fast body weight loss causes liver to secrete extra cholesterol to bile
	Bile salt hyposecretion which originates from enterohepatic cycle
Female Sex hormones	Estrogen stimulates hepatic lipoprotein receptor, thus causing increase in the uptake of dietary cholesterol and secretion of biliary cholesterol
	Progesterone causes decreased contraction of gall bladder
Long parental nutrition, fasting	Gall bladder hypomotility
Pregnancy	Progesterone inhibit gall bladder contractility

such as mucin and immunoglobulin or deficiency of anti-nucleation factors such as apolipoprotein AI and AII. The third mechanism is the presence of gallbladder hypomotility. Gallbladder emptying disorder which contains crystal or bile acid which undergo supersaturation with cholesterol supports gallstone formation.[9] Factors that support cholesterol gallstone formation can be seen in Table 1. Picture of cholesterol gallstone is shown in Figure 2.

Figure 2. Cholesterol gallstone[18]

PIGMENT GALLSTONE

In normal condition, hemoglobin breakdown product, the bilirubin, will undergo conjugation in the liver into conjugated bilirubin which is more soluble in water. Conjugated bilirubin will be hydrolysed by beta glucoronidase bacteria in the colon into unconjugated bilirubin which can precipitate as calcium salt or even catabolised by bacteria into urobilinogen. Pigment stone is formed due to the increase of unconjugated bilirubin in the bile.[19] Predisposition factors and mechanism of pigment stone formation can be seen in Table 2.

Pigment gallstone is classified into black and brown pigment stone. Black stone is small in size, irregular, and formed in the gallbladder. Black pigment stone is formed from pure bilirubinate calcium or polymer like complex with calcium and glycoprotein mucin. Black pigment stone is associated with chronic haemolysis or cirrhosis.[9] Brown pigment stone is formed in the bile duct. Components of brown pigment stone consist of calcium salt and unconjugated bilirubin with varied number of cholesterol and protein. Brown pigment stone is associated with bacterial or parasitic infection in the bile duct.[9,13] Picture of black and brown pigment stone can be seen in Figure 3.

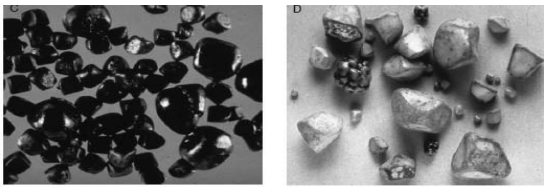

Figure 3. Pigment stone[18]

Table 2. Predisposition of pigment stone[7,9,13,19,20]

Predisposition factors	Mechanism
Demography (Asia)	Genetic factor
Chronic haemolysis	Increased of biliary unconjugated bilirubin which can precipitate with calcium
Cirrhosis	Hypersplenism and the presence of changes in the composition of erythrocyte lipid membrane which leads to haemolysis
Pernicious anaemia	Ineffective erythropoiesis due to vitamin B12 or folate deficiency
Chronic infection of biliary tract	Bacteria or parasite induce β glucuronidase which deconjugated saturated bilirubin and cause increased of unconjugated bilirubin which can form complex with calcium
Ileal disease, ileal resection/ bypass	Malabsorption of bile acid in the ileum which increase bile acid in the colon and saturate unconjugated bilirubin and increase its passive absorption in the colon (pathological enterohepatic recirculation of bilirubin).

GALLSTONE ANALYSIS

In this review, two methods of examination analysis, which are colorimetry method and gas liquid chromatography (GLC) method, will be discussed.[1,10,21]

COLORIMETRY METHOD

Colorimetry method is a technique to determine concentration of particular substance by measuring the absorption in sample in the form of coloured solution. The colour can be produced from the substance itself or the coloured product formed through a chemical reaction which is then measured with particular wavelength. Gallstone component chemical analysis with colorimetry method includes cholesterol, bilirubin, and calcium examination. The percentage of cholesterol in the stone determines the type of gallstone. Stone is classified as cholesterol stone if the cholesterol content is > 80%, pigment stone if cholesterol content is < 20% and mixed stone if the cholesterol content is 25-80%.[10,22]

Cholesterol Analysis

In cholesterol analysis of gallstone, there are two processes, which are sample extraction and the process of cholesterol level measurement using colorimetry principle. The first process is sample extraction process which can be conducted in 2 ways. The first method is as follow: Gallstone is washed and dried, from which 0.1 gram is taken. Stone is trimmed in the mortar and 30 mg of the stone powder is transferred to a tube. Extraction is performed by adding 10 mL ethanol, which is later incubated at 50^{0}C for 10 minutes. It is then left standing in room temperature for 10 minutes

and tubes are centrifuged 2000 g for 10 minutes and the supernatant is being extracted.[9] The second one is as follow: stone is made into powder using pestle and mortar. Extraction is conducted by adding 3 mL chloroform to 30 mg stone powder in a tube which is boiled in waterbath for 2 minutes.[12]

The second process is the measurement of cholesterol level using colorimetry method. Extraction result which is obtained from the first or second process is used as the substance to determine its cholesterol level.[10,12] Chemical reactions in cholesterol examination is shown in Figure 4.

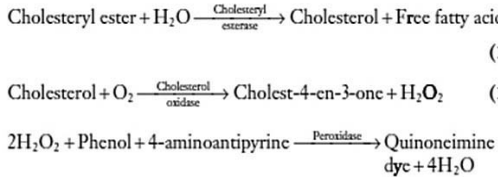

Figure 4. Chemical reaction of cholesterol examination[10]

Reaction results (quinone-imin) is read at 500 nm wavelength.[23] Cholesterol level in molarity unit (mmol/L). Formula below is used to obtain cholesterol weight in stone:

$$\text{Molarity} = \frac{\text{weight (gram)}}{\text{molecular weight } (\frac{\text{gram}}{\text{mol}})} \times \frac{1000}{\text{Volume (mL)}}$$

Cholesterol weight can later be compared with total weight of the stone which is examined to obtain its percentage.[10,24]

Unconjugated Bilirubin Analysis

In the analysis of total bilirubin in gallstone, there are 2 processes: the sample extraction and the process of bilirubin level measurement using colorimetry principle. First process is sample extraction process from the gallstone which is performed by the following procedure: gallstone is being washed and dried, from which 0.1 gram is taken. The stone is crushed using mortar and 10 mg of the stone powder is placed in a tube. Add 200 µl dimethylsulphoxide (DMSO) to dissolve unconjugated bilirubin and incubate for half of an hour. Add 10 µl HCl (12 mol/L) to the tube and incubate again for another half of an hour to separate bilirubinate calcium. Add 5 mg EDTA to bind calcium. Add 100 µL NaOH (1.2 mol/L) to obtain neutral pH. Tube is made until 5 mL with bovine albumin solution (4 g/L) in TRIS buffer (0.1 mol/L, pH 7.4).[10,25]

The second process is measuring bilirubin level by using colorimetry principles. Results of extraction from the first process is used as the ingredient to determine total bilirubin concentration. Chemical reaction in total bilirubin examination is shown in Figure 5.[10,25-26]

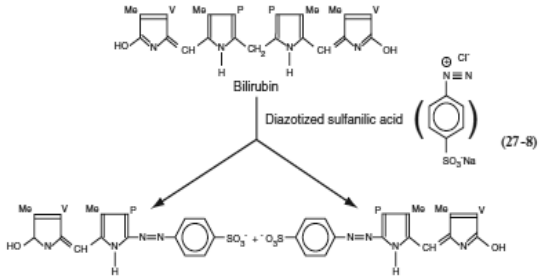

Figure 5. Chemical reaction of bilirubin examination[10]

Results from the chemical reaction is examined at 546 nm wavelength. The bilirubin level is in molarity unit (µmol/L). Bilirubin weight can be obtained by inserting the result to the formula of cholesterol examination with colorimetry method. Bilirubin weight is then compared to total stone weight which is evaluated to obtain its percentage.[10,24-25]

Calcium Analysis

Calcium analysis in gallstone with colorimetry method can be performed using 3 methods, which are o-cresolphthalein complexon (non-enzymatic colorimetry), atomic absorption spectrometry and modification of o-cresolphthalein complexon (after ashing the sample).[10]

The first method is o-cresolphthalein complexon (non-enzymatic colorimetry) with sample preparation as follows: 25 grams of stone powder is transferred to volumetric flask. Add 5 drops of concentrated HCl and 1 mL of water. This mixture is used to analyse calcium.[10] Principle of examination using o-cresolphthalein complexon method is calcium ion will react with o-cresolpthalein complex which in alkaline solution will form violet coloured complex which is absorbed at 577 nm wavelength. Addition of 8-hydroxyquinoline eliminates the interference of magnesium and iron. Chemical reaction in calcium analysis is shown in Figure 6. Increased of absorbance in reaction is line with calcium concentration in the sample.[10,27]

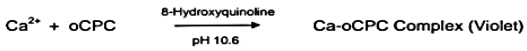

Figure 6. Chemical reaction of calcium examination[10]

The second method, the atomic absorption spectrometry, uses similar sample preparation with o-cresolphthalein method. Sample is heated in flame, thus the element in the sample will dissociate from

its chemical bond (atomised) and is in ground state. Ray of hollow cathode light pass through the flame and further is absorbed by ground state calcium atom. Monochromator will isolate ray which is emitted (in 422.7 nm wavelength) and later continued to the detector. The signal is processed by computer system to acquire its concentration and result is displayed in the screen (Figure 7).[28-29]

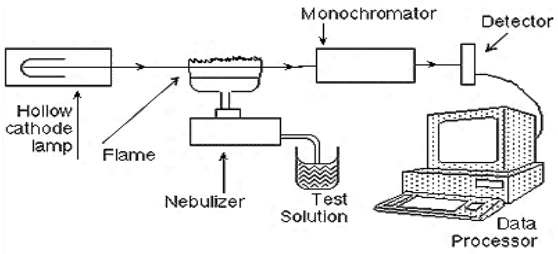

Figure 7. Examination process using atomic absorption spectrometry method[29]

The third method, o-cresolphthalein complexon after sample has been turned into ash, using the same reaction with the first method, only the substance being used has been turned into ash. Sample preparation uses 25 mg of gallstone powder which is dissolved in 250 µl of H_2SO_4 (1 mol/L) in porcelain bowl. Sample is turned into ash by heating it at 600^{0}C until it is colourless and then cooled in room temperature. Add HCl (250 µL, 370 g/kg) to the residue, and then add 10 mL double-distilled water. The sample is then transferred to 50 mL volumetric flask and calcium is analysed similarly with the first method o-cresolphthalein complexon.[10]

GAS LIQUID CHROMATOGRAPHY (GLC) METHOD

Fatty acid in the gallstone may originate from phospholipid bile acid which is hydrolysed due to the presence of phospholipase. Fatty acid (particularly saturated fatty acid such as palmitic acid, lauric acid, stearic acid) may precipitate and decrease the capacity of cholesterol solution which supports the formation of cholesterol gallstone. Examination using this method is to obtain the fatty acid content and saturated to unsaturated fatty acid ratio (S/U) in cholesterol or pigment gallstone. Gallstone analysis using GLC method can be performed by fatty acid chain separation and evaluation of fatty acid quantity in the form of methylester derivatives which is fatty acid methyl estered.[21]

Sample preparation in examination using GLC method is as follows: gallstone is washed using deionized distilled water until it is clean. The stone is crushed using mortar into a homogenous powder. Fifteen mg of the stone powder is added to 20 µg myristic acid and is heated using 5 mL hexane and butyl alcohol solution (2:1) at 65^{0}C for 1 hour. After being centrifuged, the supernatant is taken and then the residue is being extracted again using hexane-butyl alcohol solution. The mixture is evaporated until it is dried and fatty acid content is obtained. Myristic acid in hexane-butyl alcohol is used as internal standard.[22]

Fatty acid in the form of methylester derivatives (fatty acid methyl estered) is made by following method: into 20 µg fatty acid sample which is made before, 50 mL hydrochloric acid is being added and heated in 60^{0}C in boiling water for 4 hours. Take 1 mL of solution and add to 2 mL of the main reagent (mixture of 0.1 mL acetylchloride and 30 mL methanol). 1 µL filtrate is analysed using gas liquid chromatography.[21]

Chromatography is a method to separate and identify components in a mixture. The process is as follow: sample is injected and the sample component will be transferred by helium gas flow as mobile phase through the column. The column contains chemically bound fused silica 5% OV1 (polysiloxane) in cromosrb W as its stationary phase which is used to separate fatty acids. Each component will move together with the mobile phase in different velocity which is later detected by a detector. Detector signals are then being plotted as chromatogram. Process of GLC method examination can be seen in Figure 8. [21,30]

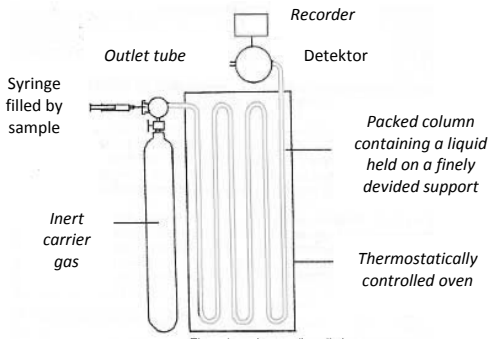

Figure 8. Examination process using gas liquid chromatography method[30]

The duration of component inside the column in this condition is called retention time. Retention time which is produced in each fatty acid can identify fatty acid in the sample. Quantification is performed by comparing sample peak area with standard peak area. Fatty acid chromatogram in gallstone sample can be seen in Figure 9.[21,30] Table 3 showed fatty acid content in different type of gallstone.

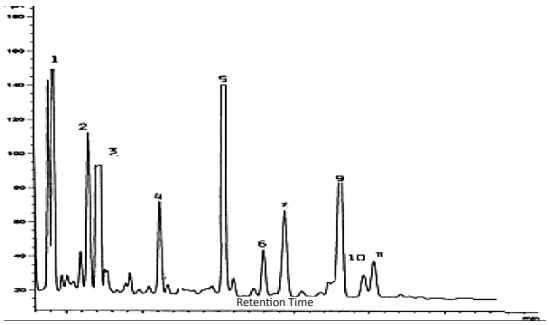

Figure 9. Fatty acid chromatogram in gallstone patients with cholelithiasis. Peak identification: (1) Butyric acid; (2) Lauric acid; (3) Palmitoleic acid; (4) Palmitic acid; (5) Myristic acid; (6) Oleic acid; (7) Stearic acid; (8) Linoleic acid; (9) Linolenic acid; (10) Archidic acid; (11) 12-hydroxy stearic acid.[21]

Table 3. Free fatty acid content (mg/g) in gallstone.[21,31]

Fatty acid (mg/g)	Saturated/ unsaturated	Cholesterol stone	Pigment stone
Butyric acid	Saturated	33.1± 8.43	33.7 ± 7.21
Lauric acid	Saturated	0.09 ± 0.01	0.32 ± 0.1
Palmitic acid	Saturated	67.8 ± 5.19	12.8 ± 3.72
Palmitoleic acid	Unsaturated	97.1 ± 27.01	3.5 ± 0.78
Stearic acid	Saturated	31 ± 5.01	0.96 ± 0.08
Oleic acid	Unsaturated	53 ± 7.71	1.69 ± 0.11
Linoleic acid	Unsaturated	25.33 ± 5.5	0.83 ± 0.15
Linoleinic acid	Unsaturated	0.03 ± 0.01	0.6 ± 0.098
Archidic acid	Saturated	0.35 ± 0.1	0.35 ± 0.1
12- Hydroxy stearic acid	Saturated	0.02 ± 0.001	0.00

A study conducted by Mohammed AM et al found that the fatty acid content in cholesterol stone (310.09 ± 49.7 mg/gram) was higher compared to pigment stone (55.59 ±7.71 mg/gram). Saturated to unsaturated fatty acid (S/U) ratio in cholesterol stone (8.6 ± 3.1) was higher compared to pigment stone (4.8 ± 1.5).[21]

CONCLUSION

Gallstone is a crystal deposit which is formed in the gallbladder or bile duct. Gallstone is classified into cholesterol stone, pigment stone (black and brown), and mixed stone. Cholesterol gallstone formation can involve supersaturation mechanism of bile with cholesterol, monohydrate cholesterol nucleation, and gallbladder hypomotility. Pigment stone happen due to increase of unconjugated bilirubin in bile. Laboratory examination in gallstone disease is unspecific, consisting of haematology examination (complete blood count), liver function test, amylase, and lipase. Chemical analysis of gallstone can give information regarding chemical composition of stone. Methods to analyse gallstone component can be performed by colorimetry method or gas liquid chromatography (GLC) method. Information regarding stone chemical component will help in the management and prevention of recurrence.

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