

# CHAPTER I

## INTRODUCTION

### I. Bilirubin metabolism

Bilirubin is a family of bile pigments that are the major waste product of heme metabolism (Roskoski, 1996). Heme is degraded in cells of the reticuloendothelial system. Heme oxygenase, assisted by O<sub>2</sub> and NADPH, opens the protoporphyrin ring to release iron, carbon monoxide, and a linear tetrapyrrole namely biliverdin. Biliverdin is metabolized to form bilirubin which is very hydrophobic in nature and thus highly insoluble in aqueous solution (Schreiber, 1989). In adults, the breakdown of heme-containing proteins generates about 250 to 400 mg of bilirubin per day. Approximately 80-85 % of bilirubin in serum comes from the breakdown product of heme in reticuloendothelial system and the remaining 15 % is derived from the catabolism of other heme-containing proteins such as myoglobin, cytochromes and peroxidase (Burtis and Ashwood, 1996).

Newly formed bilirubin is transported from the extrahepatic sources as bilirubin-albumin complex and removed very rapidly from circulation by the liver. Normally, the plasma bilirubin concentration is less than 1 mg/dL. Serum bilirubin is occurred in hepatocyte by four-step processing: the uptake, cytosolic binding, conjugation, and secretion (Cui *et al.*, 2001 ; Kaplowitz, 1996 ; Routh, 1982).

The first processing step of serum bilirubin is the uptake of bilirubin from circulation by the hepatocyte. This step occurs with the dissociation of the albumin-bilirubin complex facilitated by plasma membrane proteins with subsequent translocation of bilirubin into the hepatocyte through a saturable protein carrier, which also binds other organic anions but not bile salts. The hepatic uptake system operates well below saturation, and the uptake does not limit bilirubin excretion. Approximately 40 % bilirubin taken up by the hepatocyte after a single pass refluxes unchanged back to the plasma. This reflux may increase in hyperbilirubinemia.

Binding of cytosolic in the hepatocyte is the second step of serum bilirubin processing. In this step, bilirubin binds to two cytosolic proteins: ligandin and Z protein. The binding limits

the reflux of bilirubin back to the plasma and delivers it to the endoplasmic reticulum for conjugation.

The third step involves bilirubin esterification with glucuronic acid, first to form bilirubin glucuronide, a monoglucuronide, and then a diglucuronide. The principal enzyme involved is UDP-glucuronyl transferase. Administration of microsomal enzyme inducers such as phenobarbital, glutethimide and clofibrate causes increased activity of this enzyme. Conjugation renders bilirubin water soluble and is essential for its elimination from the body via bile and urine. Most of the conjugated bilirubin excreted into human bile is diglucuronide with lesser amount of monoglucuronide.

The final step is secretion of conjugated bilirubin from hepatocytes to the bile canaliculi. This step involves a specific carrier and occurs against concentration gradient. The carrier is shared by other anions including anabolic steroids and cholecystographic agents but not bile acids. In fact, bile acids facilitate bilirubin secretion. Secretion is the rate-limiting step in transferring of bilirubin from plasma to bile. Conjugated bilirubin (Bc) is excreted in bile as a micellar complex with cholesterol, phospholipids and bile salts. Bacteria in the colon deconjugate and convert it to a large number of urobilinogens. A minor portion of these pigments is absorbed into plasma through the enterohepatic circulation and is excreted in the urine. The rest is excreted in the stool.

## II. Chemistry and structure of bilirubin in circulation

Bilirubin is a family of bile pigments. All of them contain a common asymmetric tetrapyrrole structure. There are three major classes of bilirubin in blood: unconjugated bilirubin (Bu), sugar-conjugated bilirubin (Bc) and bilirubin covalently linked to albumin also called delta bilirubin (B<sub>δ</sub>) or biliprotein (Doumas and Wu, 1991).

### Unconjugated bilirubin (Bu)

Unconjugated bilirubin is extremely apolar and practically insoluble in water at physiologic pH and temperature. The apolar nature of Bu presented at two propionic acid chains. The main form of Bu (the IX- $\alpha$  isomer) is not a linear tetrapyrrole, but a tightly internally folded structure in which the propionate groups are linked to the pyrrole nitrogens (Fog and Jellum, 1963). This kind of structure, which accounts for the poor solubility of Bu, was confirmed by X-

ray crystallography (Bonnert *et al.*, 1976). Bu assumes the *Z,Z*-configuration, the folded conformation being stabilized by six intramolecular H-bonds, and from this model it can be deduced that partial or complete rupture of the H-bonds, as in irradiation of Bu with blue light, could lead to partially open (*Z,E* and *E,Z*) or completely unfolded (*E,E*) geometric isomers (Fig. 1). As expected, these isomers are more polar and more water-soluble than native Bu. Thus, geometric isomerization provides a plausible mechanism for the excretion of Bu in bile as a soluble pigment upon phototherapy (McDonagh *et al.*, 1980). It should be noted that, apart from X-ray data, there has been no direct proof that Bu actually occurs as a *Z,Z*-isomer *in vivo*. Also, the photon-induced geometric isomers of Bu appear less diazo-reactive and bind to albumin less avidly than native Bu (Onishi *et al.*, 1980). On the basis of these findings, one may infer that the overall configuration and conformation of Bu (or of its isomer) plays an important role in determining its solubility, binding affinity for albumin and diazo-reactivity.

#### **Sugar-conjugated bilirubins (Bc)**

Bc is formed by the enzymatic addition of one to two molecules of a sugar (principally glucuronic acid) onto either one ( $C_{12}$  or  $C_8$ ) or both ( $C_{12}$  and  $C_8$ ) of the propionic acid side chains of Bu. The resulting mono-(mBc) and di-(dBc) sugar conjugates (Fig. 2) are more polar than Bu, nontoxic to cells, and are excreted against a concentration gradient across the canalicular membrane of the hepatocyte in the bile. The glucuronide nature of Bc was proposed independently in 1956 and 1957 (Talaftant, 1956 ; Schmid, 1957 ; Billing *et al.*, 1957). It appears that while dBc is the predominant bilirubin in human bile, mBc is often the chief bilirubin conjugate in jaundiced sera in which the direct fraction is greater than 50 % of total bilirubin (Wu and Sullivan, 1982). The fact that mBc seems to be much less stable than dBc *in vitro* may be among the reasons why mBc has been particularly difficult to isolate and purify. One should also note that bilirubin can migrate from C-1 to C-2, C-3, and C-4 hydroxyls of glucuronic acid to form acyl-migrated isomers of both mBc and dBc (Compernelle, 1982) with potentially profound consequences for the formation of delta bilirubin (McDonagh *et al.*, 1984).

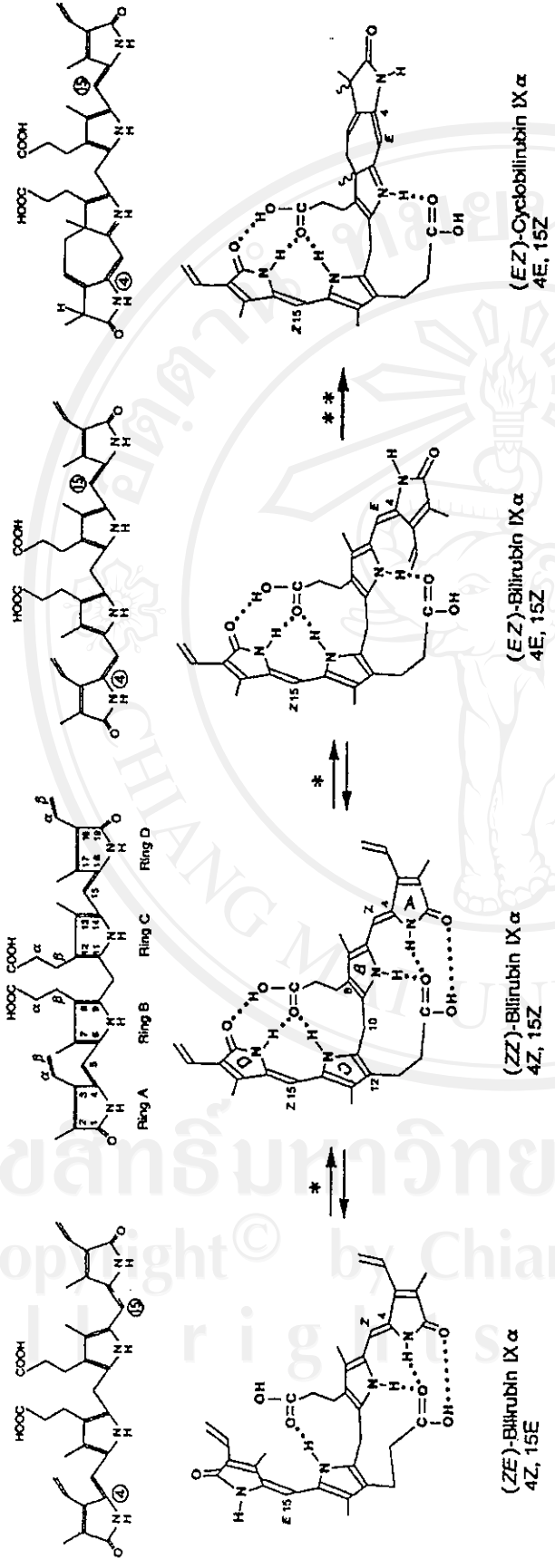


Figure 1. Bilirubin subfractions and photoisomers during phototherapy for unconjugated hyperbilirubinaemia \* = configurational photoisomerization, \*\* = structural photoisomerization (Itoh *et al.*, 2000).

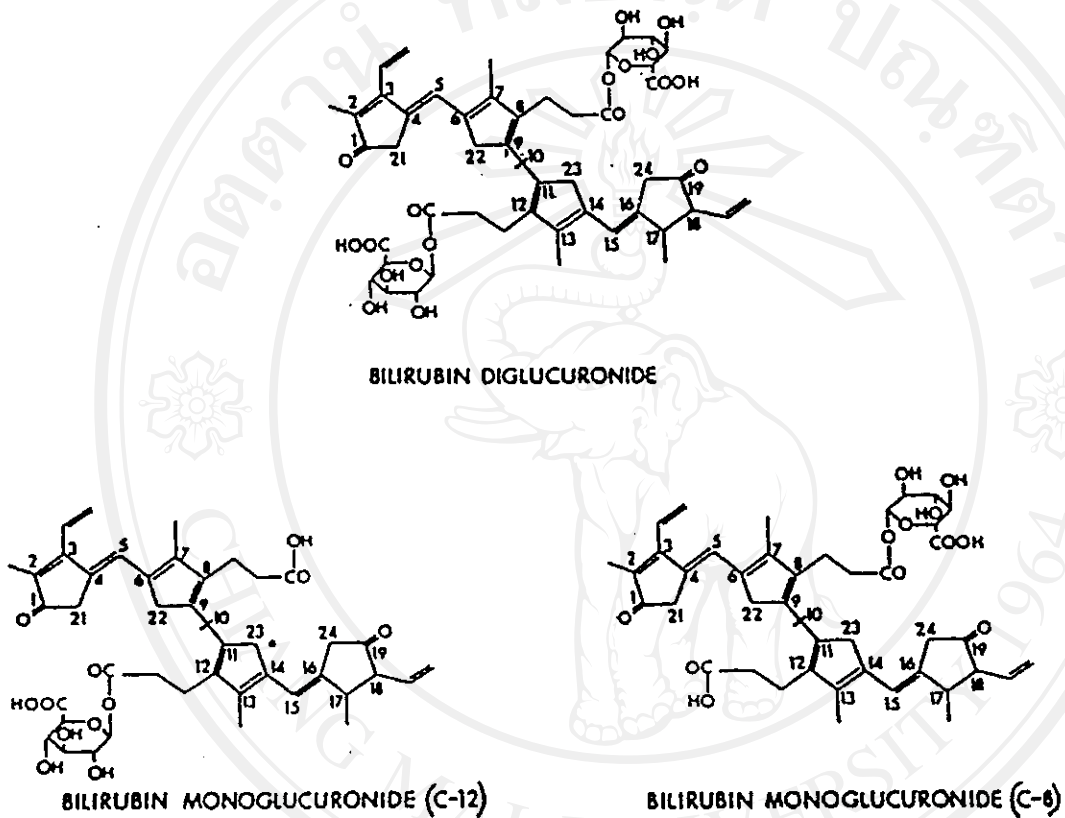


Figure 2. Structure of bilirubin glucuronides. Monoglucuronides can exist as two molecular species, depending on whether the C-12 or C-8 propionic acid side chain is esterified (Doumas and Wu, 1991).

### Bilirubin covalently linked to albumin ( $B_{\delta}$ )

The possible presence of a strongly protein-bounded bilirubin was first proposed in 1966 (Kuenzle *et al.*, 1966) but it was not until 1981 that  $B_{\delta}$  was identified (Wu *et al.*, 1981) to be an entity distinct from Bu and Bc. On the basis of current knowledge,  $B_{\delta}$  is a bilirubin covalently linked to albumin through an amide bond between one of its two propionic acid side chains and an  $\epsilon$ -amino group of a lysine residue on albumin (Wu, 1987).  $B_{\delta}$  has been synthesized *in vitro* by incubating (at 37 °C) rat serum with dBc or a mixture of mBc and dBc, or human serum albumin with a mixture of mBc and dBc (McDonagh *et al.*, 1984). Formation of  $B_{\delta}$  *in vivo* appears to be largely nonenzymatic and it involves acyl migration of bilirubin from a bilirubin-glucuronic ester to a nucleophilic site on albumin. Unconjugated bilirubin does not react with albumin to form  $B_{\delta}$ . Further evidence for the formation and structure of  $B_{\delta}$  is as follows: (1) in unconjugated hyperbilirubinemia (hemolytic jaundice) there is no more than a trace of  $B_{\delta}$  in serum (Brett *et al.*, 1984), and analbuminemic rats are unable to synthesize  $B_{\delta}$  (Kambe *et al.*, 1987), (2) in obstructive hepatobiliary disease, the increase in the concentration of  $B_{\delta}$  in serum parallels the increase in Bc (Lauff *et al.*, 1983), (3)  $B_{\delta}$  is the slowest fraction to clear from serum following resolution of severe hepatobiliary disease, presumably owing to its albumin moiety, which has a half-life of approximately 19 days; (4) the half-life of bilirubin, covalently bound to rat albumin, injected into the rat was 2 days, identical with that of rat albumin, while the half-life of unconjugated bilirubin linked to rat albumin was 6 min (Reed *et al.*, 1988), (5) when  $B_{\delta}$  is coupled with diazo reagent, one half of the azopigment is not ultrafilterable presumably because it is still bound to albumin (Lauff *et al.*, 1982).

An interesting attribute of  $B_{\delta}$  is that this irreversible bilirubin-albumin complex reacts readily, as does Bc, in the direct diazo reaction (Lauff *et al.*, 1982 ; Wu, 1987). It could suggest that the strength of binding to albumin is not a basis for distinguishing between "direct" and "indirect" bilirubins. Rather, such terms may refer to bilirubins of different solubilities in water. From an examination of the azopigments of authentic human  $B_{\delta}$ , it could be deduced that about 65 % of  $B_{\delta}$  molecules are nonesterified (not conjugated to sugar), while 30 to 35 % could be sugar conjugated (Lauff *et al.*, 1982).  $B_{\delta}$  arising from dBc will tend to be sugar conjugated on the carboxyl group not linked to protein, whereas  $B_{\delta}$  arising from mBc will tend to be unconjugated (Yoshida *et al.*, 1987). Eventually, elucidating the exact binding site(s) of bilirubin on the

albumin will be important to our understanding of its chemistry and structure. Results from chemical and enzymatic cleavage of B $\delta$  indicate the presence of one or two bonding regions with very similar amino acid sequence on albumin for the covalent linkage (Wu, 1983). Because the two regions are located in different sites within the protein, one of the sites might be preferentially involved in the linkage with bilirubin. This remains to be verified.

### III. Clinical significances of bilirubin

Hyperbilirubinemia, a condition characterized by an increase of bilirubin in the blood is that interfered in bilirubin metabolism. Five mechanisms that can lead to hyperbilirubinemia are listed as follows; overproduction, impaired uptake by liver cells, defects in the conjugation reaction, reduced excretion into bile, and obstruction to the flow of bile (Whitby and Smith, 1988).

In adults, serum bilirubin assays are largely used as a test of liver function, which may be compromised by inflammatory or obstructive lesions. The latter are more likely to give rise to increases in the conjugated and protein-bound bilirubin species in serum but in practice the differential diagnosis of hepatic disease on this basis is rarely clear cut, and only the measurement of total bilirubin is widely used at the present time.

In the neonate, red cell life is shorter than in adult, and this leads to a greater bilirubin burden for hepatic metabolism, which may already be compromised by immaturity of the glucuronyl transferase system. Jaundice is therefore common in the neonate, and in some may be sufficiently severe to require treatment by phototherapy which requires frequent monitoring by serum bilirubin assays. A very wide range of neonatal disorders may exacerbate the hepatic compromise by increasing bilirubin formation. In some severely ill infants, especially if premature, exchange transfusions may be required. Inborn errors of bilirubin metabolism and congenital disorders of the bile duct (biliary atresia) may also present in childhood and consequently bilirubin and the conjugated species are required (Muraca *et al.*, 1987).

It has long been held, but never unequivocally proven, that it is the "free" bilirubin in serum which is toxic, and that kernicterus is consequently prevented by albumin-binding and potentiated by drugs which displace bilirubin from its binding site (Levine, 1979). However, there are clinical and experimental inconsistencies with this argument (Levine *et al.*, 1982), and

albumin-bound bilirubin may itself have a role in cerebral icterus when the patency of the blood-brain barrier is diminished (Wennberg and Hance, 1986 ; Robinson and Rapoport, 1987). Consequently, free bilirubin assays do not yet have an established place in clinical chemistry laboratories.

#### IV. Methods for determination of serum bilirubin

Serum bilirubins are one of the laboratory analysis routinely employed in the diagnosis and treatment of hepatobiliary disease and hemolytic jaundice. Assays for total and direct reacting bilirubin can be performed by several methods and used for various diagnostic purposes.

##### Direct spectrophotometry

Bilirubin is yellow in colour by virtue of its absorbance at about 450 nm. Its concentration can be measured in serum by spectrophotometric methods (Westwood, 1991). In most methods two absorbances are measured, one near the bilirubin peak and another at a higher wavelength as a blank against a number of possible interfering compounds such as hemoglobin,  $\beta$ -carotene, lipochromes and other pigments that increase the absorbance at 455 nm (Roskoski, 1996). This method is used for determining total bilirubin in serum at 455 and 555 nm. The difference in absorbance between these two wavelengths represents absorbance of bilirubin only (Sherwin and Sobenes, 1996). The spectrophotometric methods require little manipulation of the specimen and have been developed into simple analyzers called "bilirubinometers" which have been widely used in both clinical chemistry laboratories and in baby units for the rapid assessment of neonatal jaundice. In some methods the wavelength difference may be compared with spectrophotometric (e.g. filter) standards or with sera of known bilirubin concentration, and in others the measured absorbances may be inserted into a formula for conversion to a bilirubin concentration based upon an estimated molar absorbance of bilirubin (Hertz *et al.*, 1974 ; Blijenberg *et al.*, 1987b)

##### Diazotization method

The reference method is the Jendrassik and Grof (Jendrassik and Grof, 1938 ; Doumas and Wu, 1991) that recommended by the Committee on Standards of the American Association for Clinical Chemistry and "credentialed" by the National Reference System for the Clinical Laboratory (National Committee for Clinical Laboratory Standards, 1989). The reaction



involved coupling of bilirubin with the diazo reagent. By this principle, the diazotization of Bc in serum or plasma is performed in the absence of "accelerator" in a one-minute reaction so called "direct bilirubin" (DB). In total reaction, Bu in serum or plasma is determined by the addition of one of accelerators such as caffeine/sodium benzoate (Jendrassik and Grof, 1938), methanol (Malloy and Evelyn, 1937), or dimethyl sulfoxide (DMSO) (Gambino, 1965 ; Walter and Gerade, 1970). The azopigment color product has indicator properties at different acid (red color) or alkaline (blue color) pH condition, and thus are absorbed spectrophotometrically at 540 nm (Malloy and Evelyn, 1937 ; Gambino, 1965 ; Walter and Gerade, 1970) or 600 nm (Jendrassik and Grof, 1938) that can be measured for the value of "total bilirubin". Value for Bu (indirect) is obtained by subtracting Bc (direct) from the total bilirubin value.

#### Enzymatic method

The enzymatic method for the measurement of bilirubin is based on its oxidation to biliverdin (colorless) by bilirubin oxidase (BOD) isolated from *Myrothecium verrucaria*. The resulting decrease in absorbance at 450 nm is proportional to the bilirubin concentration. The oxidation rates for various bilirubin depend on pH of the reaction mixture and on the presence of surfactants (Kosaka *et al.*, 1987). In the absence of detergent, Bc and B<sub>g</sub> are oxidized at acidic pH, while Bc is oxidized at alkaline pH, which in the conditions of the assay, only 5 % of Bu is oxidized and B<sub>g</sub> is not oxidized (Doumas *et al.*, 1999). Bu is oxidized in the presence of sodium dodecyl sulfate in 0.1 mol/L Tris buffer, pH 8.2 (Kosaka *et al.*, 1987).

The bilirubin oxidase method for bilirubin determination in serum has been developed in 1998 (Kurosaka *et al.*, 1998). In the conjugated bilirubin assay, conjugated bilirubin is selectively oxidized by bilirubin oxidase at pH 5.5 in the presence of reagents such as sodium fluoride (NaF) and *N*-acetylcysteine (NAC) which can decrease bilirubin oxidase reactivity to unconjugated and delta bilirubins. On the other hand, bilirubin oxidase can oxidize all three bilirubin species (unconjugated, conjugated and delta bilirubins) to non-coloured corresponding substances at pH 7.8 in the presence of an anionic detergent. The decrease in absorbance at 450 nm which occurs upon the oxidation is linearly related to the concentration of bilirubin species in serum. These assays perform well within an appropriate dynamic range of measurement, good reproducibility of results and good correlation of the results with diazo assay results.

### High Performance Liquid Chromatography (HPLC)

The HPLC is the method of choice used for the separation of bilirubin species in serum. By this technique, bilirubins are resolved on a reversed-phase column, and eluted in order of decreasing polarity. Four distinct bilirubin fractions in serum were reproducibly separated on the column, the delta bilirubin ( $B_{\delta}$ ), as well as the more familiar bilirubin diglucuronide (BDG), bilirubin monoglucuronide (BMG), and Bu respectively (Lauff *et al.*, 1982 ; 1983). The concentration of each fraction is calculated from the peak areas by using a calibration curve. However, this method is flawed by: (1) the need to pretreat serum to remove the globulins, which may entail a variable loss of  $B_{\delta}$ ; (2) reliance on the untested assumption that all of the bilirubins have absorptivities identical to that of Bu at one wavelength (450 nm); (3) errors in measurement of each fraction may be cumulative, and may result in a large total errors; (4) the method is relatively insensitive at total bilirubin concentrations below 1 mg/dL; (5) the procedure is laborious and not readily amenable to routine clinical analysis (Doumas and Wu, 1991).

### V. The problems of the measurement of bilirubin fractions in serum and urine

An accurate method for specific determination of bilirubin and its conjugates in biological fluids is not yet available. Most clinical laboratories measure serum bilirubin by direct-indirect reaction with diazo reagent in the presence or absence of an accelerator (Jendrassik and Grof, 1938 ; Malloy and Evelyn, 1937 ; Gambino, 1965 ; Walter and Gerade, 1970). Although diazo procedures are useful for assessing the concentration of serum bilirubin, these methods have been found to be inadequate for accurate determination of the unconjugated and conjugated pigmented fractions.

The measurement of total bilirubin (Bu, Bc and  $B_{\delta}$ ) in a sample by a diazo method is needed for determination of the actual concentration of each individual pigment. Moreover, urinary pigments distinct from bilirubins, such as mesobilifuscins and uroerythrin, have been found to interfere with conventional diazo procedures, and the present findings suggest that similar interfering substances may be present in serum. Total bilirubins as determined by diazo methods correspond exclusively with bilirubin and its ester conjugates, the presence of diazo-positive material that is distinct from bilirubin and its ester conjugates in biological samples has not been excluded (Blanckaert, 1980). In the absence of a definitive method for total bilirubin, it

is impossible to assess the accuracy of the reference method. It is reasonable to conclude that three of four bilirubin fractions (Bu, mBc and dBc) are measured accurately by this method because a 3 % underestimation of total bilirubin occurred when the B<sub>g</sub> concentration in serum is high (Doumas *et al.*, 1985a).

The development of a HPLC procedure which the various fractions of bilirubin can be quantitated has led to a better understanding of bilirubin compositions. The method shows that plasma from healthy individuals contains very little (that is, less than 5 %) conjugated bilirubin. This finding indicate that most diazo methods for measurement of conjugated bilirubin greatly overestimate the actual concentration of this fraction. Measurement of bilirubin using chemical assays that give a low proportion (15 % or less) of direct-reacting bilirubin in the serum of healthy individuals probably gives the most accurate results. The amount of direct-reacting bilirubin in a particular diazo method is depend on the time and temperature of the reaction.

Direct diazo reagent reacts with not only conjugated bilirubin, but also some unconjugated bilirubin and delta bilirubin. Their contribution to the direct-reacting fraction appears to depend on the reaction condition. In the Jendrassik and Grof method, 76-89 % of delta bilirubin was reported to be detected as direct-reacting bilirubin (Lauff *et al.*, 1982). The lack of reference materials for the measurement of direct-reacting bilirubin has necessitated the use of Bu as a calibrator for direct-reacting bilirubin method. In such methods, the calibrator (Bu) must be analyzed by a procedure for total bilirubin in the presence of an accelerator, whereas serum specimens are assayed without an accelerator. Calculations of direct-reacting bilirubin values are based on the assumption that azopigments produced from Bu and Bc have identical molar absorptivities. By this assumption, it is true for the azopigment that conjugated with glucuronic acid. The use of Bu as the calibrator in direct bilirubin assays was said to be impractical and ditaurobilirubin (DTB) is suggested to be used as a calibrator for direct bilirubin assay (Lo and Wu, 1983). DTB is easily synthesized and behaves spectrally like natural Bc in the Jendrassik and Grof total bilirubin assay (Wu, 1984). For diagnostic purposes, specificity is more important than accuracy in measuring direct bilirubin. A falsely increased direct bilirubin can lead to unwarranted diagnostic procedure, conclusions, and therapies. The most important factors affecting accuracy in direct-reacting bilirubin assay are calibration, concentration of HCl in the

final reaction mixture, reaction time, specimen blanking, bichromatic correction techniques, and possibly the presence of wetting agents (Lott and Doumas, 1993).

$B_{\delta}$  gave a large direct reaction in the diazo analysis procedure for bilirubin. By an Ektachem spectrophotometric methods,  $B_{\delta}$  is underestimated because it is retained in the spreading layer of film slide (Westwood, 1991).  $B_{\delta}$  concentration has been shown to be accurately quantitated by the reversed phase HPLC techniques. This techniques can detect both free and protein-bound species as well as bilirubin photoderivatives (Adachi *et al.*, 1988). However, this technique is blemished by same reasons and unsuitable to routine clinical analysis.  $B_{\delta}$  was found to react with direct diazo reagent to produce two azodipyrrolic fragment, where one of two fragments has no protein attached to it and the other fragment remains firmly attached to albumin (Lauff *et al.*, 1982).  $B_{\delta}$  can also be determined by a more simple anion-exchange chromatographic technique combined with the diazo-reaction (Seligson *et al.*, 1985). The diazo-products of  $B_{\delta}$  are in protein bound and free form in solution in equal amounts. The former can be determined because it remains in solution after treatment with the ion-exchange resin. Enzymatic methods using bilirubin oxidase from *Myrothecium verrucaria* are described for the determination of delta bilirubin fraction in serum. The determination is based on the different reactivities of the enzyme to bilirubin fraction at different pH in the presence or absence of anionic detergent such as sodium dodecyl sulfate (SDS) or sodium cholate. In the absence of detergents, Bc and  $B_{\delta}$  are oxidized at acidic pH, while Bc is oxidized at alkaline pH reaction. Bu is not oxidized at either acidic or alkaline pH (Kosaka *et al.*, 1987). Thus, subtraction of Bc and  $B_{\delta}$  concentration analyzed in acid pH detection with Bc concentration in the alkaline pH reaction yields the  $B_{\delta}$  concentration in a serum sample.

## VI. Literature reviews

The analysis of bilirubin in serum has frequently been requested by the physicians to help with diagnosis of liver diseases. The most widely used method for bilirubin determination in most clinical chemistry laboratories is the diazo coupling method. However, it was reported that the direct diazo reagent reacts with some unconjugated bilirubin in serum (Killenberg *et al.*, 1980). It also has less specificity to estimate the albumin bound conjugated bilirubin ( $\delta$ -bilirubin) which reacts slowly in the direct reaction determination (Doumas *et al.*, 1987). The enzymatic

method, which was proposed to have more specificity than the diazotized method, is still has some limitation because of the variation of the assay conditions in various instruments (Dumas *et al.*, 1999) and the enzymatic kit is too expensive to be used in the routine clinical chemistry laboratories (Nakayama, 1995).

A routine assay for conjugated bilirubin is available only to those who have certain types of clinical analyzers. The more generally available measurement is that of direct-reacting bilirubin (diazo reagent principle). The availability of a practical conjugated bilirubin determination method adaptable to variety of clinical analyzers might increase recognition of the clinical value of routine conjugated bilirubin testing.

The preparation of suitable materials for standardization of bilirubin assays is compounded by variability and instability of bilirubin reference solution preparations, the nature and variable concentration of the different species present in serum and their different behaviour in virtually all methods. Only unconjugated bilirubin is readily available in pure (crystalline) form. It is poorly soluble in water and unstable, especially to light and oxidation. It is more soluble but still unstable in DMSO, chloroform, alkali, or aqueous protein solutions, in addition its spectral behaviour is very dependent on the nature of the solvent and any impurities present. Similarly, while bilirubin is more soluble in aqueous solutions in the presence of proteins, its absorbance peak and absorptivity are dependent on both the source and nature of the proteins (Blijenberg *et al.*, 1987a). Even different preparations of the same protein may yield different results, and for the spectrophotometric methods human serum or protein based standards are essential. Indeed, no synthetic standard can be considered entirely satisfactory for these methods, and some regard the use of pools of neonatal serum were also essential for older diazo methods using methanol as an accelerator. But those based upon the method of Jendrassik and Grof are more robust in this respect and standards in either human or bovine albumin can be used satisfactorily though the human material is preferable.

A number of methods have been described for dissolving the currently available purified bilirubin preparations in serum or protein solutions. These generally involve adding an accurately weighed quantity of crystalline bilirubin into a suitable solvent before diluting it into the serum or protein solution. However, bilirubin is unstable in strong alkali. DMSO solutions are less easily added to the protein diluent, and some preparations are very difficult to completely dissolve in

sodium carbonate. The preparation of primary standards in this way requires care and attention to detail, and for this reason many laboratories use commercially available secondary standards such as control serum.

There are currently no standards available for diazo-direct bilirubin assays, and this together with the variety of reaction conditions in use account for the apparently very poor performance of the methods. At present, there seems no alternative to the use of DTB or unconjugated bilirubin as standards (Doumas *et al.*, 1987). The former is at least direct-reactive, but neither species is actually a true direct-reacting component of serum.

The lack of reference materials for the measurement of direct bilirubin has necessitated the use of unconjugated bilirubin as a calibrator. The preparation of suitable materials for use in internal quality control suffers from exactly the same problems as those mentioned for standards. However, the precise bilirubin concentration is obviously less critical, and the main requirement is behaviour corresponding to that of the characteristic specimen.

Preparation of conjugated bilirubin was reported in 1979. Bilirubin 1-*O*-acyl  $\beta$ -D-mono- and diglucuronides were prepared from rat bile and isolated by thin-layer chromatography (Blanckaert *et al.*, 1979). The purity of these preparations was assessed by the alkaline methanolysis procedure followed by normal-phase HPLC. The monoglucuronide preparation contained 92 % authentic monoglucuronide, 6 % diglucuronide, and 2 % unconjugated bilirubin. Neither unconjugated bilirubin nor monoglucuronide were detectable in the diglucuronide preparation.

The concentration of conjugated bilirubin (Bc) was found higher in native bile than unconjugated bilirubin (Donovan and Carey., 1993). In bile, there are species-specific differences in bilirubin conjugation (Cornelius *et al.*, 1975), and alterations can occur in the normal pattern of bilirubin conjugation within a given species. Isolation of bilirubin glucuronide from human gall-bladder bile was performed primarily by Lucassen in 1961 (Lucassen, 1961). The isolation protocol modified by Wu *et al.* gave more consistently yield than the original protocol of Lucassen (Wu *et al.*, 1980). The presumptive Bc isolated by the modified method showed physical characteristics of dark-brownish yellow, fluffy, very hygroscopic, totally water-soluble and turns green in humid air or in the light. The appearance of visible spectrum is broad peak

over 420-450 nm with maximum absorbance between 420-425 nm. The isolated obtained, quantitated by nuclear magnetic resonance (NMR), ranged in purity from 15-40 %.

The *in vitro* biosynthesis of Bc was also reported by Wu *et al.* The method performed from fresh human liver homogenate in the presence of Bu substrate and D-glucaro-1,4-lactone (a glucuronidase inhibitor). It was deduced from direct analyses with 270-MHz proton NMR and field desorption mass spectrometry that a major conjugated bilirubin species in bile isolate is a diglucuronide, whereas in liver biosynthesis, a diconjugate containing glucuronic acid and possibly glucuronolactone co-esterified to the bilirubin backbone were obtained (Wu *et al.*, 1980).

The synthesis of di- and mono-taurobilirubin was reported by Jirsa *et al.*, in 1956 (Jirsa *et al.*, 1956). Both forms are water-soluble compound which reacted directly without accelerator with diazo reagent and produced azopigments with an absorption spectrum similar to that of azobilirubin (Jirsa and Vecerek, 1958 ; Jirsa and Jirsova, 1959).

In 1985, Dumas *et al.* (Dumas *et al.*, 1985) examined purified and commercial preparations of putative ditaurobilirubin with regard to purity, spectral characteristics, and reactivity in the total and direct diazo reactions. The structural studies showed that the DTB as supplied is largely a disodium salt of ditaurobilirubin with more than 95 % purity in purified preparation and 85 % purity in the commercial-grade material. DTB could be used as a surrogate calibrator because of its chemical and physical properties. DTB was suggested to be a suitable material for calibrating direct-reacting bilirubin and total bilirubin assays based on, but not restricted to, the Jendrassik and Grof principle. It is freely soluble in water and stable in the powder form when kept dry. The DTB, commercial-grade was stable for three years at 4 °C without any change in its unconjugated bilirubin content or the ratio of direct-reacting bilirubin to total bilirubin. Nowadays, ditaurobilirubin is used by several manufacturers in the preparation of calibrator for direct bilirubin assays and control serum.

The separation of conjugated bilirubin from native bile and in model bile system by using HPLC was described by Spivak and Yuey. The method involved the use of a Perkin-Elmer 3 $\mu$  C<sub>18</sub> column and a methanol/ sodium acetate/ aqueous ammonium acetate buffer system. It was an extremely efficient 12-min HPLC method for the separation of native bile pigments without sample preparation. The method permitted the separation of bilirubin diglucuronide (BDG),

bilirubin monoglucuronide (BMG), and unconjugated bilirubin using only 6-10  $\mu$ L of sample (Spivak and Yuey, 1986).

Inaccurated determination of bilirubin in serum can be caused by some sources of error associated with the most commonly used method. Inadequate standardization appears to be the most common error. Availability of pure or certified Bu standard has not improved the accuracy of Bc analysis because Bu reacts very slowly with diazo reagent in direct reaction. As described before, Bc could be prepared by at least three different methods (Lucassen, 1961 ; Wu *et al.*, 1980 ; Spivak and Yuey, 1986). In this study, the rapid and efficient HPLC method was used to isolate Bc from native bile. The isolated Bc would be identified and characterized for its chemical and physical properties. The application of Bc for being used as a standard and preparing a control material was also demonstrated.

## VII. Objectives

1. To isolate the conjugated bilirubin (Bc) from fresh bovine, chicken and human gall-bladder bile and compare with those biosynthesized from bovine, chicken and human livers.
2. To identify the chemical characteristic and investigate the physical properties of conjugated bilirubin separated from the selected bile source.
3. To prepare conjugated bilirubin standard and quality control serum containing conjugated and unconjugated bilirubin.