

## CHAPTER IV

### DISCUSSION AND CONCLUSION

The effective and quantitative HPLC method for fractionation of bilirubin species in native bile (Spivak and Yuey, 1986) was modified and used in this study. In bile system, bilirubin species were resolved on a reversed-phase C<sub>18</sub> column, eluted in order of decreasing polarity by gradient mixtures of methanol-sodium acetate / aqueous ammonium acetate buffer. In this study, this mobile system was also used to fractionate bilirubin subfractions in conjugated hyperbilirubinemia serum, which showed good separation and agreed with that found in the human bile. This was differed from other serum fractionation system which used other mobile solvents, most frequently, acetonitrile containing (Itoh *et al.*, 2000 ; Bhutani *et al.*, 2000) one. The acetonitrile that has been used as solvent is known as a carcinogen (Doumas and Wu, 1991). Routine direct injection of 6 or 10  $\mu$ L of crude undiluted or diluted (1:1) bile sample was sufficient for analytical purposes. In this study, native bile in a volume of 100  $\mu$ L was injected because the eluate must be collected and the material in the eluate must be identified and used for further experiments. Bc from bile was gradually separated by this technique at the retention time approximately 6, 7 and 8 min respectively. Performances of peak areas and retention times were pretty good for repeating the same bile sample fractionation. For different bile samples particularly those from chicken, variation of peak areas and retention times were observed. These variations were caused by biological variations of bile pigments concentrations in bile specimens and variation of analytical performances. By using equal volume of bile injection in these studies, the higher peak areas were always observed mostly in chicken bile fractionations. However, it was difficult to collect the purely Bc eluate from separated peaks because chicken bile contained more species of separated Bc which were not resolved completely from each other than those obtained from bovine and human bile. Since the concentrations of Bc collected from chicken bile samples gave higher yields than that obtained from bovine bile, therefore chicken bile was chosen for using as raw material for Bc preparation. Although human gall bladder bile contained large amount of Bc as observed after HPLC separation but it must be achieved by autopsy. Bc in different eluates collected from different bile sources showed similar absorption

spectra with broad peaks of those frequently obtained from between 410 and 450 nm whereas Bu gave a sharp peak of absorption near 450 nm. These observations were agreed with that found by Wu (Wu, 1984) which demonstrated that Bc and Bu in serum were spectrally distinct with absorption maxima at ~ 420 and ~ 460 nm, respectively.

For HPLC separated technique with lack of Bc commercial standard used to determine the Bc retention time, eluate under bilirubin oxidase treated condition in 0.1 mol/L Glycine-NaOH buffer, pH 10.0 was necessitated. By the principle of the enzymatic method, the reaction of various species of bilirubin with the enzyme occur at pH 10.0. Bc oxidation rate was immediately performed at this pH (Perry *et al.*, 1986 ; Kurosaka *et al.*, 1998), when approximately 5 % of Bu is oxidized and there was no reactivity of the enzyme with B $\delta$  (Dourmas and Wu, 1991). Therefore decrease in peak observed at the separated position clarified Bc retention time of the bile pigment fractionations. However, types of Bc (mBc or dBc) conjugation were unable to identify by this method. In addition of retention time analysis, the experiments of azopigment formations were performed to show the possibility that putative Bc in eluate reacted with the diazo reagent in direct bilirubin reaction. Because the eluting buffer for Bc fractionation contained significant amount of methanol, the Malloy-Evelyn diazo method (Malloy and Evelyn, 1937) was preferably used to identify and determine the conjugated bilirubin concentrations in the fractionated bile eluate. Alternative method for Bc identification in eluate separated using this mobile system was described by Spivak and Carey (Spivak and Carey, 1985), by which the initial quantification of bilirubin diglucuronide (BDG) and bilirubin monoglucuronide (BMG) was performed automatically by a post column analysis using the ethyl anthranilate azo dye and AZO UCB as a standard.

Bc is unstable (Wu, 1984). It is easily oxidized by light and oxygen. To protect the degradation of Bc, all eluates obtained from analytical column reported in this experiment were promptly chilled in an ice bath, under nitrogen and in the dark. Although it gave satisfactory yield but for further application uses the preparative HPLC technique has been suggested (Spivak and Carey, 1985).

Bc obtained from biosynthesis from different types of liver homogenates were similar to those found in their corresponding gall-bladder biles. Bc is preferentially extracted into aqueous media. The absorption peak increased between 410-420 nm demonstrated newly synthesized Bc

from biosynthesis of liver homogenate. During extraction, the concentration of Bc from biosynthesis of liver was degraded by oxygen and light. In this process, saccharolactone, an inhibitor of  $\beta$ -glucuronidase was incorporated into the reaction medium which then help improving the rate, extent, and overall yield of the putative Bc formation (Wu *et al.*, 1980). In different types of liver homogenate, the rates of synthesized Bc observed in this experiment were different. The chicken liver homogenate showed more or less the same Bc concentrations (as shown by absorbance measurements) of both before and after incubation periods and the kinetic patterns of liver biosynthesis in mammalian livers. Human and bovine liver are higher and different from chicken liver. Therefore, it could be suggested that the rate of enzyme bilirubin-IX $\alpha$ -UDP-glucuronyltransferase in chicken liver might be relatively slow as compared with those from bovine and human sources. This was reported by Fevery *et al.*, (Fevery *et al.*, 1977) that activity of bilirubin-IX $\alpha$ -UDP-glycosyltransferase were varied in different species of mammals and in chicken. Wu *et al.* also reported the considerable differences in the nominal conjugation activity between different types of liver (Wu *et al.*, 1980) which resulted in different concentration of Bc accumulated in gall-bladder bile. Despite the presence of higher Bc concentrations in chicken bile, the *in vitro* biosynthesis was limited. This suggested that the assay conditions (pH, incubation time and conditions, activators) used may not have been optimal for chicken liver homogenate.

In chicken bile, glucoside and glucuronide conjugates were of equal importance because of bilirubin-IX $\alpha$ -UDP-glycosyltransferase activities in digitonin-activated liver homogenate and the ratio of transfer rates (glucuronyl / glucosyl /xylosyl) were 1:1:1 (Fevery *et al.*, 1977). In this study, type of sugar conjugated to bilirubin isolated from chicken bile at the retention time approximately 6.6 min was shown to be bilirubin glucuronide with molar ratio of bilirubin to glucuronic acid of 1:1.13. To confirm the type of this Bc conjugation, bilirubin structure should be further examined under mass spectrometry (MS) or nuclear magnetic resonance (NMR) study. As shown on chromatogram, there are other types of Bc conjugation in chicken bile separated at different retention times (identified by reacting with bilirubin oxidase at pH 10.0). However, they are out of the interest because they were expected to have lower concentration of conjugated bilirubin (lower absorbance).

Bilirubin and its conjugates are very labile: they readily undergo oxidation, dipyrrolic scrambling and photochemical breakdown (Lightner, 1982). When unconjugated bilirubin is exposed to light, whether *in vivo* or *in vitro*, it is rapidly converted from a nonpolar internally hydrogen-bonded structure to a variety of more polar isomeric forms and these polar isomers must be excreted (*in vivo* study) (Ennever *et al.*, 1987). Three types of photochemical reactions of bilirubin occur during photooxidation, conjugational isomerization, structural isomerization and photooxidation (lumirubin) (McDonagh *et al.*, 1982a ; 1982b). For conjugation of bilirubin photoproduct, Ennever *et al.* (Ennever *et al.*, 1987) reported that no lumirubin was found in bile samples obtained before exposing to light or 24 hours after ending of exposure. Identification of material eluted at 6.6 min as lumirubin based on chromatographic retention time and absorption spectrum of the material, both of which were identical to that of lumirubin prepared *in vitro* as described by McDonagh and co-workers (McDonagh *et al.*, 1982b). All bile samples contain 450 nm absorbing material with chromatographic retention times similar to that of unconjugated bilirubin (native 4Z, 15Z form) and mono- and di-glucuronide conjugated bilirubin (~ 6.9 and ~ 5.3 min, respectively). The major yellow pigment formed in all samples of bile obtained from infants undergoing phototherapy was lumirubin. *In vitro* photoirradiation of conjugated hyperbilirubinemia serum was investigated by Ihara *et al.* (Ihara *et al.*, 1992). Using HPLC separation technique, it was found that when the serum was photoirradiated *in vitro*, all the peak areas of the bilirubin subfractions, except for the bilirubin diglucuronide subfractions, decreased remarkably. No photobilirubin had been found but more polar bilirubin peaks were formed which were observed at the retention times between 4 and 6 min and it was assumed that the polar peaks were bilirubin photoisomers. In contrast to this study, forms of Bc products after exposing to light or oxygen were corresponded to that of biliverdin which the absorption spectra were shifted (overnight exposure) from 412 nm (yellow) to 380 nm (green). Change of maximum absorption spectra of Bc exposed to light overnight may be caused by some oxygen contaminated in Bc solution tubes (used the same Bc solution tube to follow rate of photooxidation). However the lower absorbance peak at 380 nm observed presumably occurred because of Bc degradation. Unfortunately the Bc photoproducts had not been assayed. The photodegradation rate of Bc found in this study and other investigation seemed to be slower than that of *in vitro* photoirradiation of Bu (Ihara *et al.*, 1992). The degradation rate of Bc in 0.1 mol/L Tris-HCl

buffer, pH 7.4 and 4 % BSA by light was faster than oxygen oxidation rate and the data suggested that Bc was more stabilized in protein (BSA) matrix than in Tris-HCl buffer.

Molar absorptivity ( $\epsilon$ ) of Bc (as Bu equivalent) prepared in 0.1 mol/L Tris-HCl buffer, pH 7.4 and 4 % BSA was approximately equaled. These data have never been reported in any literatures before. From these results, molar absorptivity of Bc was higher than that of Bu prepared in the same protein matrix. Molar absorptivity of conjugated bilirubin such as DTB varied, depending on buffers or protein matrix, which were used to prepare those bilirubin solutions. For example, the molar absorptivity of purified DTB (as Bu equivalent) in BSA dissolved in Tris or phosphate buffer were 56,700 and 43,200 (at 460 nm)  $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ , respectively (Doumas *et al.*, 1985).

The recovery of bilirubin added in control serum was used to determine the accuracy and efficiency of control serum preparation. In this study, % recovery obtained from Bu standard and Bc recovered were still varied. It has been suggested by Tonk (Tonk, 1970) that the percentage recoveries of the method should be greater than 85 %. The unaccepted % recovery values reported in this study may have caused by the instability of bilirubin in control serum.

Evaluation of control serum containing Bc isolates and enriched with commercial Bu standard which performed by direct and total bilirubin assays based on Jendrassik and Grof diazo reagent method were accepted by the OCV-RCV principle criteria of WHO (Whitehead, WHO doc. Lab 76/10). The OCV-RCV precisions of both direct and total bilirubin reactions were within 6 and 12 %, respectively. However, most % CVs obtained from commercial control serum (Randox, UK) were better than that obtained from control serum prepared in this laboratory, which probably depended on the numbers of observation (n) and the mean concentration values of bilirubin levels in control serum. For direct reaction, the concentration of Bc in commercial control was greater than that prepared in this study, which was contrasted for Bu concentration in the total reaction. In this study, one abnormal level of bilirubin control serum was performed because of limitation of Bc supply (eluates). For further studies, at least two levels of Bc in control serum should be prepared in order to control the precision of wider assay conditions, such as neonatal and adult hyperbilirubinemia in serum. These could be accomplished by using a preparative HPLC to isolate more Bc from the rich selected bile source.

**In conclusion,** Bc isolated from chicken gall-bladder bile by the reverse phase HPLC method showed similar properties to that obtained from human bile. The Bc in eluates demonstrated the absorption spectra at approximately 410 and 450 nm and reacted positively in the direct diazo reaction based on Malloy and Evelyn method. Type of bilirubin conjugation of Bc isolated from chicken bile was identified as bilirubin glucuronide. Degradation rate of Bc by light was faster than oxygen and Bc was more stable in protein (albumin) matrix than in 0.1 mol/L Tris-HCl buffer, pH 7.4. Molar absorptivities (as Bu equivalent) of Bc prepared in 0.1 mol/L Tris-HCl buffer, pH 7.4 and 4 % BSA were approximately  $49,735 \pm 3,126.27$  and  $51,155 \pm 469.11 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ , respectively. Bilirubin control serum prepared by adding Bc eluate to human serum was accepted by the criteria of WHO for using as internal quality control sample.