CHAPTER II

MATERIALS AND METHODS

Materials

I. Specimen

Chicken and bovine gall-bladders were obtained from the slaughterhouse and fresh market. They were stored at $-20^{\circ}C$ in the dark until used.

Human bile and liver were obtained from autopsy at the Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University with permission of the relatives of the deceased. The liver with normal appearance, without tissue necrosis and hepatitis was cut approximately 20 g from the autopsy and put into a plastic bag. Two milliliter of bile was collected from gall-bladder from the same autopsy. The tissues were kept frozen and stored at $-20^{\circ}C$ before analysis.

II. Instruments

HPLC apparatus equipped with autosampler (Varian 9100), solvent delivery system (Varian 9012Q), and UV-VIS detector (ProStar 9050) were used for fractionation of bilirubins in bile. Analytical column, Inertsil ODS-3 and Guard column, Inertsil ODS-3 were obtained from GL Sciences Inc., Japan. Absorbance measurements were performed by Shimadzu 160-A UV-Visible spectrophotometer (Shimadzu Corp., Kyoto, Japan). Freeze-drying of Bc and Bc products were performed by Lioalpha-10 Lyophilizer (Telstar, Spain) (Appendix A).

III. Chemicals and reagents

All solvents used were HPLC grade. Other chemicals and reagents were all analytical grade (See Appendix B). Composition of reagents prepared and used in this study were shown in Appendix C.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Methods

I. Separation of Bc in bile

The method for separation of Bc in bile was based on the procedure described by Spivak and Yuey (Spivak and Yuey, 1986). The protocol for separation of bilirubin fractions from bile was as followed.

Stationary phase was the ODS analytical column packed with C_{18} packing material, 250 mm in length and 4.6 mm inner diameter. Guard column was connected to the separative column in order to protect the column from bile protein, which might interfere with the separative capacity. The column was C_{18} ODS with an inner diameter of 4.0 mm and the length of 10 mm. The HPLC mobile phase were solvent A; 0.04 M sodium acetate in methanol and solvent B; 1 % ammonium acetate in water, pH 4.5. The amount of sample used in each injection was 10-100 µL. Before analytical separation, the HPLC column was equilibrated for 5 min with initial gradient mixture. Solvents A and B were used for the analytical bile-pigment separations, a linear gradient of 65 % A 35 % B to 95 % A in 7 min, 95 % A to 75 % A in 5 min and then maintaining the gradient at 75 % A for an additional 3 min was performed. The pressure of a pump was in range of 100-300 atm, while running, and the flow rate of mobile phase was 1.0 mL/min. The separative fractions were detected at 450 nm using UV-visible detector. A solfware intergrater system was adapted to a PC to calculate the area of the peak.

II. Identification of bilirubin fractions separated from bile using HPLC

a. Determination of the bilirubin fractions retention time

Unconjugated bilirubin (Bu) standard (20 mg/dL) prepared in 4 % BSA was subjected to HPLC separation and used as reference for Bu in bile fractionation.

Bilirubin glucuronide in bile separated by HPLC was identified after oxidation reaction with bilirubin oxidase. The unknown HPLC chromatogram peaks of the bilirubin glucuronide in bile were compared to the bilirubin glucuronide detected in the serum of obstructive jaundice patients. Bilirubin oxidase treated protocol was carried out by incubating 100 µL of serum or bile with 0.825 U bilirubin oxidase and 1.0 mL of 0.1 mol/L Glycine-NaOH buffer, pH 10.0. A hundred microliters of distilled water was used instead of enzyme in untreated control. Then the absorbances of the reaction mixture were measured for 15 min at 450 nm in a

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Shimadzu UV-160A spectrophotometer. After that, the reactions were stopped by keeping the reaction tubes in an ice bath. Bilirubin in the reaction mixture of treated sample was further analyzed by using the HPLC compared with its untreated control sample (Doumas *et al.*, 1999 modified).

For re-examining whether peaks obtained from bile fractionation by HPLC were bilirubin peaks or not, the bilirubin oxidase treated protocol for determining Bc retention time as described before was performed but using 0.1 mol/L Tris-SDS buffer, pH 8.0 (buffer used for determination of total bilirubin by bilirubin oxidase method) instead of 0.1 mol/L Glycine-NaOH buffer, pH 10.0. The reduction or disappearance of peaks of the fractionated bile sample treated with enzyme as compared with the untreated control revealed positions and retention times of total bilirubin species in bile separated by the HPLC method.

b. Post column identification

Determination of absorption spectra

Eluates containing different bilirubin species from HPLC individual peaks were collected and the absorption spectra were scanned at the wavelength between 350-650 nm in the Shimadzu UV-160A spectrophotometer.

Diazotization reaction of bilirubin

Identification of bilirubin pigments in the collected eluate of HPLC-peaks were performed by diazotization reaction (Malloy and Evelyn, 1937).

For direct bilirubin determination, 1.0 mL of eluate was added into 0.25 mL diazo reagent which has previously been placed in a direct reaction tube. In the blank tube, equal volume of diazo blank was added instead. After mixing, the absorbance of 1 min reaction was measured at 540 nm against the blank tube in Shimadzu UV-160A spectrophotometer.

Total bilirubin reaction was carried out by adding 1.25 mL of methanol in the above direct reaction and blank tubes. After the tubes were thoroughly mixed and stood for 30 min, the absorbances at 540 nm were measured. Concentrations of Bu and Bc in each bile specimen were calculated using total Bu standard graph prepared by the same method as the unknown sample.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

III. Biosynthesis of Bc from different types of liver

The conjugated bilirubin were prepared by biosynthesis using homogenates of chicken, bovine and human (from autopsy) liver. The *in vitro* synthesis of Bc was performed with liver homogenates in the presence of D-glucaro-1,4-lactone, a glucuronidase inhibitor (Wu *et al.*, 1980).

In preparation of liver homogenate, the liver was briefly rinsed with distilled water, blotted on paper towels, then sliced into smaller portions with a sharp knife. Before homogenization, the liver lobes contained much fibrous connective tissue, were gently excised. Each portion of liver (50-200 mg) was homogenized in about five volumes of ice-chilled 0.05 mol/L phosphate buffer, pH 7.4 which had been bubbled with nitrogen for about 30 min beforehand. The homogenization was carried out by a Waring blender with 20 sec bursts interspersed with 30 sec pauses during which the partial homogenate was cooled in an ice bath. Homogenized process was stopped when an even suspension was obtained, which was then quickly filtered through two layers of cheesecloth and was divided into 50-mL aliquots. It was kept at 0-4 °C for less than an hour before used.

Before *in vitro* incubations, equal aliquots of the freshly prepared liver homogenate were separated into two tubes. One tube was used as zero hr incubation. Another tube was mixed thoroughly but gently with stock solution of Bu and uridine diphosphateglucuronide (UDPGlcUA) to give final concentrations of 60 mg/L bilirubin and 1.5×10^{-3} mol/L UDPGlcUA. The pH range of the incubation mixture was 7.0-7.8. The pH of reaction containing saccharolactone (D-glucaro-1,4-lactone) was kept nearer 7.0 (because the compound is less stable in the mixture at higher pH). For incubations, 10-15 g/L Triton X-100 surfactant and 10^{-4} mol/L $MgCl_2$ were also added into the medium. The entire reaction mixture was kept in a nitrogen-rich atmosphere, shielded from strong light at 37 °C (with constant, gentle swirling during the 3-4 hrs incubation).

Separation of *in vitro* Bc from the incubation mixture was done by using a simple semi quantitative protocol. At two hours after incubation, one volume of the reaction mixture was diluted with three volumes of ice-chilled 0.1 mol/L phosphate buffer, pH 6.8 and immediately mixed, under nitrogen, with an equal volume of ice-chilled chloroform. The mixture was briefly shaken and centrifuged at 3,500 rpm for 10 min at 4 °C. The resulting chloroform and aqueous

Copyright© by Chiang Mai University
All rights reserved

phases were separately scanned and measured for their absorbance at 450 and 425 nm, respectively, each referenced against its appropriate solvent blank. A major assumption in this assay was that Bu is preferentially extracted into chloroform while Bc is preferentially extracted into aqueous media. Absorption spectrum of Bc biosynthesis using different types of liver was compared with the spectrum of the respective Bc fractionated from its native bile.

IV. Preparation of lyophilized Bc product

One milliliter of the Bc eluate (0.6 AU) was mixed with 2.0 mL of 0.1 mol/L Tris-HCL buffer, pH 7.4 or 1.0 mL of 0.1 mol/L Tris-HCl buffer, pH 7.4 which previously mixed with 1.0 mL of 4 % BSA (4 % in 0.1 mol/L Tris-HCl buffer, pH 7.4) before filling in lyophilized vials. The lyophilization was performed by the Lioalpha-10 lyophilizer using 12-hour cycle protocol. Lyophilization parameters were set as follow: -

Freezing process	-45 °C for 3.5 hours	
Primary drying	Max- pressure	7.5×10^{-1} mbar
	Min- pressure	1.9×10^{-2} mbar
	Shelf-temperature	-45 °C
	Time	10 hours
Secondary drying	Shelf-temperature	25 °C
	Time	2.5 hours

Lyophilized Bc products were used for further investigations.

V. Determination of chemical properties of conjugated bilirubin

a. Absorption spectra

The absorption spectra of the lyophilized Bc products fractionated by the HPLC and those obtained from the biosynthesis were scanned from 350 to 650 nm in Shimadzu UV-Vis spectrophotometer against their respective blanks. The maximum peaks of Bc spectra were compared with Bu standard prepared in 4 % BSA (Wu *et al.*, 1980).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

b. Diazotization

Total bilirubin was determined in the total reaction by using caffeine sodium benzoate as accelerators. In the test and blank tubes, 0.2 mL of the eluate or sample was added with 2.0 mL caffeine solution.

In the direct reaction, Bc was determined by adding 0.2 mL of eluate or sample in 2.0 mL of 0.05 N HCl. In the test reaction tubes of both total and direct reaction, 0.5 mL of diazo reagent was added and incubate at room temperature exactly for 10 and 1 min, respectively. The diazotization reaction was stopped by adding 100 µL of ascorbic acid. In each blank reaction tube, equal volume of sulfanilic acid was added instead of diazo reagent and followed with 100 µL of ascorbic acid. After that, 1.0 mL of alkaline tartrate were added into both test and blank tubes and mixed. After standing for 10 min, the absorbances at 600 nm were measured using Shimadzu UV-Vis spectrophotometer.

The concentration of bilirubin was calculated using Bu standard curve of total reaction prepared by the same procedure as the unknown eluate.

VI. Determination of physical properties of conjugated bilirubin

a. Effects of photooxidation on Bc product

The experiment was performed by exposing the HPLC Bc product to irradiation (Ihara *et al.*, 1992 modified). The lyophilized Bc products prepared in 4 % BSA or 0.1 mol/L Tris-HCl buffer, pH 7.4 were reconstituted with 1.0 mL of distilled water. The absorbances of Bc prepared in 4 % BSA and 0.1 mol/L Tris-HCl buffer, pH 7.4 were measured prior to exposing to irradiation (control-without irradiation). After that, the tubes containing reconstituted Bc solution were deoxygenated with pure (99.9 %) N_2, then irradiated for 2.5 hours (or overnight for the different set) with a 100-W projection lamp which had a radiation spectrum between 340 and 700 nm at 25 °C. Rate of Bc oxidation by irradiation was measured by interval scanning of the absorption spectra to observe the change in absorbance values and peak of absorption spectra.

Copyright© by Chiang Mai University
All rights reserved

b. Effects of exposing Bc product to oxygen (air) oxidation

The lyophilized Bc products prepared in 4 % BSA or 0.1 mol/L Tris-HCl buffer, pH 7.4 were reconstituted with 1.0 mL of distilled water. The absorbances of Bc prepared in 4 % BSA and 0.1 mol/L Tris-HCl buffer, pH 7.4 were measured prior to exposing to oxygen (in atmospheric air). Then the tubes containing Bc solution were left opening for 2.5 hours (or overnight for the different set). Rate of Bc oxidation by oxygen was measured the same way as described in photooxidation protocol.

VII. Determination of type of sugar conjugated to bilirubin

Bilirubin glucuronide

The lyophilized conjugated bilirubin was identified for bilirubin glucuronide by the method of Wu *et al.* (Wu *et al.*, 1980). The lyophilized conjugated bilirubin product was reconstituted with 1.0 mL of 0.1 mol/L potassium phosphate buffer, pH 6.8. The absorbance of the Bc solution was scanned at wavelength between 350-650 nm which then the maximum absorption was also recorded. After that, equal volume of 10 g/L β-glucuronidase was added and mixed. The reaction was incubated at 37°C for 2 hours under nitrogen and protected from light. At the end of the incubated time, 1.0 mL of ice-chilled chloroform was added and mixed. The reaction mixture was then centrifuged at 3,500 rpm for 10-15 mins at 0-4 °C. The resulting chloroform and aqueous phases was separated to contain unconjugated bilirubin and glucuronic acid, respectively. The glucuronic acid concentration in 0.1 mol/L potassium phosphate buffer, pH 6.8 was determined by the colorimetric method described by Blumenkrantz and Asboe-Hansen (Blumenkrantz and Asboe-Hansen, 1973). Briefly, 0.2 mL of sample or standard was added with 1.2 mL of sulfuric acid / tetraborate reagent. The tubes were refrigerated in crushed ice. The mixture was shaken in Vortex mixture and the tubes heated in a water bath at 100°C for 5 min. After cooling in a water-ice bath, 20 µL of the m-hydroxydiphenyl reagent was added (for blank tube, 20 µL of 0.5 % NaOH was added instead). The tubes were shaken and the absorbance at 520 nm were measured within 5 min in a Shimadzu UV-160 A spectrophotometer. The concentrations of glucuronic acid were compared with glucuronic acid standard prepared in 0.1 mol/L potassium phosphate buffer, pH 6.8.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Bilirubin ditaurate (ditaurobilirubin, DTB)

Conjugation of bilirubin with taurine was examined by using alkaline hydrolysis method to separate bilirubin from taurine (Doumas *et al.*, 1985). By this method, 0.5 mL conjugated bilirubin eluate was mixed with 5.0 mL of 3.0 mol/L NaOH, then refluxed for 6 hours. At the end of alkaline hydrolysis process, pH of the mixture was acidified by adding glacial acetic acid. After that, taurine was extracted three times using equal volume of methylene chloride. The taurine in aqueous solution was lyophilized by using Lioalpha-10 lyophilizer. After reconstitution, the taurine residue was quantified by ninhydrin reaction (Fowler, 2002).

VIII. Application for using conjugated bilirubin isolated from bile

a. Molar absorptivity of conjugated bilirubin prepared for use as a standard

Lyophilized conjugated bilirubin prepared in 4 % BSA or 0.1 mol/L Tris buffer, pH 7.4 were reconstituted in 1.0 mL of distilled water. After mixing, the absorbance of each solution was scanned at the wavelength between 350-650 nm by using a Shimadzu UV 160-A spectrophotometer. The absorbance at the peak was used to calculate the molar absorptivity (ε, $L.mol^{-1}.cm^{-1}$) of the conjugated bilirubin prepared in 4 % BSA or 0.1 mol/L Tris-HCl, pH 7.4 solution. The molar absorptivity of Bc solution was calculated as Bu equivalence (Doumas *et al.*, 1987) using a formula $A = \varepsilon bc$, where A = absorbance (AU), b = path length (cm) and c = concentration (mol/L), respectively (Wu , 2000).

b. Application of conjugated bilirubin for preparation of control serum

The method was performed by adding known amount of conjugated bilirubin eluate and commercial unconjugated bilirubin to a pooled human serum which was previously screened for HIV, HbAg as recommended by WHO (For details see Appendix D) (Browning *et al.*, WHO doc. Lab/86 4). The concentrations of unconjugated and conjugated bilirubin in human serum based matrix were adjusted to 15.0 and 0.8 mg/dL respectively. After 4.0 mL of liquid bilirubin control serum was dispensed in lyophilized vials, the control serum was lyophilized by the Lioalpha-10 lyophilizer using the parameter described previously in the method of lyophilization of Bc solution. These conjugated bilirubin together with the commercial unconjugated bilirubin in control serum were evaluated for monitoring the precision of bilirubin analysis in serum in routine laboratory.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved