CHAPTER III

RESULTS

I. Separation of Bc in bile

Fractions of different bile pigments were rapidly separated by reverse-phase HPLC in 12 min.

Figure 3 shows the retention time of 20 mg % Bu standard. The pigment identification of Bu was based on the retention time of Bu demonstrated at 11.132 min.

Figure 4 illustrates three HPLC chromatogram profiles of bile pigments fractionated from bovine, chicken and human bile. There is only one major peak of bile pigment at retention time of 8.292 min in bovine bile. There are several peaks detected in chicken bile. However, the peak at the retention time of 7.102 min was the major peak. In human bile, two major peaks at the retention times of 6.108 and 8.157 min were obtained. The minor peak at the retention time of 10.039 min was also observed in human bile. The retention times of bile pigments obtained from different bile sources were summarized in Table 1. The with-in run CV of repeated fractionation of the same bovine bile sample peak areas was 8.61% and within-run CV of peak areas of different specimen separation was 16.77 %. Although chicken bile showed more separated peaks than bovine and human bile, the precision of repeated fractionation was better (% CV = 2.92). However, different specimen fractionations gave varied peak areas of highest peak separations (% CV = 10.18). The within-run precision of peak area fractionations of human native bile demonstrated the retention times ranged from 8.095-8.184 yielding % CV of 13.68. The between-run precision of the same bile fraction could not be performed because of instability of bile pigments in the related bile specimens.

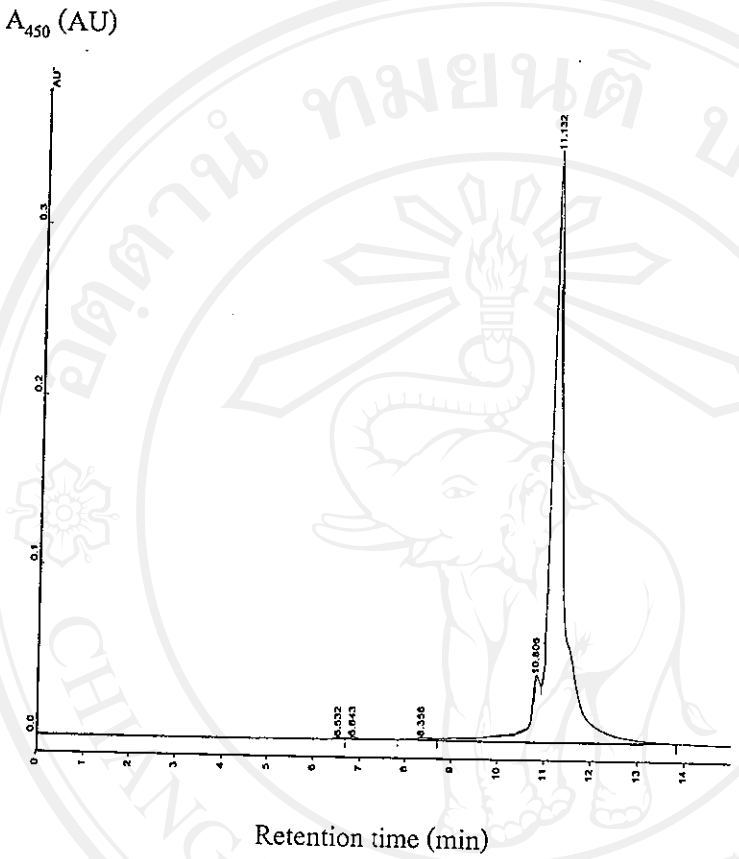

Figure 3. Retention time of 20 mg/dL Bu standard in 4 % BSA.

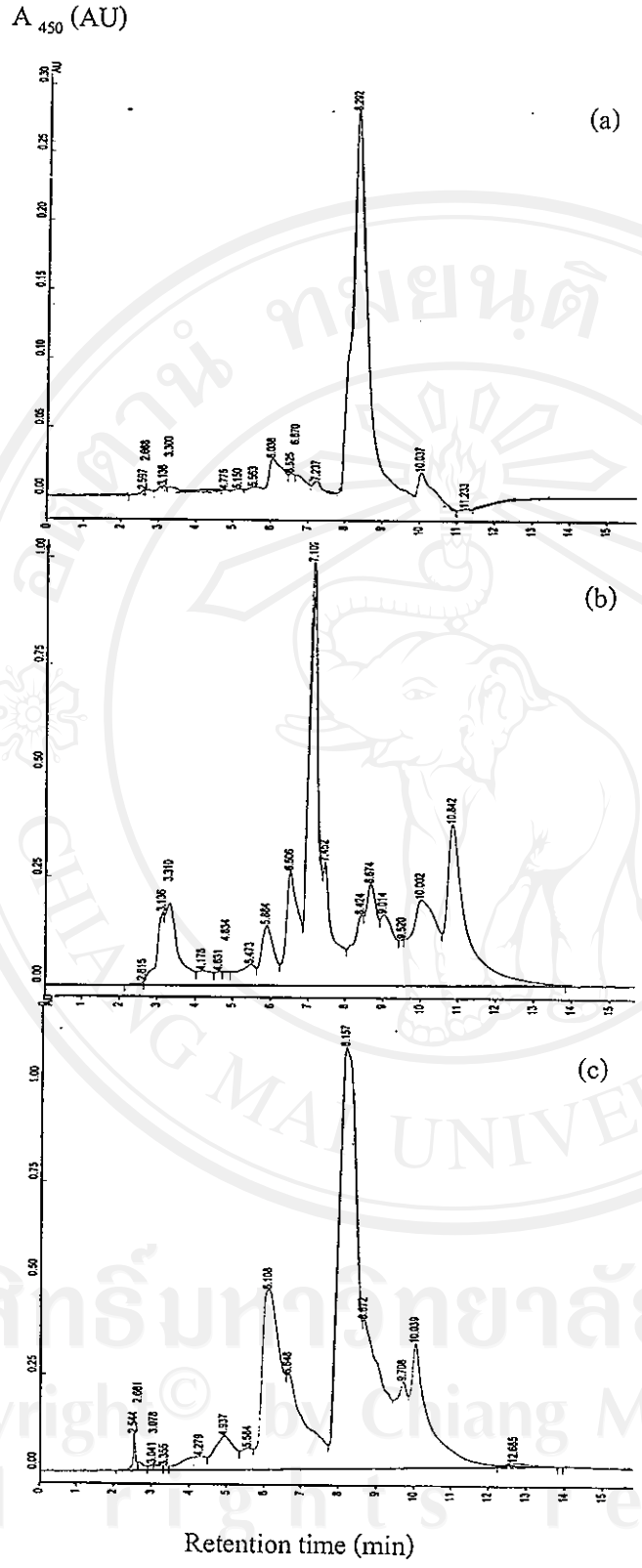

Figure 4. HPLC separation profiles of bilirubin species in different types of bile: (a) bovine bile; (b) chicken bile; (c) human bile.

Table 1. Precisions of peak areas and retention times of bile pigments separated from different bile sources.

Source	Retention time (min) (No.)			Absorbance (AU) (No.)			Peak areas (counts) (No.)			Statistical values of peak areas		
	1	2	3	1	2	3	1	2	3	$\overline{X}$	SD	%CV
Bovine (repeated specimen)	8.675	8.613	8.236	0.17	0.17	0.15	3480921	3681449	3101267	3421212	294663	8.61
(different specimens)	8.675	8.233	8.292	0.17	0.10	0.30	3480921	4601219	4829409	4303850	721752	16.77
Chicken (repeated specimen)	6.779	6.752	6.674	0.50	0.43	0.50	6029570	5705941	5774186	5836566	170594	2.92
(different specimens)	6.605	6.674	6.662	0.50	0.50	0.46	4770135	5774186	5660029	5401450	549706	10.18
Human (repeated specimen)	8.157	8.184	8.095	1.10	1.00	0.98	18906750	24251370	24200168	22452763	3071044	13.68
(different specimens)	-	-	-	-	-	-	-	-	-	-	-	-

II. Identification of bilirubin fractions separated from bile using HPLC

a. Determination of the bilirubin fractions retention time

The present method for separating bilirubin fractions from native bile was adapted from the HPLC method described by Spivak and Yuey (Spivak and Yuey, 1986).

Bu retention time of the fractionated bile was identified by comparing with the retention time of Bu standard (11.132) in Fig. 3. As seen in Fig. 4a-c, no Bu fractions were detected in bovine and human bile separations. It seems that chicken bile contained Bu which fractionated at the retention time of 10.84.

Estimation of Bc retention times which performed by using bilirubin oxidase treated Bc fractions were shown in Fig. 5-7. The retention time analysis under treated and untreated conditions (Doumas and Wu, 1991) with bilirubin oxidase was used to identify the retention time of Bc separated fractions. The retention times of Bc and Bu fractionated from bile were confirmed by the retention times of patient serum and Bu standard separated by the same fractionated conditions.

The retention times of Bc from chicken bile in untreated reaction (sample without bilirubin oxidase treatment) appeared at 2.790, 6.784, 7.936 and 8.610 min (6.0, 6.5, 1.0 and 1.5 mAU), respectively. For treated samples (sample with bilirubin oxidase treatment), all separated peaks at the retention times of 6.784, 7.936 and 8.610 min were absent (Fig. 5). Figure 6 revealed the peaks represented the separation of bilirubin species in serum of patient with obstructive jaundice. Two peaks having the retention times approximately at 6 and 8 min were markedly decreased after treating with bilirubin oxidase. Peak of the same serum sample separated at the retention time of 11 min was slightly influenced by the enzyme and was identified as Bu (Fig. 7). Therefore, it could be concluded that the retention times of decreased (or absent) peaks resulting from specific oxidation of bilirubin by bilirubin oxidase in Glycine-NaOH buffer at pH 10.0 was corresponding to Bc fraction while Bu fraction was unaffected (the retention time approximately 11 min).

Figure 8-10 shows the effect of bilirubin oxidase on the oxidation of total bilirubin species in chicken bile, human patient serum and Bu standard. All samples were treated with bilirubin oxidase in 0.1 mol/L Tris-SDS buffer, pH 8.0 following by HPLC separation. It was shown from the results that all peaks of the enzyme treated samples were absent (or

decreased). Chicken bile (Fig. 8), patient serum (Fig. 9) and Bu standard before and after treatment with bilirubin oxidase showed the same pattern of peak separations (Fig. 10). The peak at the retention time of 2.7 for chicken bile (Fig. 8) was insensitive to the enzyme treatment. Bu standard dissolved in 4 % BSA, eluted at retention time of 11 min, was slightly decreased in treated as compared with the untreated standard control (Fig. 10).

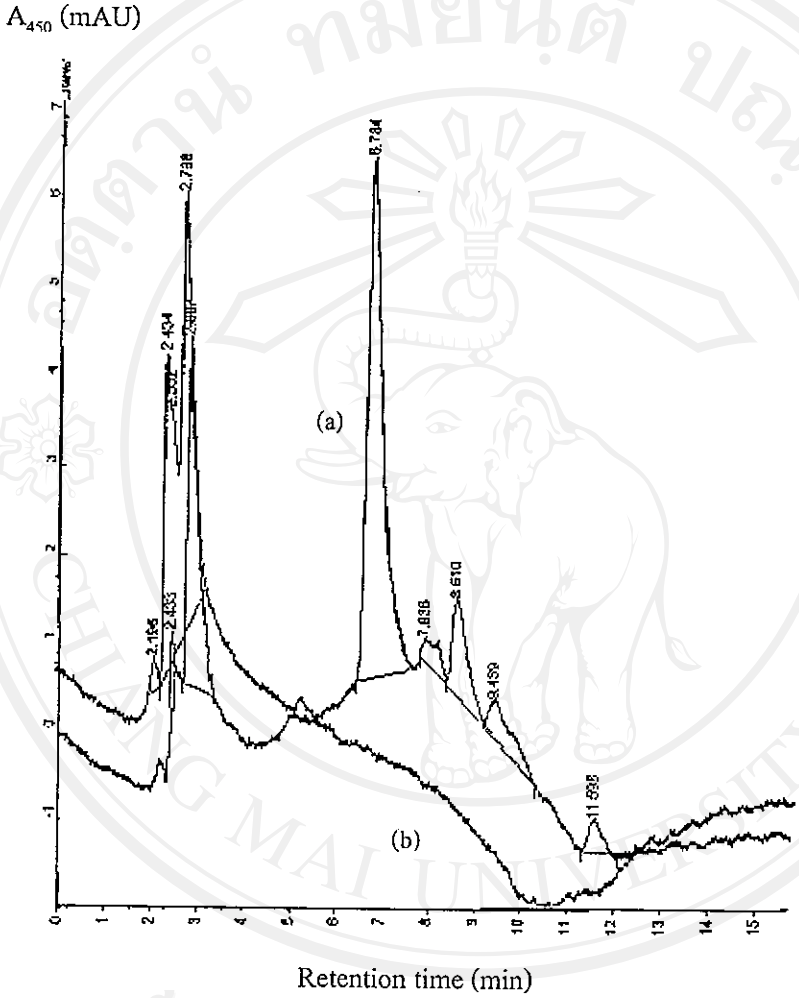

Figure 5. Comparison of Bc retention time of fractionated chicken bile untreated (a) and treated (b) with bilirubin oxidase in 0.1 mol/L Glycine-NaOH buffer, pH 10.0.

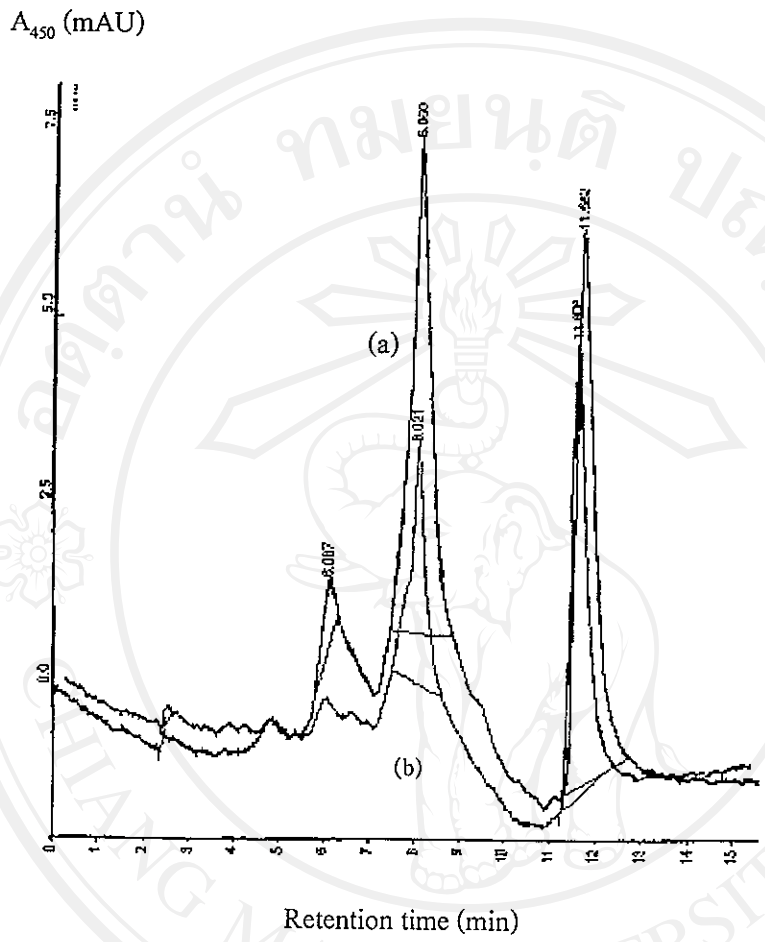

Figure 6. Comparison of retention time of fractionated human obstructive jaundice patient serum untreated (a) and treated (b) with bilirubin oxidase in 0.1mol/L Glycine-NaOH buffer, pH 10.0.

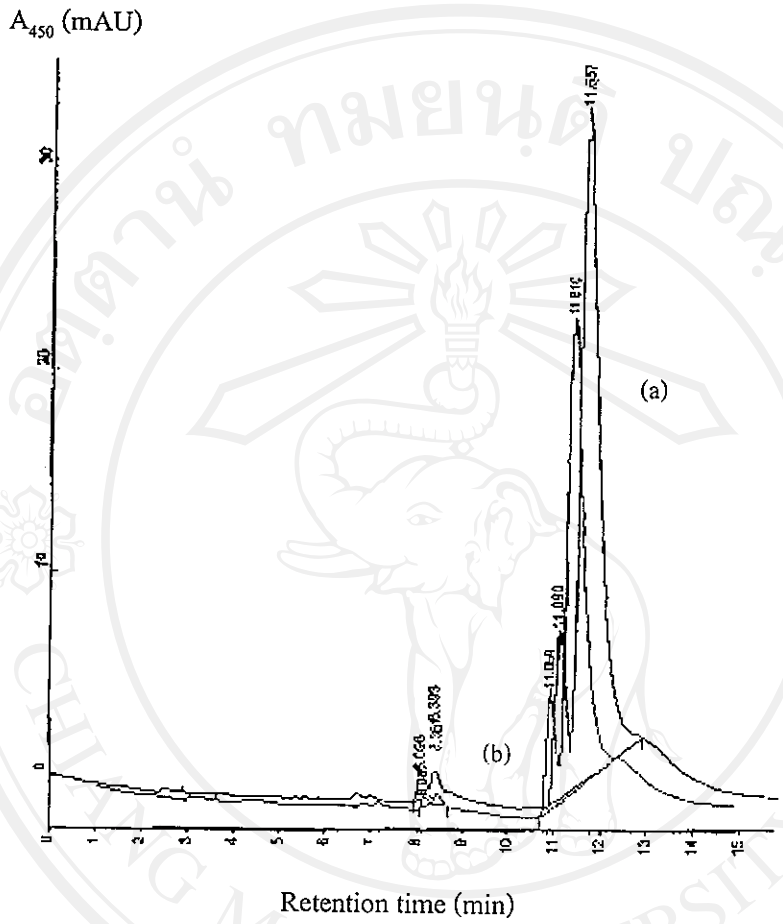

Figure 7. Comparison of Bu retention time of Bu standard untreated (a) and treated (b) with bilirubin oxidase in 0.1 mol/L Glycine-NaOH buffer, pH 10.0.

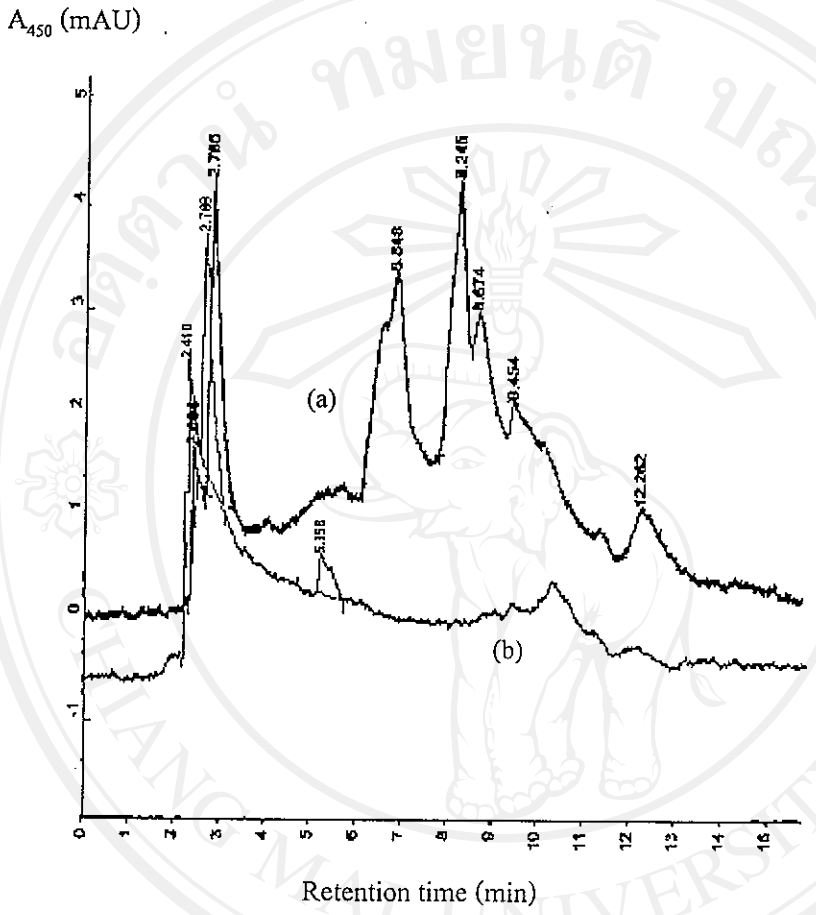

Figure 8. Comparison of Bc and Bu retention times of fractionated chicken bile untreated (a) and treated (b) with bilirubin oxidase in 0.1 mol/L Tris-SDS buffer, pH 8.0.

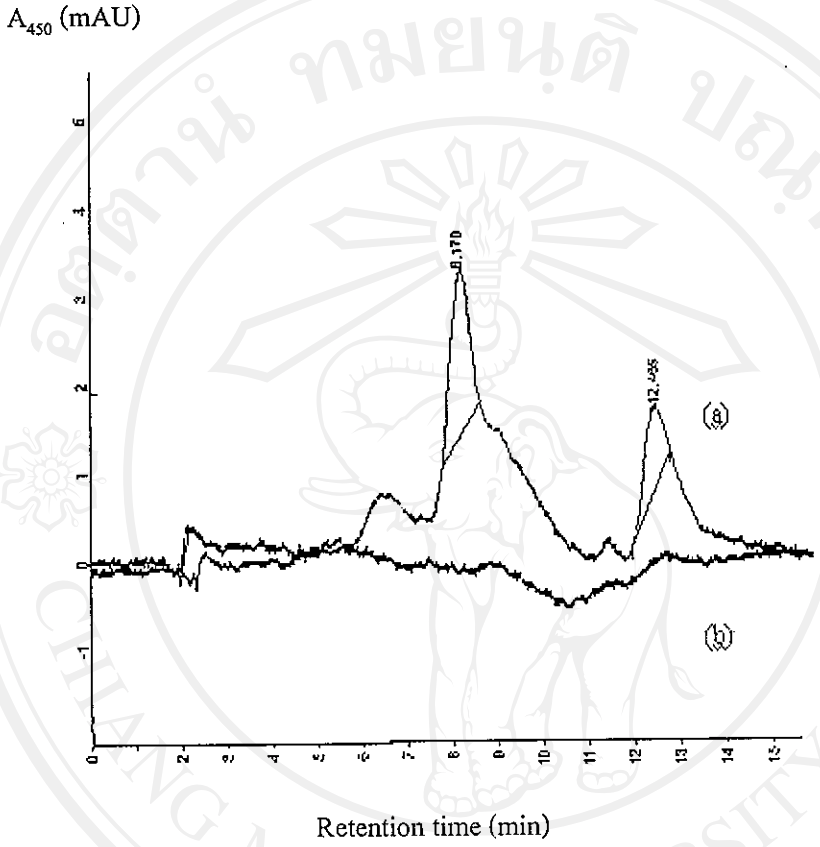

Figure 9. Comparison of Bc and Bu retention times of fractionated human patient serum untreated (a) and treated (b) with bilirubin oxidase in 0.1mol/L Tris-SDS buffer, pH 8.0.

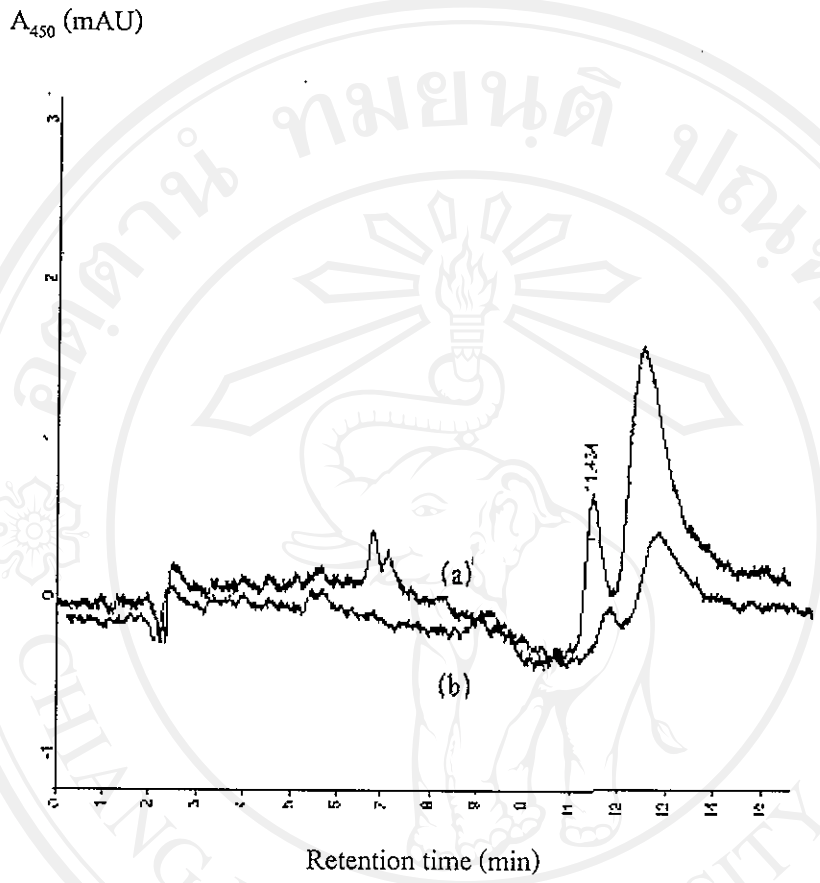

Figure 10. Comparison of retention time of Bu standard dissolved in 4 % BSA untreated (a) and treated (b) with bilirubin oxidase in 0.1 mol/L Tris-SDS buffer, pH 8.0.

b. Post column identification

Determination of absorption spectra

In this study, collected Bc eluates from HPLC column were scanned to investigate the absorption spectra between 350-650 nm. All absorption spectra of Bc eluate from bovine, chicken and human bile demonstrated biphasic characteristic between 410 and 450 nm. The absorption spectrum of Bc in bovine bile eluate (Fig. 11-a) gave the absorbance peaks at 401 (0.33 AU) and 450 nm (0.30 AU), respectively. The same characteristics of peaks as Bc in bovine bile were observed in bile eluates of chicken and human. The maximum absorbance of peaks of chicken bile eluate were 0.50 AU (406 nm) and 0.48 AU (448 nm) (Fig. 11-b). Human bile eluate which was used as a reference bilirubin fractionation showed the high absorbance peaks at 413 nm (0.69 AU) and 448 nm (0.74 AU) (Fig. 11-c).

Diazotization reaction of bilirubin

The diazo reagent reaction based on the method of Malloy and Evelyn (Malloy and Evelyn, 1937) was used to investigate azopigment (pink or red pigment) formation of the pigment in bile eluate reacted with diazo reagent.

Figure 12-14 showed the maximum absorption peaks of azopigment form direct and total reactions scanned between 350-650 nm. Bovine bile eluate gave the lowest absorption peaks of direct and total bilirubin as shown in Fig. 12. The absorbances at 540 nm of azopigment of direct and total bilirubin in chicken bile eluate were shown to be 0.507 and 0.271 AU, respectively (Fig. 13). Human bile eluate which was used as a reference bile sample also elucidated the azopigment absorption peaks of direct and total bilirubin at 540 nm (0.328 AU and 0.187 AU, respectively)(Fig. 14). Since the absorption spectrum of azobilirubin forming by the reactivity of Bu standard with diazo reagent demonstrated the maximum absorbance peak at 540 nm (Fig. 15), therefore the color products obtained from the reactivity of pigment in bile eluates with diazo reagent were resembled the azopigment products of bilirubin (Fig. 12-14).

Table 2 shows the results of direct and total diazo reactions of three types of bile eluates. The positive tests were found in bile fractions collected from the highest peak separated from each source. The concentrations of direct and total bilirubin were also calculated. The concentrations of direct and total bilirubin of chicken bile at the retention time of 6.912 min

was the highest concentration obtained and the ratio of direct to total bilirubin (DBIL / TBIL) is approaching 1.

Concentrations of direct and total bilirubin calculated per 100 mL of bile were shown in Table 3. The concentrations of both bilirubin species in bile eluate of chicken were found to be the highest as compared with those obtained from the other two eluates (Appendix E).

It could be concluded from results in Table 2 and 3 that the appropriate sample for preparing Bc for using as standard and adding to a control serum was chicken bile. Although human bile also gave high yield concentration of Bc separation but by the ethic reason it was not allowed to be used.

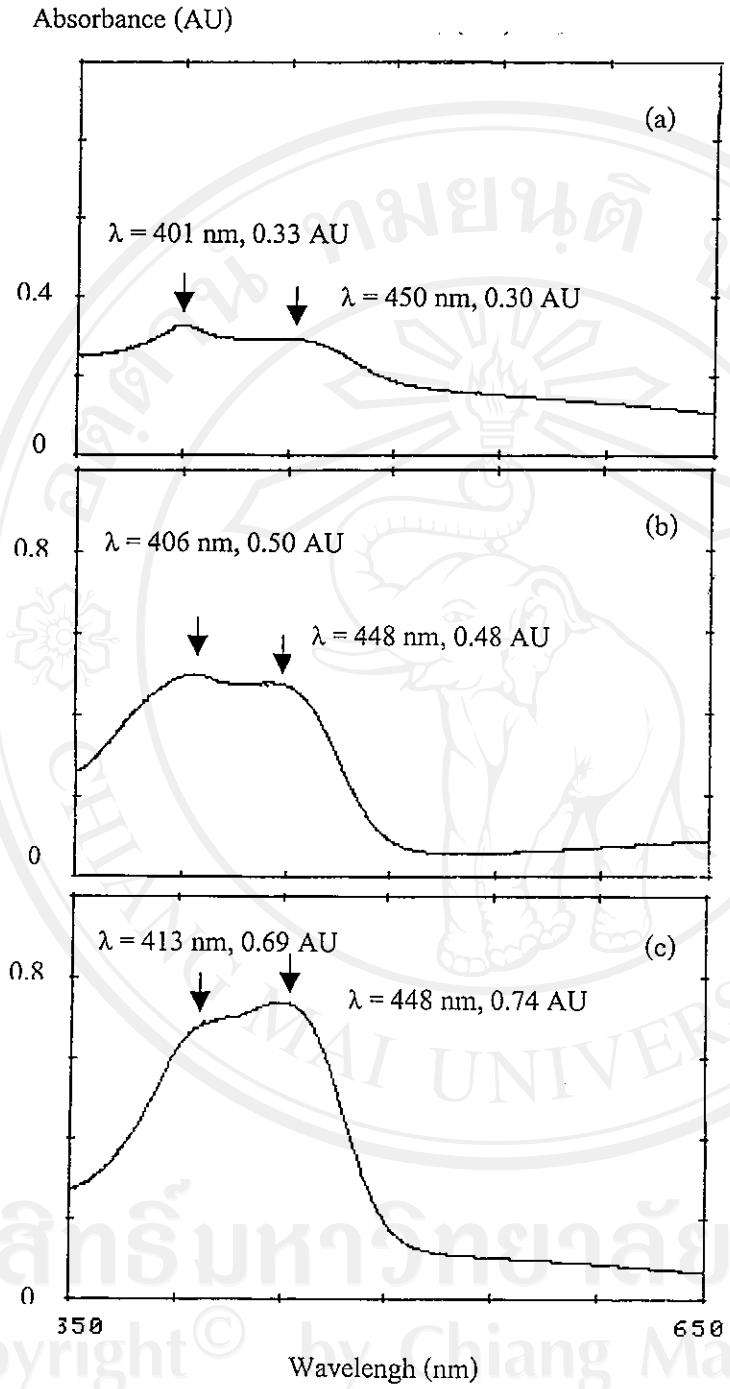

Figure 11. The absorption spectra of Bc eluate from HPLC separation :

(a) bovine bile eluate; (b) chicken bile eluate; (c) human bile eluate.

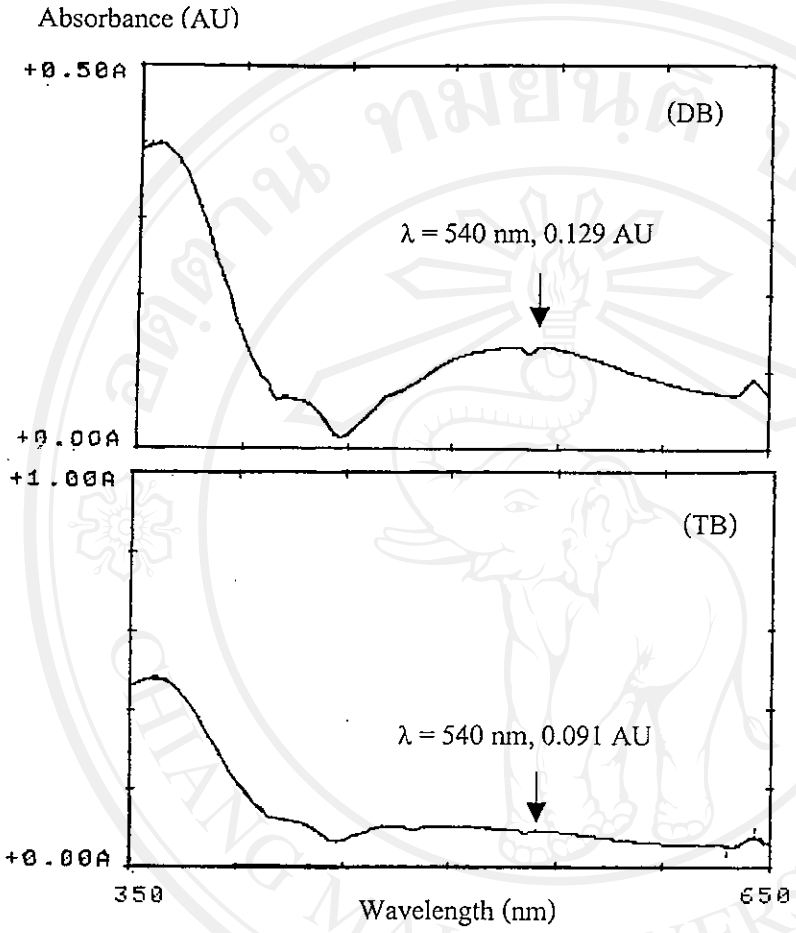

Figure 12. The absorption spectra of azobilirubin formed by the reaction of bile pigment in bovine eluate with diazo reagent (M-E method).

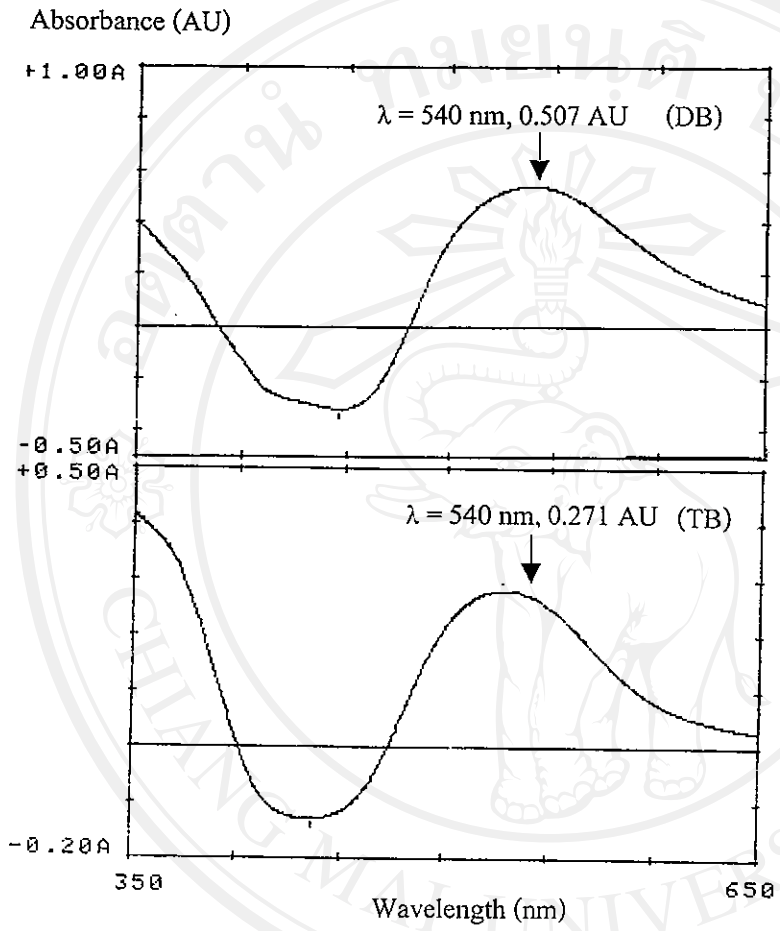

Figure 13. The absorption spectra of azobilirubin formed by the reaction of bile pigment in chicken eluate with diazo reagent (M-E method).

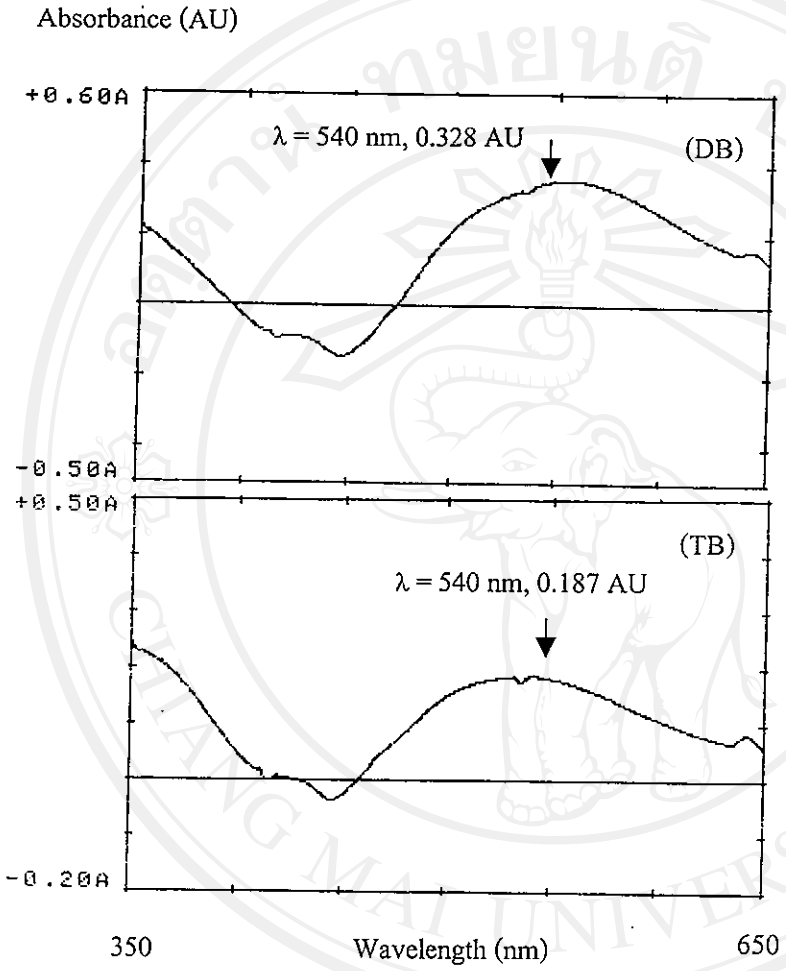

Figure 14. The absorption spectra of azobilirubin formed by the reaction of bile pigment in human eluate with diazo reagent (M-E method).

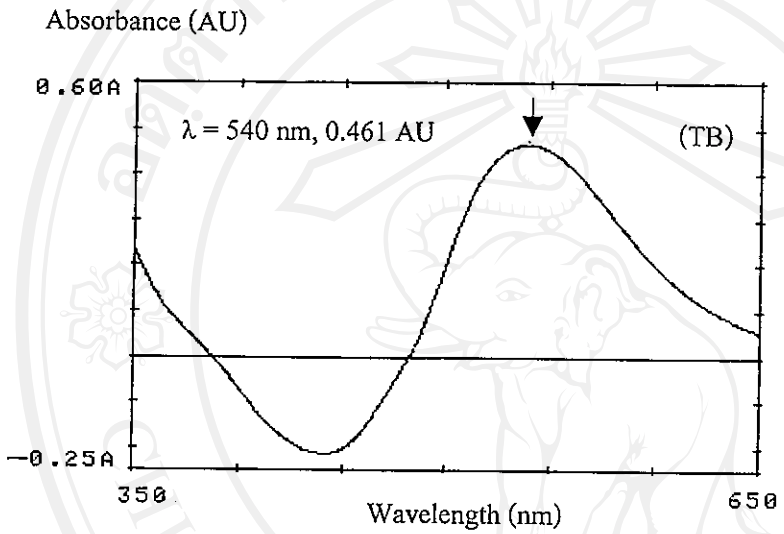

Figure 15. The absorption spectra of azobilirubin formed by the reaction of Bu standard with diazo reagent (M-E method).

Table 2. Concentrations of bilirubin calculated from the absorbance of azopigment formation according to the Malloy-Evelyn diazo reagent reaction (Malloy-Evelyn, 1937).

Source	Peak of retention time	Direct bilirubin		Total bilirubin	
		Azopigment formation	Concentration (mg/dL)	Azopigment formation	Concentration (mg/dL)
Bovine bile	8.292	(+)	2.19	(+)	3.09
	10.037	(+)	2.02	(+)	4.28
Chicken bile	6.359	(+)	1.51	(+)	1.84
	6.912	(+)	7.64	(+)	8.16
	7.326	(+)	3.45	(+)	3.98
	8.993	(+)	3.00	(+)	3.61
Human bile	4.937	(+)	2.58	(+)	4.01
	6.108	(+)	3.85	(+)	5.44
	8.157	(+)	5.57	(+)	6.35
	10.039	(+)	3.00	(+)	5.13

Table 3. The concentrations of direct and total bilirubin in the most prominent peak of bovine, chicken and human bile eluate.

Bilirubin species	Bilirubin concentrations (mg/dL)		
	Bovine bile	Chicken bile	Human bile
Direct bilirubin	21.9	76.4	55.7
Total bilirubin	30.9	81.6	63.5
DBIL / TBIL	0.709	0.936	0.877

III. Biosynthesis of Bc from different types of liver

Rate of biosynthesis of conjugated bilirubin in liver homogenate was depended on the UDP-glucuronyl transferase activity which catalyzed the formation of bilirubin glucuronide in its respective bile. After biosynthesis process, the resulting bilirubin extracted in chloroform and aqueous phases were separately scanned and measured for their absorbances at 450 and 425 nm, respectively. The rate of appearance of the water-soluble species or newly synthesized Bc was shown in Fig.16 (180 min incubation). With the same conditions of incubation, the lowest formation of new Bc was found in chicken liver homogenate. However it seems that the 180 min incubation reaction for Bc formation was not the optimal reaction time for all those kinetic rates because Bc formations were not reached their saturation curves. This result was confirmed by the absorbance scanning shown in Fig. 17. The absorption peak approximately at 410-420 nm of the extract Bc in aqueous phase, the absorbance of Bc extract from chicken liver homogenate was slightly changed after incubating for 150 min as compared with before incubation (Fig. 17-c). In contrast, the absorbance of Bc aqueous extracts from bovine and human liver homogenates were approximately 1 : 1.5-2, respectively, as compared with the reactions before incubation.

Figure 18 shows the scanning of chloroform extracts obtained from three sources of liver homogenates. The peaks of Bu spectra were at 450 nm. There was small amount of Bu detected in chloroform extract at zero time before incubation. At 150 min after incubation reaction, Bu presented in the chloroform extracts was derived from the substrate remaining after the biosynthesis reactions.

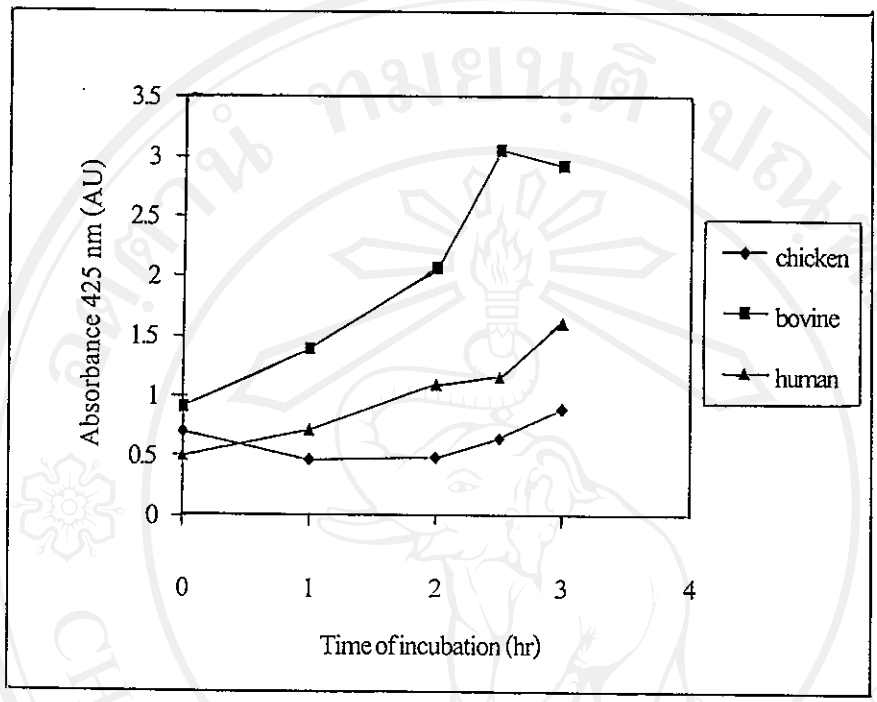

Figure 16. The kinetic of liver biosynthesis from different types of liver homogenate. Liver homogenates were sampled at various times during 180 min incubation.

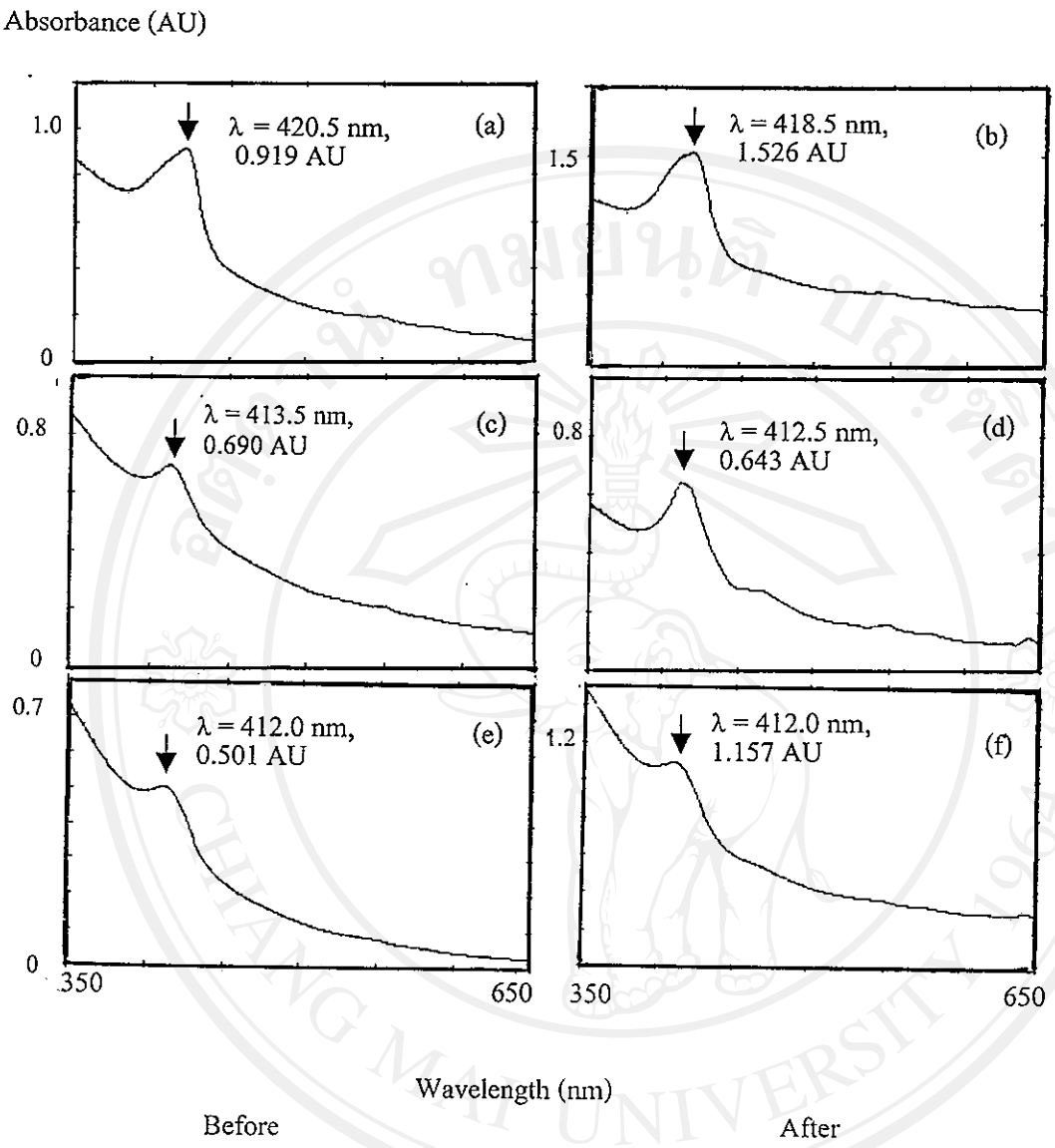

Figure 17. The comparison of Bc absorption spectra from different types of liver biosynthesis : (a, b) bovine liver ;(c, d) chicken liver ; (e, f) human liver. All figures shown on the left-side were Bc in liver homogenate before the biosynthesis reaction and all figures on the right-side were absorption spectra of Bc a fter 150 min biosynthesized activity.

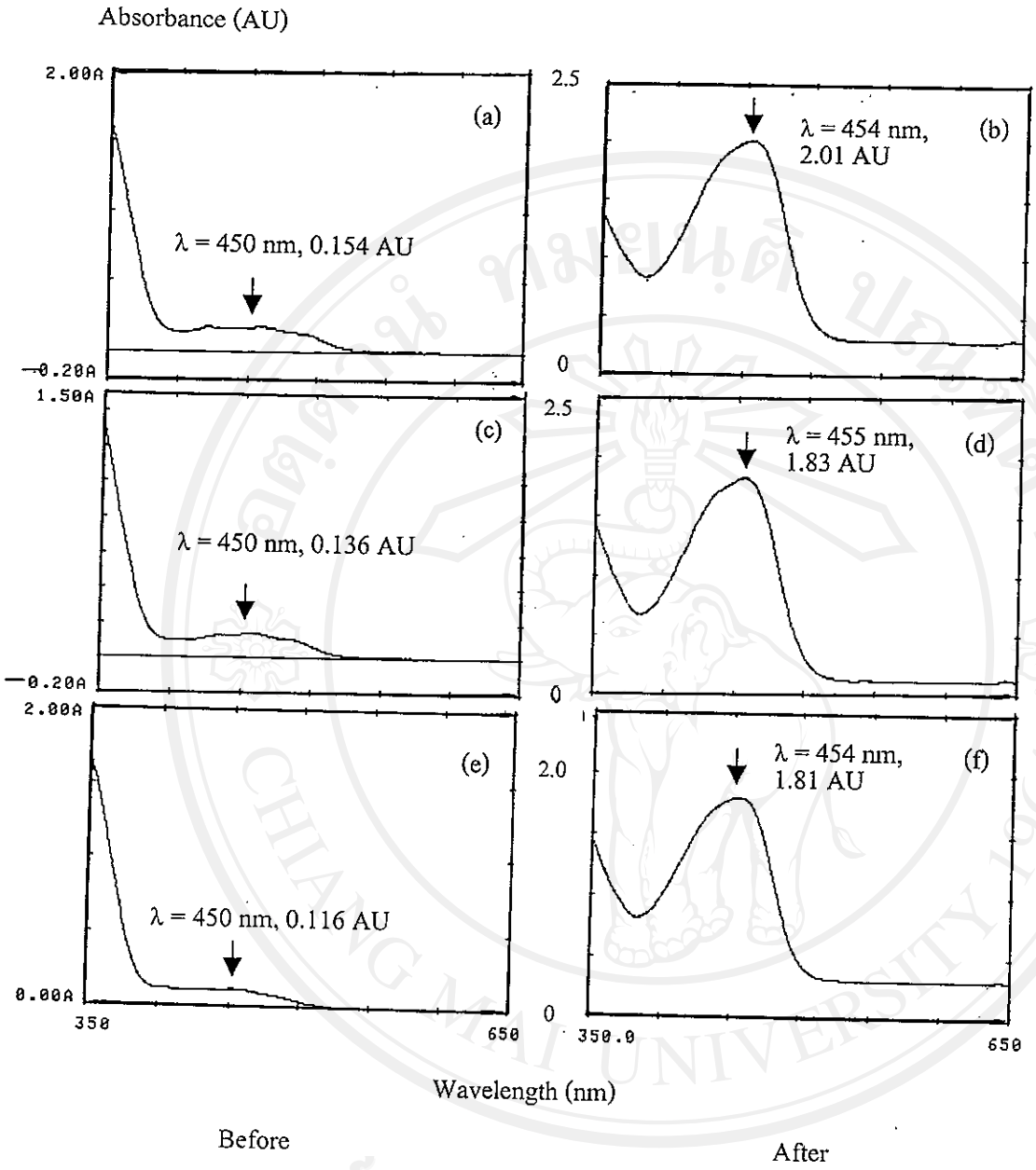

Figure 18. The comparison of Bu absorption spectra from different types of liver biosynthesis: (a, b) bovine liver ; (c, d) chicken liver ; (e, f) human liver (diluted 1:4). All figures shown on the left-side were Bu in liver homogenate before the biosynthesis reaction and all figures on the right-side were absorption spectra of Bu after 150 min biosynthesized activity.

IV. Preparation of lyophilized Bc product

Bc products were successfully lyophilized by the Lioalpha-10 lyophilizer within 12 hrs cycle. The products were used to evaluate chemical, physical properties and molar absorptivity of Bc as shown in the next parts of results.

V. Determination of chemical properties of conjugated bilirubin

a. Absorption spectra

Bc products reconstituted with 3.0 mL distilled water. The maximum absorption of Bc spectra prepared in 0.1 mol/L Tris-HCl buffer, pH 7.4 appeared as biphasic peaks at 429.5 and 470 nm (resembled Fig. 11), which was different from that of Bu standard which gave the maximum absorption at 462.5 nm.

b. Diazotization

The diazotization reaction of Bc in total reaction gave positive result but for direct reaction, the concentration of Bc was demonstrated further in the application of uses (Table 9).

VI. Determination of physical properties of conjugated bilirubin

a. Effects of photooxidation on Bc product

The relationship of absorption spectra of Bc in 0.1 mol/L Tris-HCl buffer, pH 7.4 or 4 % BSA with the oxidation time were plotted (Fig. 19-22). Figure 19 shows the effect of light on oxidation of Bc solution in 0.1 mol/L Tris-HCl buffer, pH 7.4. The significant decrease in absorbance and change in maximum absorbance of peak of the absorption spectra was observed after 6 hours of the irradiation time. As seen in Fig. 20-a, the rate of photooxidation measured at wavelength 412 nm was slightly changed during 4.5 hours of irradiation. The overnight exposure to light of Bc (Fig. 20-b), however, showed the decrease in absorbance at 412 nm by about 34.43 %. The rate of photooxidation of Bc solution in 4 % BSA was demonstrated in Fig. 21. There was no change in maximum peak of absorption spectra of Bc during the first 4.5 hours of the exposition to light. The decreased rate of oxidation was found after 6 hours of the irradiation time. Alike the Bc solution prepared in 0.1 mol/L Tris-HCl buffer, pH 7.4, the final maximum peak of absorption spectra after overnight exposure of Bc in 4 % BSA

was changed from 412 to 380 nm. Figure 22-a demonstrated that there was no effect of light on Bc molecule dissolved in 4 % BSA during 4.5 hours of irradiation. After overnight exposition, however, the absorbance at 412 nm decreased approximately 16.94 %.

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

Figure 23 shows the relationship between the absorbance of peak and the exposure time to oxygen of the Bc product. The oxidation rate of Bc solution in 0.1 mol/L Tris-HCl buffer, pH 7.4 exposed to oxygen was demonstrated by decreasing in the absorbance at 412 nm and change in the maximum absorbance of peak of the absorption spectra. After 6 hours of exposure to oxygen, the absorbance at 412 nm was slightly changed. The final maximum peak of absorption spectra after overnight exposure was changed from 412 to 380 nm, the same as observed after Bc was irradiated. The absorbance at 412 nm was also decreased (Fig. 24-b) by about 28.84 % after 18 hours of exposure. The rate of oxidation by oxygen of Bc solution in 4 % BSA was demonstrated in Fig. 25. There was no change in maximum peak of absorption spectra of Bc during 2.5 hours after exposure to oxygen. The absorbance at 412 nm of Bc in 4 % BSA exposed to oxygen overnight decreased 12.55 % with the observation that the maximum peak of absorption spectra was then shift to 380 nm (Fig. 25 and 26-b).

In this study, reconstituted Bc prepared in 4 % BSA was less sensitive to light and oxygen than that in 0.1 mol/L Tris-HCl buffer, pH 7.4 (see Appendix F).

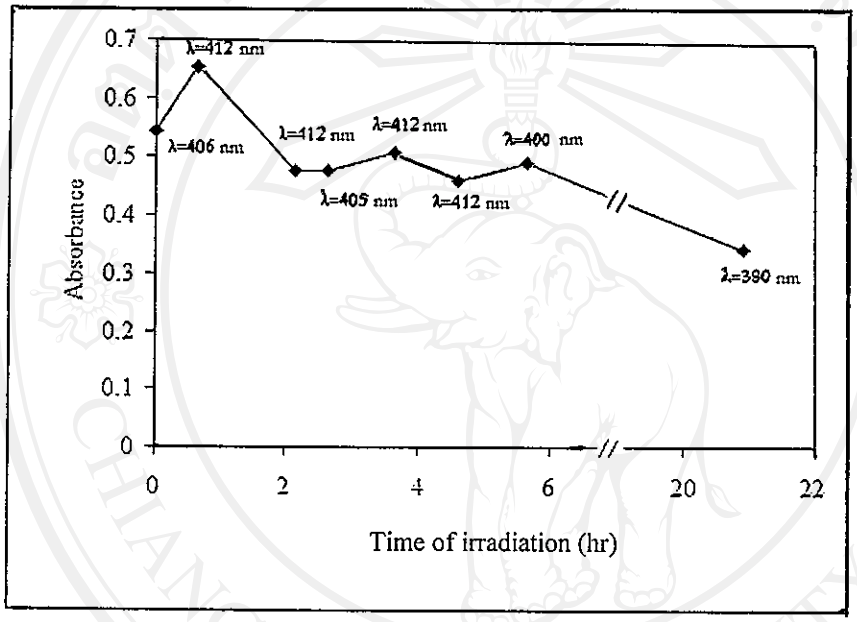

Figure 19. The relationship of the maximum peak of absorbance and irradiation time of Bc in 0.1 mol/L Tris-HCl buffer, pH 7.4.

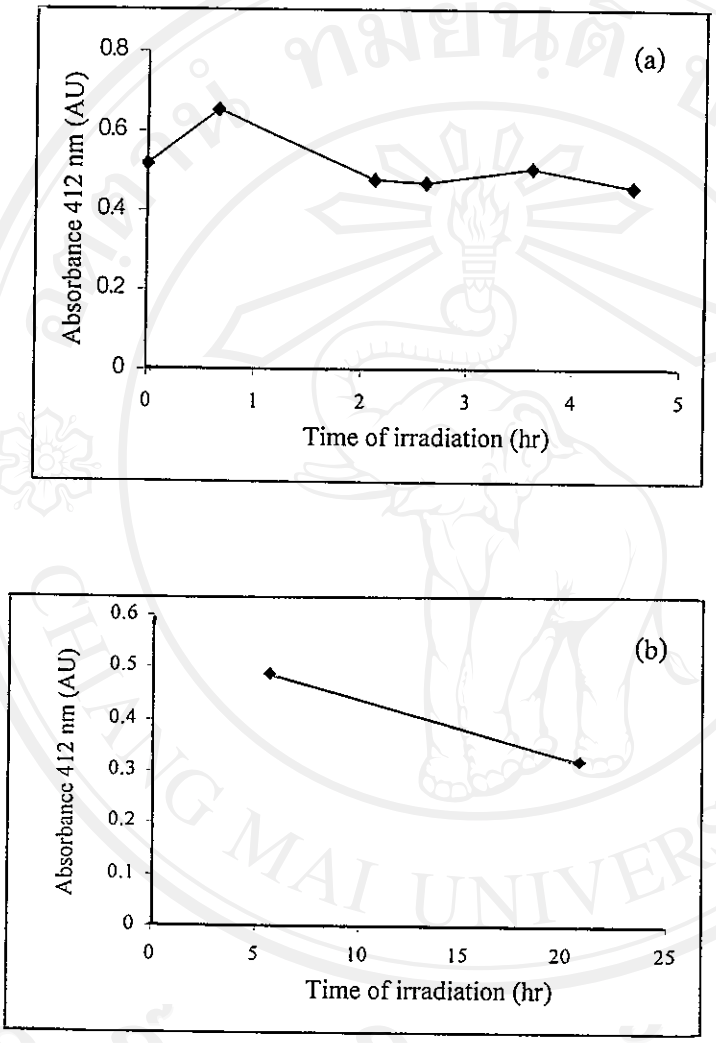

Figure 20. The photodegradation rate of Bc in 0.1 mol/L Tris-HCl buffer, pH 7.4 at 412 nm.

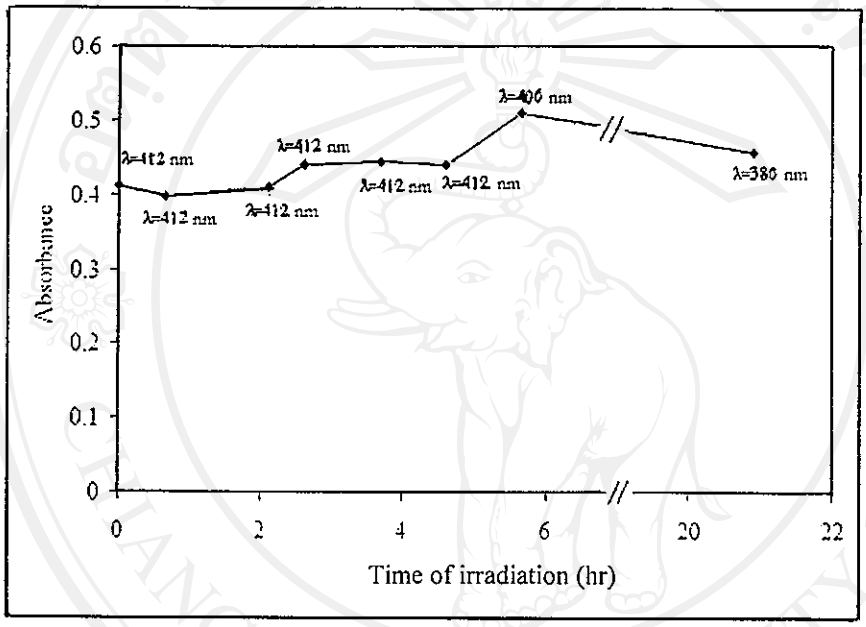

Figure 21. The relationship of the maximum peak of absorbance and irradiation time of Bc in 4 % BSA.

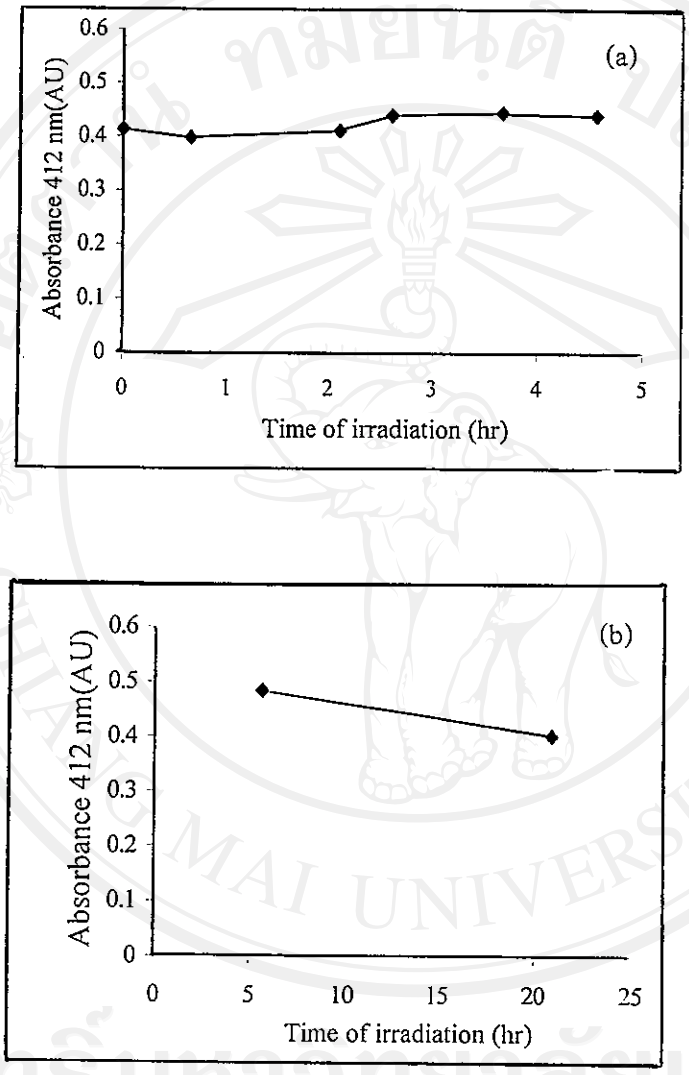

Figure 22. The photodegradation rate of Bc in 4 % BSA at 412 nm.

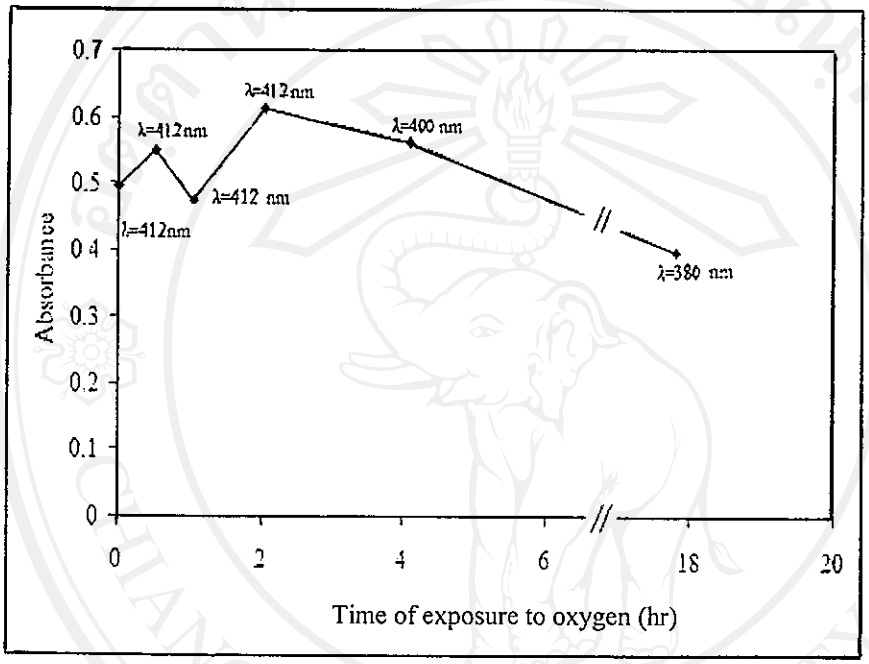

Figure 23. The relationship between the maximum peak of absorption spectra and time of exposure to oxygen of Bc in 0.1 mol/L Tris-HCl buffer, pH 7.4.

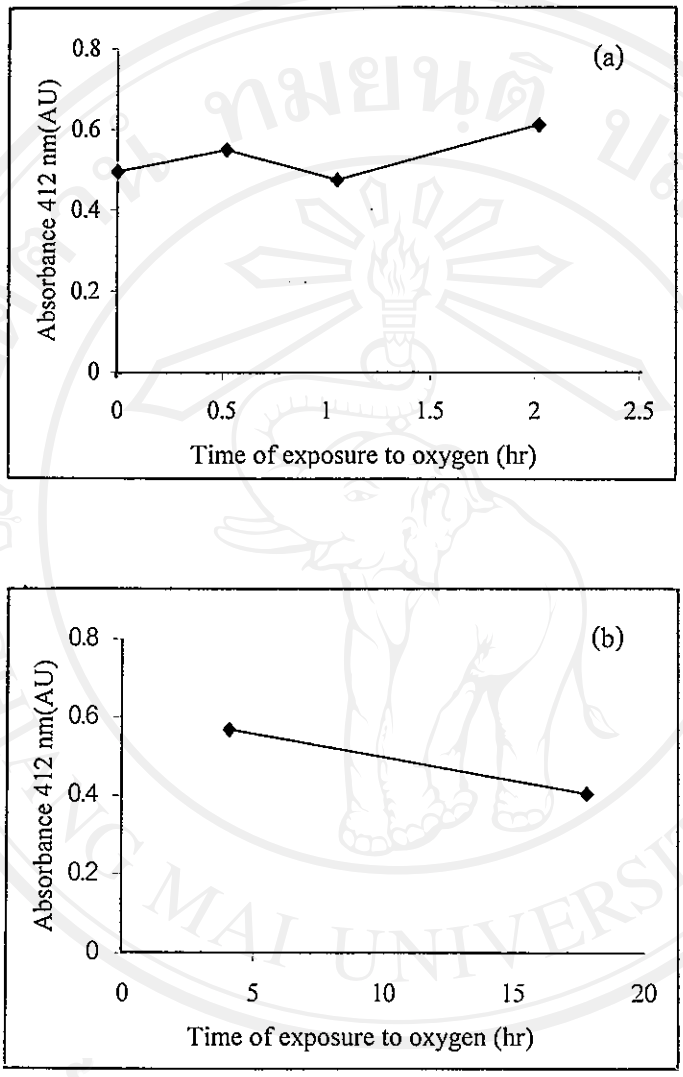

Figure 24. The rate of oxidation by oxygen of Bc in 0.1 mol/L Tris-HCl buffer, pH 7.4 at 412 nm.

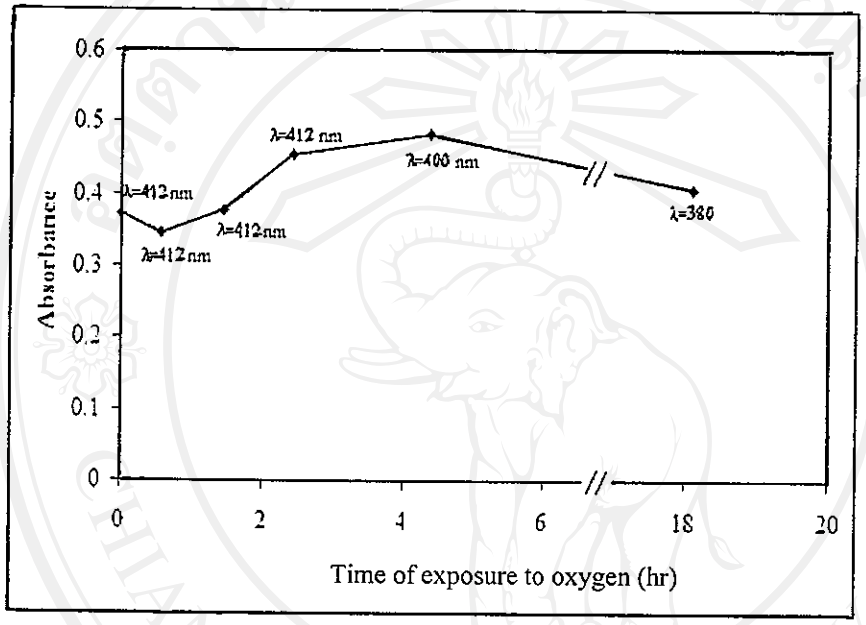

Figure 25. The relationship between absorption spectra and oxidation time of Bc in 4 % BSA.

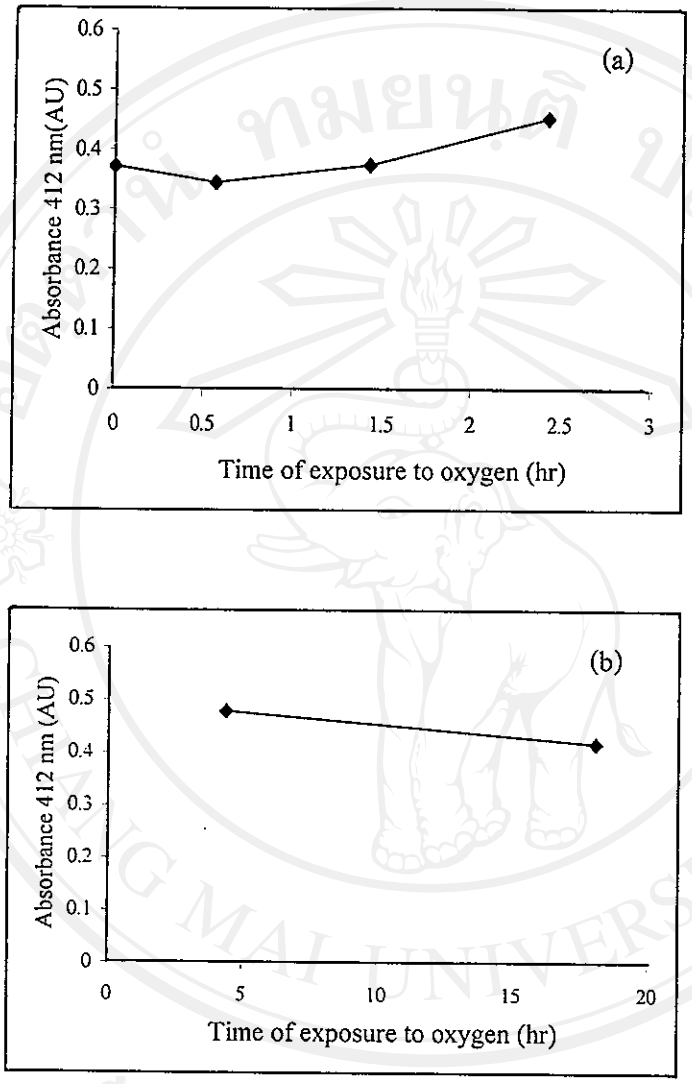

Figure 26. The rate of oxidation by oxygen of Bc in 4 % BSA at 412 nm.

VII. Determination of type of sugar conjugated to bilirubin

Bilirubin concentration of reconstituted Bc prepared in 0.1 mol/L potassium phosphate buffer, pH 6.8 was determined by direct spectrophotometry. The Bc concentration calibrated as Bu equivalent was 0.8 mg/dL. After incubation with β-glucuronidase followed by the extraction with chloroform, glucuronic acid concentration in aqueous phase was determined by comparing with glucuronic acid standard curve (Appendix G). The concentration of bilirubin and glucuronic acid were found to be 0.0136 and 0.0154 mmol/L, respectively and the ratio was calculated as 1 : 1.13. Since Bc in the interested peak was proved to be bilirubin glucuronide, therefore the DTB experiment was omitted.

VIII. Application for using conjugated bilirubin isolated from bile

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

Lyophilized Bc prepared in 0.1 mol/L Tris-HCl buffer, pH 7.4 or 4 % BSA were reconstituted with 1.0 mL distilled water. After scanning, the absorbances at maximum absorption spectra were used to calculate the molar absorptivity ($L.mol^{-1}.cm^{-1}$) of each presumptive Bc standard solution. As shown in Tables 4 and 5, the maximum absorption of Bc in both solubilizers were approximately between 398-411.5 nm. The absorbances of Bc in three separated preparations in those two solubilizers were compared for Bu equivalent concentrations in a Bu standard curve (Appendix H). Molar absorptivity of Bc (as Bu equivalence) was then calculated using the formula previously described in material and method. As shown in Tables 4 and 5, the mean molar absorptivity (ε) and standard deviation of Bc prepared in 0.1 mol/L Tris-HCl buffer, pH 7.4 (Table. 4) and 4 % BSA (Table 5) were equaled to 49,735 ± 3,126.27 and 51,155 ± 469.11 $L.mol^{-1}.cm^{-1}$, respectively.

Table 4. Molar absorptivity of Bc isolate solubilized in 0.1 mol/L Tris-HCl buffer, pH 7.4 (calculated as Bu equivalence)

No.	Peaks (nm)	Absorbance (AU) (A)	Bu equivalence (mg/dL)	Bu equivalence (mol/L) (c)	ε*(L.moL^{-1}.cm^{-1}) Bu equivalence
1	411.5	0.355	0.450	7.69625×10^{-6}	46,126
2	406.5	0.543	0.615	1.05182×10^{-5}	51,625
3	407.0	0.616	0.700	1.19720×10^{-5}	51,454

* ε = (A) / (b)(c), where (b) = path length = 1 cm

$\overline{X}$ = 49,735 L.moL^{-1}.cm^{-1}

SD = 3,126.27 L.moL^{-1}.cm^{-1}

% CV = 0.06

Table 5. Molar absorptivity of Bc isolate solubilized in 4 % BSA (calculated as Bu equivalence)

No.	Peaks (nm)	Absorbance (AU) (A)	Bu equivalence (mg/dL)	Bu quivalence (mol/L) (c)	ε*(L.moL^{-1}.cm^{-1}) Bu equivalence
1	411.5	0.335	0.380	6.49906×10^{-6}	51,546
2	399.5	0.433	0.500	8.55139×10^{-6}	50,635
3	398.0	0.518	0.590	1.00906×10^{-5}	51,285

* ε = (A) / (b)(c), where (b) = path length = 1 cm

$\overline{X}$ = 51,155 L.moL^{-1}.cm^{-1}

SD = 469.11 L.moL^{-1}.cm^{-1}

% CV = 0.009

b. Application of conjugated bilirubin for preparation of control serum

Control serum containing Bc isolate enriched with Bu commercial standard was evaluated for using as an internal quality control sample as compared with a commercial control serum (Randox multi sera elevated, Randox Laboratories. LTD, UK). Table 6 demonstrated the concentrations of TBIL and DBIL in human-based serum and two preparations of control serum analyzed by the diazo reaction based on Jendrassik and Grof method (Jendrassik and Grof, 1938). The recovery of a commercial Bu standard and Bc isolate which were added in the control serum were 78.33-97.53 % (Table 7) and 62.30-85.25 % (Table 8), respectively.

Table 6. Analytical concentration of total and direct bilirubin (TBIL and DBIL) in two preparations of control serum by the diazo reaction based on Jendrassik and Grof method (Jendrassik and Grof, 1938).

Assay of bilirubin in serum					
Sample		TBIL absorbance (AU)	DBIL absorbance (AU)	TBIL concentration (mg/dL)	DBIL concentration (mg/dL)
Control serum	no.1	0.595	0.036	13.16	0.80
	no.2	0.725	0.030	16.04	0.66
Human-based serum		0.036	0	0.80	0

Table 7. The recovery of a commercial Bu standard added to control serum analyzed by the diazo reaction based on Jendrassik and Grof method (Jendrassik and Grof , 1938).

No.	TBIL*[1] (mg/dL)	Standard added*[2] (mg/dL) (b)	Bc eluate (mg/dL) (c)	Bu standard recovered (mg/dL) (d)	% Recovery*[3]	Remark
1	0.80 [a]	none	none	-	-	Human-based serum
2	13.16 [c]	15	0.61	11.75	78.33	
3	16.04 [c]	15	0.61	14.63	97.53	

*[1] TBIL analyzed in control serum (see Table. 6)

*[2] Bu standard prepared in 4 % BSA

*[3] % Recovery = [standard recovered (d) / standard added (b)] x 100, where (d) = e-a-c

Table 8. The recovery of Bc isolates added to control serum analyzed by the diazo reaction based on Jendrassik and Grof method (Jendrassik and Grof , 1938).

No.	DBIL*[1] (mg/dL) (a)	Standard added*[2] (mg/dL) (b)	Bc eluate (mg/dL) (c)	Bc recovered (mg/dL) (d)	% Recovery*[3]	Remark
1	0	-	none	-	-	Human-based serum
2	0.80	0.28	0.61	0.52	85.25	
3	0.66	0.28	0.61	0.38	62.30	

*[1] DBIL analyzed in control serum (see Table. 6)

*[2] 20 mg/dL Bu standard reacted in direct reaction

*[3] % Recovery = [Bc recovered (d) / Bc in eluate (c)] x 100, where (d) = a-b

The application of bilirubin control serum to demonstrate the precision of bilirubin determination was shown in Table 9 (For details see Appendix I). It is shown that % CV of RCV of direct bilirubin analyzed by Jendrassik and Grof method was less than 2 times of the OCV precision (WHO limited the % CV of OCV $\leq$ 6 and for RCV $\leq$ 12, which then RCV/OCV $\leq$ 2, Whitehead, WHO doc. Lab 76/10). In analysis of total bilirubin in the same control serum it was found that the ratio of % CV of RCV to that of OCV is grater than 2, however it is still in the acceptable limit of WHO (12%). Moreover, it is the mean value of RCV is close to that of OCV (good accuracy).

Table 9. The OCV and RCV precision of direct and total bilirubin in the prepared control serum and commercial control serum analyzed by Jendrassik and Grof method.

Estimation	Prepared control serum		Commercial control serum	
	Direct bilirubin (mg/dL)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)	Total bilrubin (mg/dL)
OCV n	5	5	5	5
$\bar{X}$	0.79	16.91	2.64	7.11
SD	0.05	0.22	0.15	0.08
% CV	6.33	1.30	5.68	1.13
RCV n	4	5	5	5
$\bar{X}$	0.70	16.23	2.63	7.05
SD	0.08	0.53	0.19	0.23
% CV	11.43	3.27	7.22	3.26

When a commercial control serum was used to monitor the precision of the analytical method (Jendrassik and Grof) for bilirubin determination, it was demonstrated that the % CVs of OCV and RCV of direct and total bilirubin were accepted by the OCV-RCV criteria of WHO. When compared with the control serum prepared in this study, it is seen that the precision of analysis of direct bilirubin of the commercial control serum was better than the prepared control serum. But for total bilirubin, the % CVs of OCV and RCV were more or less the same with those found in our preparation.