CHAPTER III

RESULTS AND DISCUSSION

1. Synthesis of HRP Labeled Theophylline

1.1 To prepare HRP ready for conjugation

Chemical Reaction for aminating HRP are shown in figure 1.

The product was 84.14 mg Its appearance was raddish - brown amorphous powder. Its ultraviolet spectrum still exhibited the maximum absorbance of HRP at 403 nm (Figure 2).

The molar extinction coefficient of amino groups in HRP which reacted with TNBS was determined to be 9,800 , calculated from data in Table 2.

The ratio of amino groups incorporated to aminated IIRP was then calculated to be approximately 14 moles per one mole of HRP, calculated from data in Table 3.

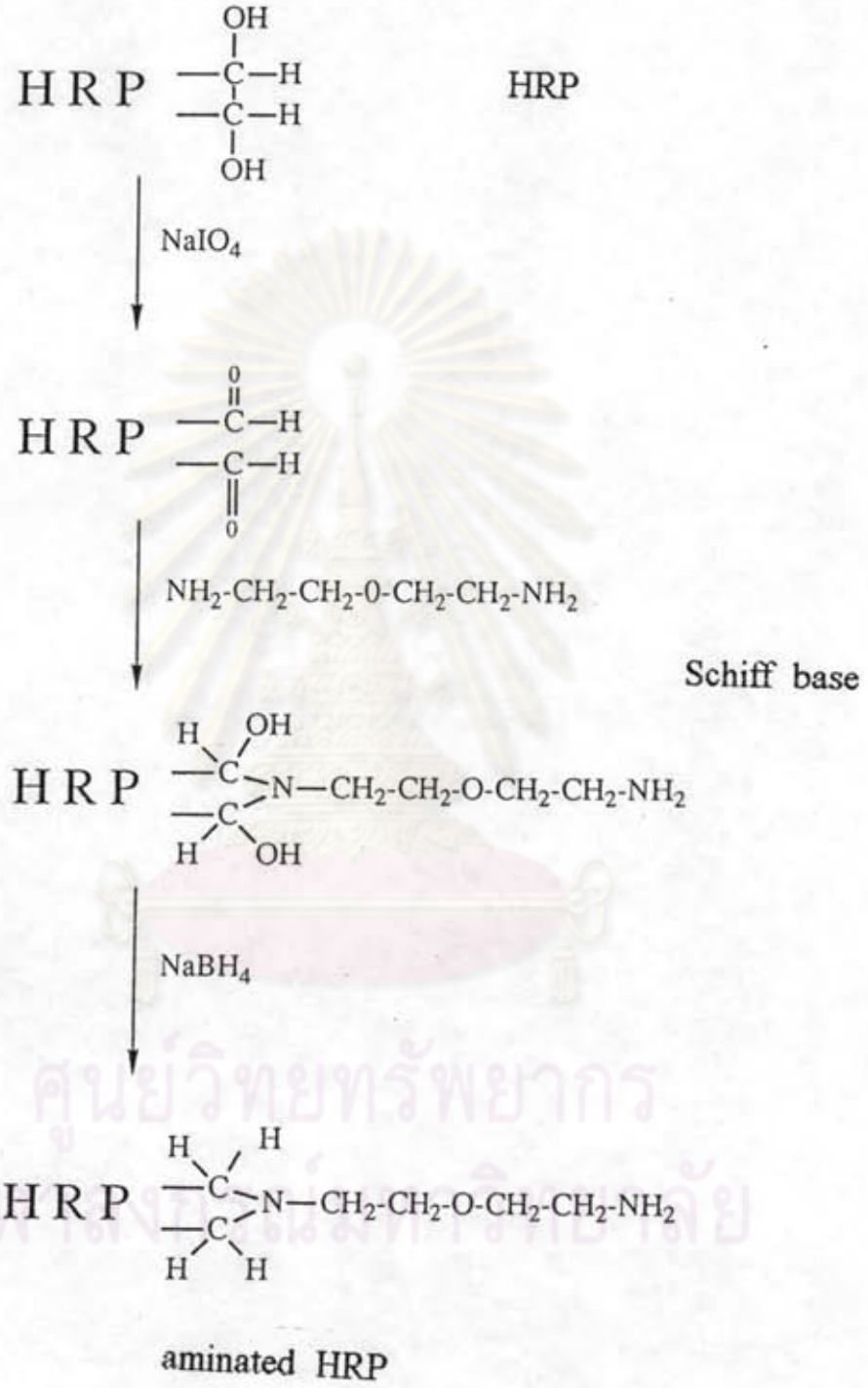


Figure 1 Chemical Reaction for Aminating HRP

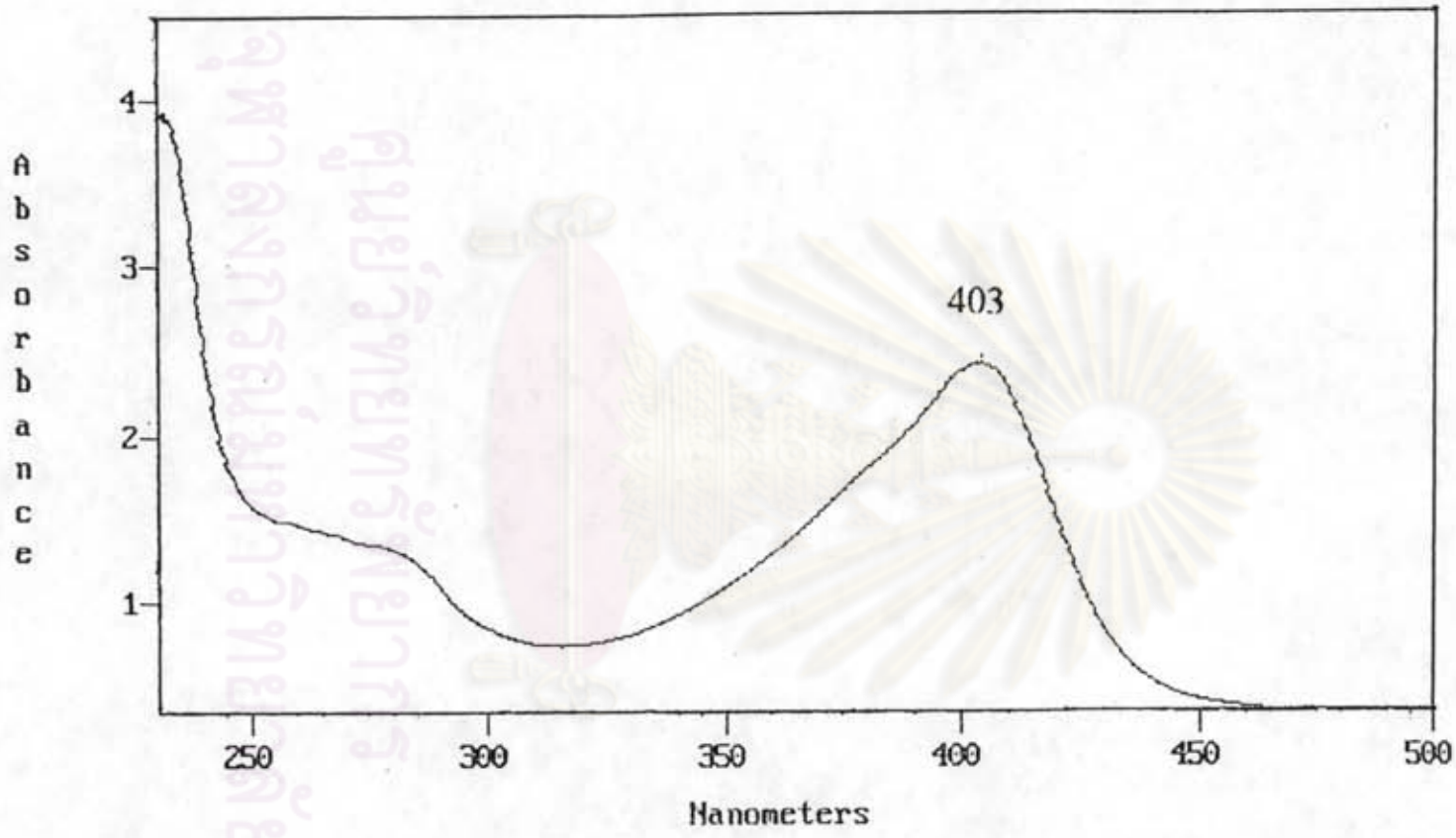


Figure 2 The Ultraviolet Spectrum of Aminated HRP aqueous solution concentration 4.0 mg /ml

Table 2 Ultraviolet Absorption Data for Amino group in HRP

CONCENTRATION (* 10^{-7} M)	ABSORBANCE 354 nm	THE MOLAR EXTINCTION COEFFICIENT (E_{354} * 10^3)
1.00	1.00	10.0
1.00	0.96	9.60
1.00	0.98	9.80
	AVERAGE	9.80

Table 3 Ultraviolet Absorption and number of amino groups data for aminated HRP

CONCENTRATION (* 10^{-7} M)	ABSORBANCE 354 nm	NUMBER OF AMINO GROUPS
1.00	2.07	14.4
1.00	2.03	14.1
1.00	2.11	14.6
	AVERAGE	14.4

The employed procedure for HRP amination has been reported to contain 12 - 18 moles of amino groups per one mole of HRP (Litman et al., 1983 ; Zuk et al., 1985). Therefore , the aminated HRP product from this study which contains 14 moles of amino groups should be satisfactory. However , to increase more amino groups per HRP molecule , more 2,2 - oxybis (ethylamine) have to be added into the reaction. This aminated HRP was further used for conjugating with theophylline.

1.2 To prepare theophylline ready for conjugation

The chemical reaction for synthesis of theophylline ester derivative are shown in Figure 3.

7- (3-carboxypropyl)-1,3- dimethylxanthine

This compound was synthesized by a modification of the method of Hu and Singh , 1980 which contained two steps. The first step involves an alkylation reaction between N-7 of theophylline and ethyl -4- bromobutyrate to yield 7-(4-ethylbutyrate) -1,3- dimethylxanthine. The second step of reaction was hydrolysis of 7- (4- ethylbutyrate) -1,3- dimethylxanthine to yield 7-(3-carboxypropyl) -1,3- dimethylxanthine. Mechanistically , this process results from a nucleophilic attack of N-7 of theophylline upon the ethyl -4- bromobutyrate and the corresponding 7- (4- ethylbutyrate) -1,3- dimethylxanthine. This ester was hydrolyzed and the corresponding 7- (3-carboxypropyl) -1,3-dimethylxanthine was obtained shown in appendix A .

Its ultraviolet spectrum showed the maximum absorption of theophylline at 275 nm (Figure 4).

The IR spectra (KBr disk) as depicted in Figure 5 and assigned to functional groups of structure shown in Table 4.

Its ^{1}H-NMR spectra (DMSO) shown in Figure 6 indicated the position of hydrogen atoms in the structure in the term of proton at the following chemical shift and assigned of proton of structure shown in Table 5.

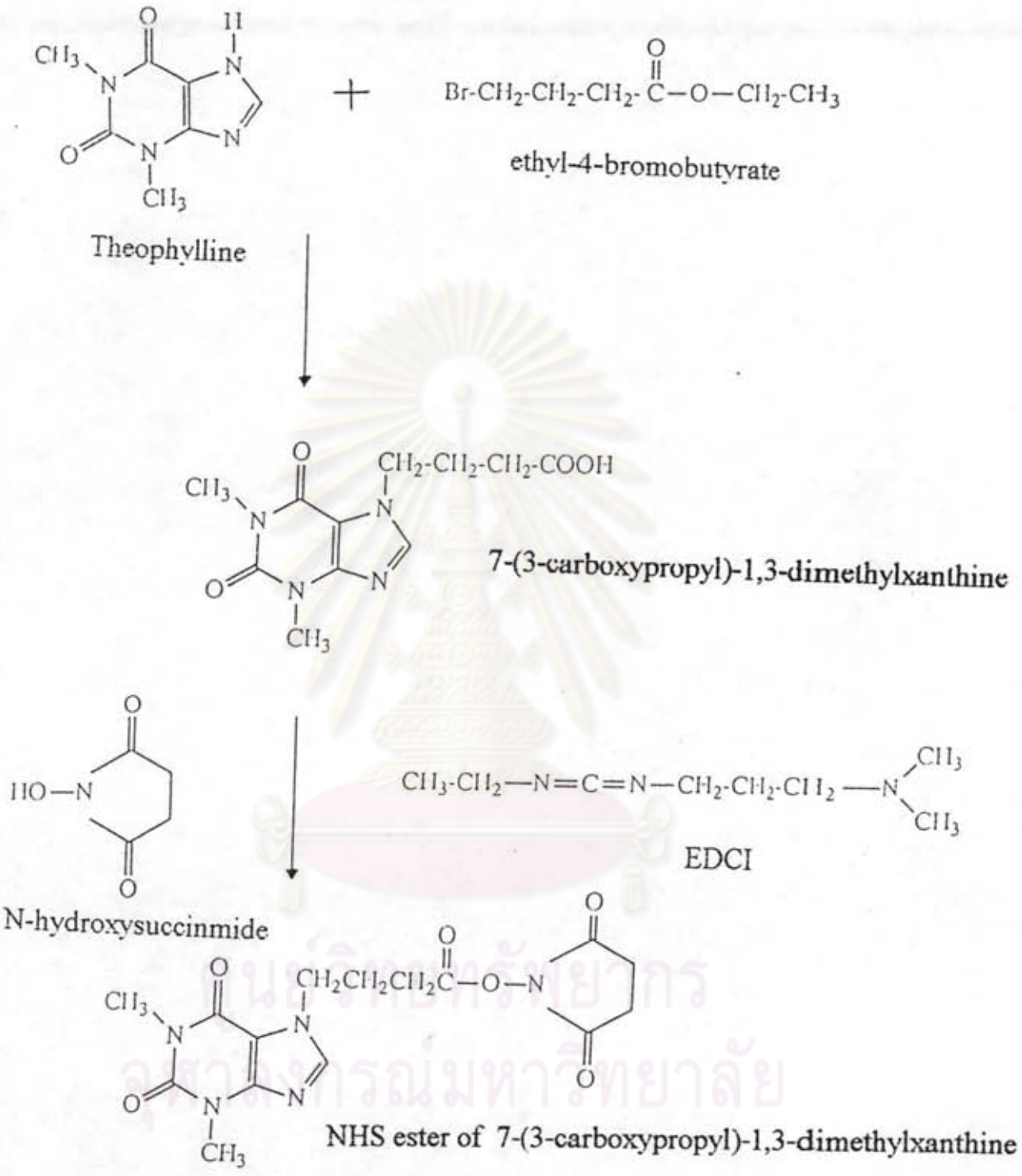


Figure 3 Chemical Reaction for Synthesis of Theophyliine ester Derivative

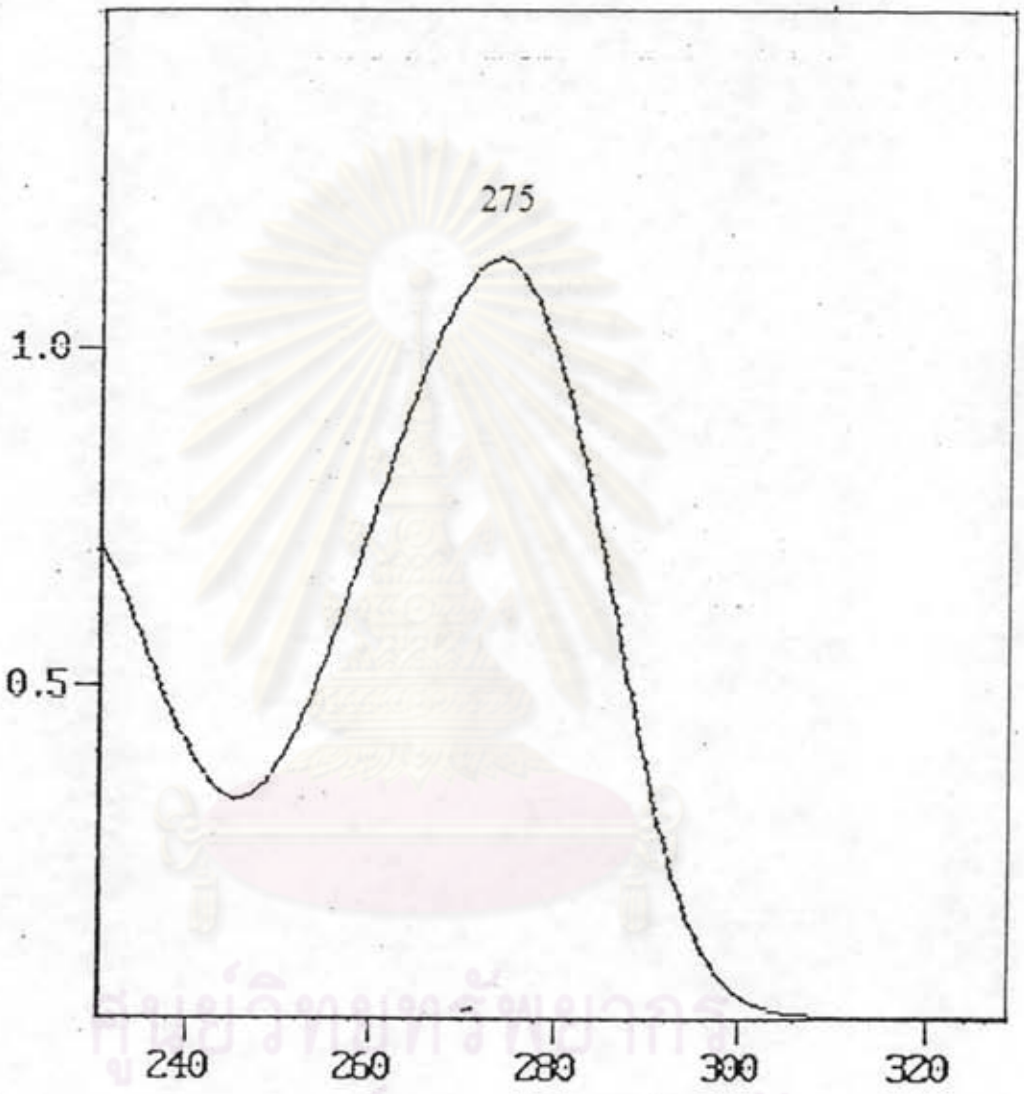


Figure 4 The UV spectrum of 7-(3- carboxypropyl)
-1,3-dimethylxanthine aqueous solution
concentration 0.03 mg /ml

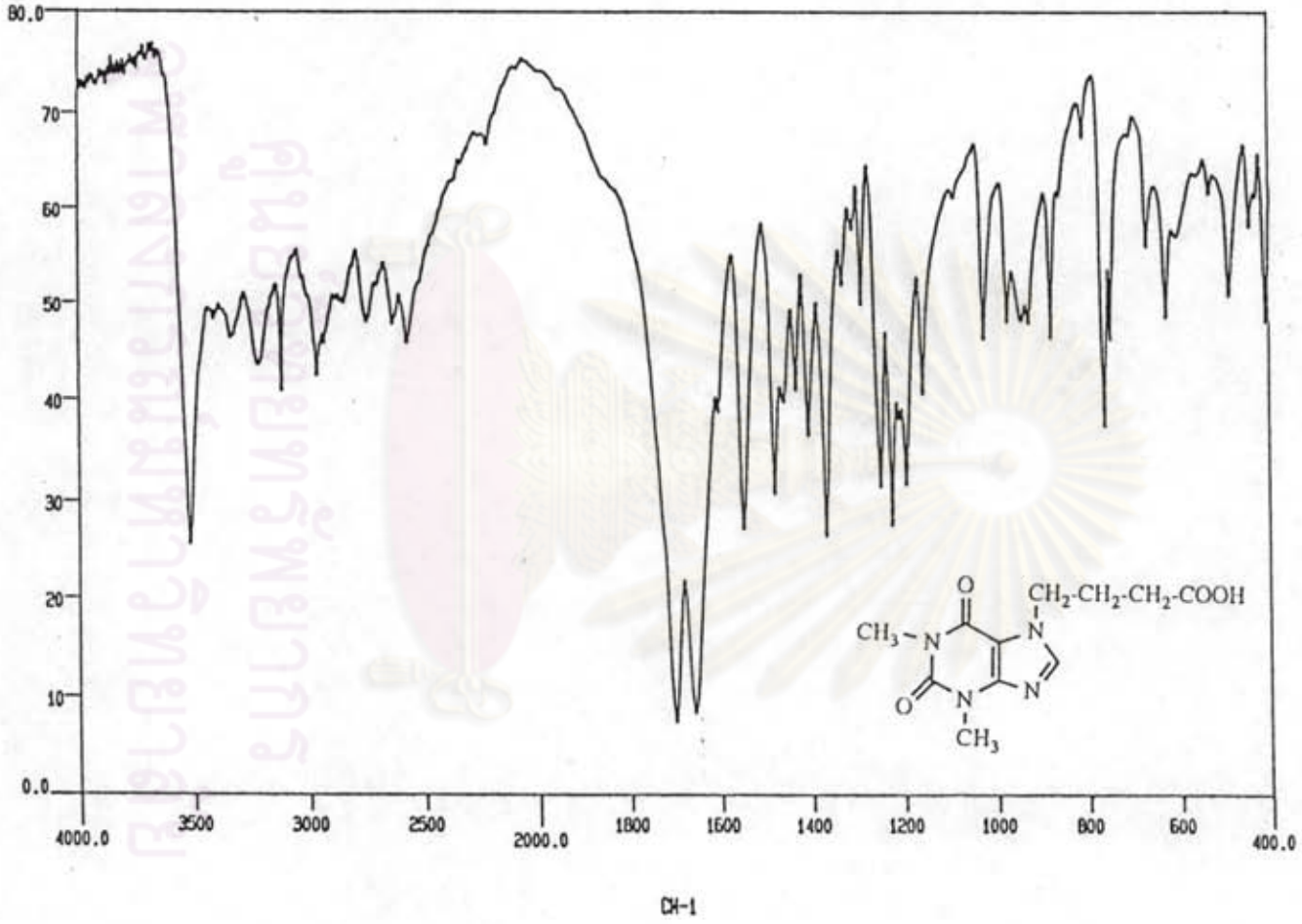


Figure 5 The IR spectrum of 7-(3-carboxypropyl)-1,3-dimethylxanthine

Table 4 Assignment of IR Spectrum of 7-(3-carboxypropyl)-1,3-dimethylxanthine

WAVENUMBERS (cm^{-1})	FUNCTIONAL GROUPS
2572 - 3521	O - H STRETCHING OF CARBOXYLIC ACID DIMERS
1657 - 1699	C = O STRETCHING
1395 - 1440	O - H BENDING
1260 - 1320	C - N STRETCHING

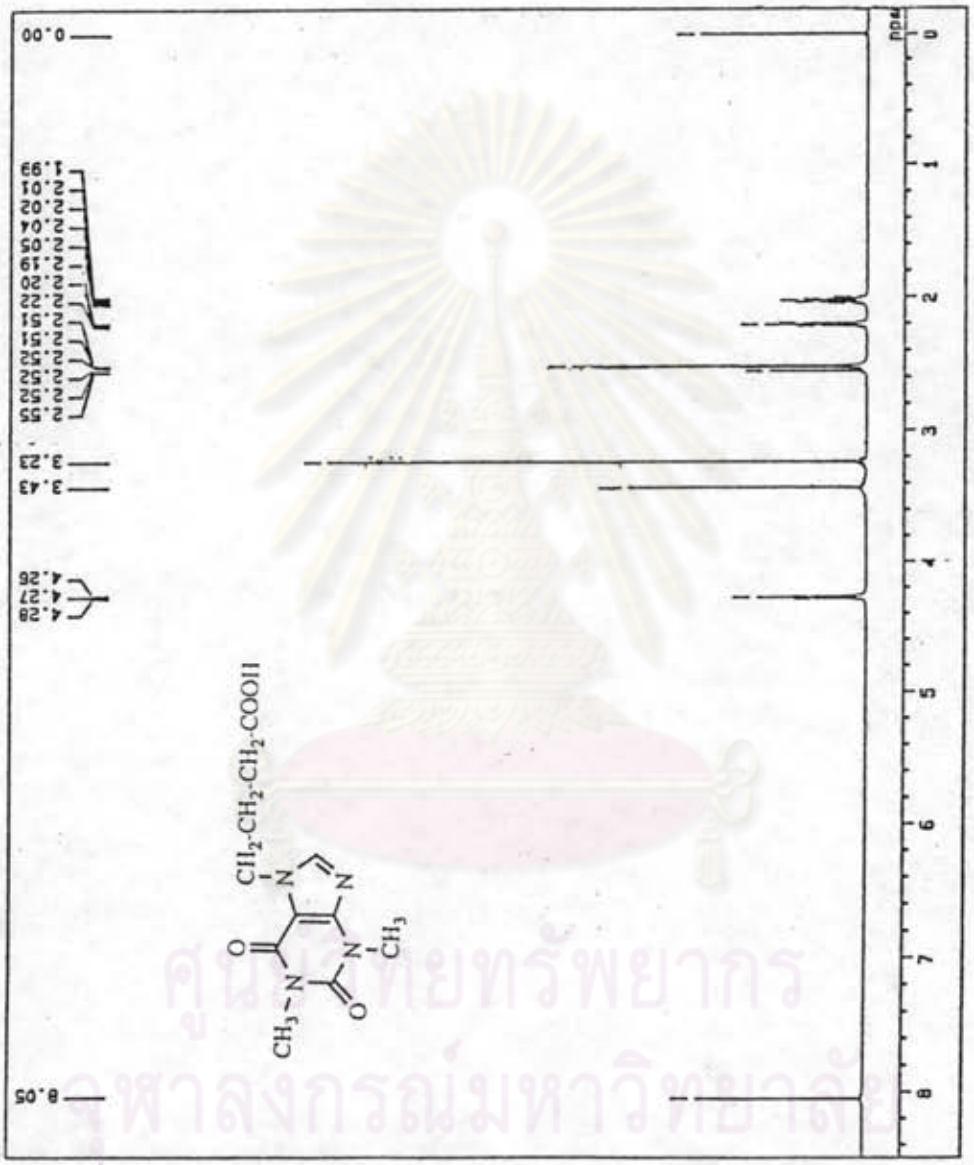
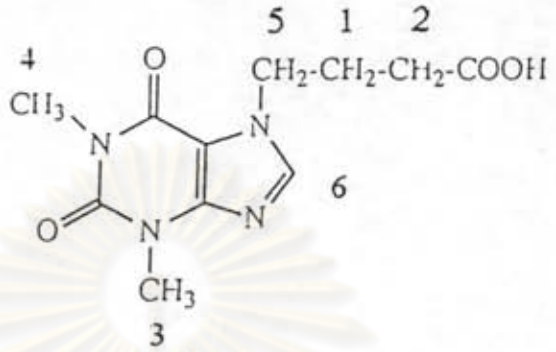


Figure 6 The ^{1}H-NMR spectrum of 7-(3-carboxypropyl)-1,3-dimethylxanthine in DMSO-d_6

Table 5 Assignment of ^{1}H-NMR chemical shift of **7-(3-carboxypropyl)-1,3-dimethylxanthine**



POSITION	CHEMICAL SHIFT	MULTIPLICITY
1	1.99 - 2.05	MULTIPLET , 2H
2	2.18 - 2.21	TRIPLET , 2H
3	3.23	SINGLET , 3H
4	3.42	SINGLET , 3H
5	4.25 - 4.28	TRIPLET , 2H
6	8.05	SINGLET , 1H

NHS ester of 7-(3-carboxypropyl)- 1,3- dimethylxanthine

This compound was synthesized according to the modification method of Peptide synthesis of L. Wackerle , 1979. The reaction step involves a condensation reaction between 7-(3-carboxypropyl)- 1,3- dimethylxanthine and N-hydroxysucinimide to yield NHS ester of 7-(3-carboxypropyl)- 1,3- dimethylxanthine . EDCI was used as carboxyl group activator of reaction. The reaction mechanism shown in appendix B.

Its ultraviolet spectra exhibited the maximum absorption of theophylline at 269 nm (Figure 7).

The IR spectra (KBr disk) as shown in Figure 8 and assigned to functional groups of structure shown in Table 6.

Its ^{1}H-NMR spectrum ($CHCl_3$) shown in Figure 9 indicated the position of hydrogen atoms in the structure in the term of proton at the following chemical shift and assigned of proton shown in Table 7.

Therefore , according to these results it is confirmed that NHS ester of 7-(3- carboxypropyl)- 1,3- dimethylxanthine was actually synthesized and ready for conjugation to HRP.

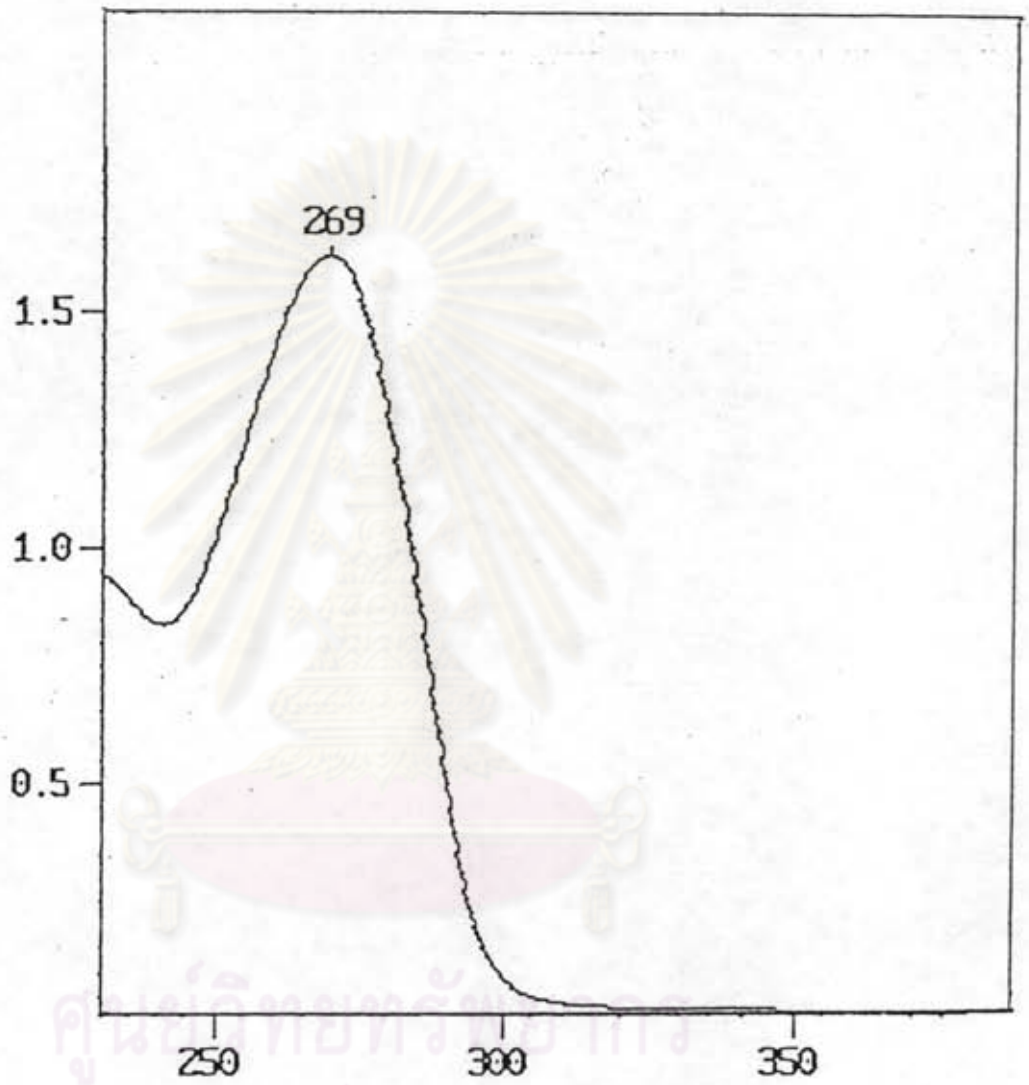


Figure 7 The UV spectrum NHS ester of 7-(3- carboxypropyl) -1,3-dimethylxanthine aqueous solution concentration 0.04 mg /ml

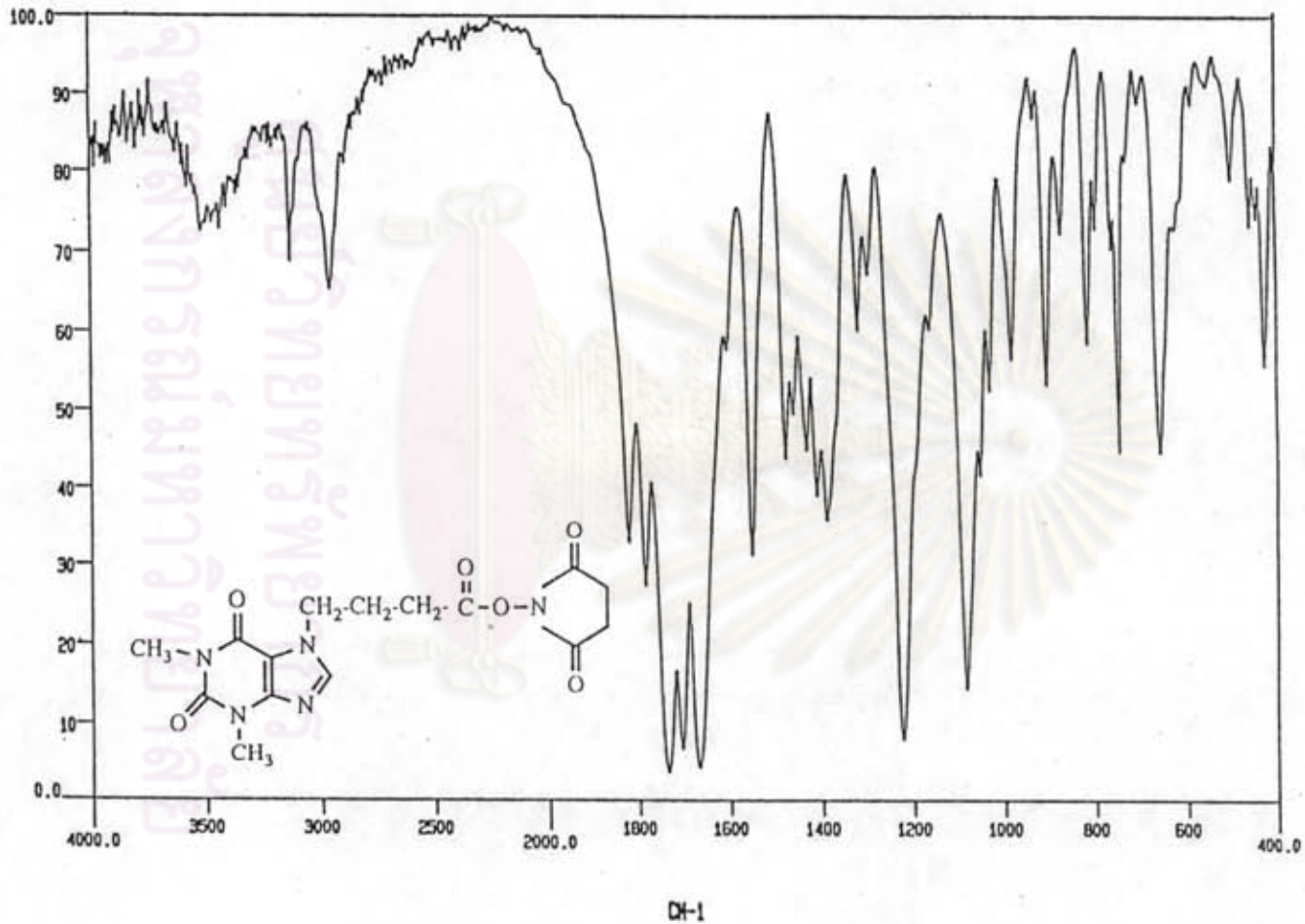


Figure 8 The IR spectrum of NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine

Table 6 Assignment of IR Spectrum of NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine

WAVENUMBERS (cm^{-1})	FUNCTIONAL GROUPS
1084 , 1223	C - O STRETCHING
1700 - 1800	C = O STRETCHING
2900 - 3150	C - H STRETCHING

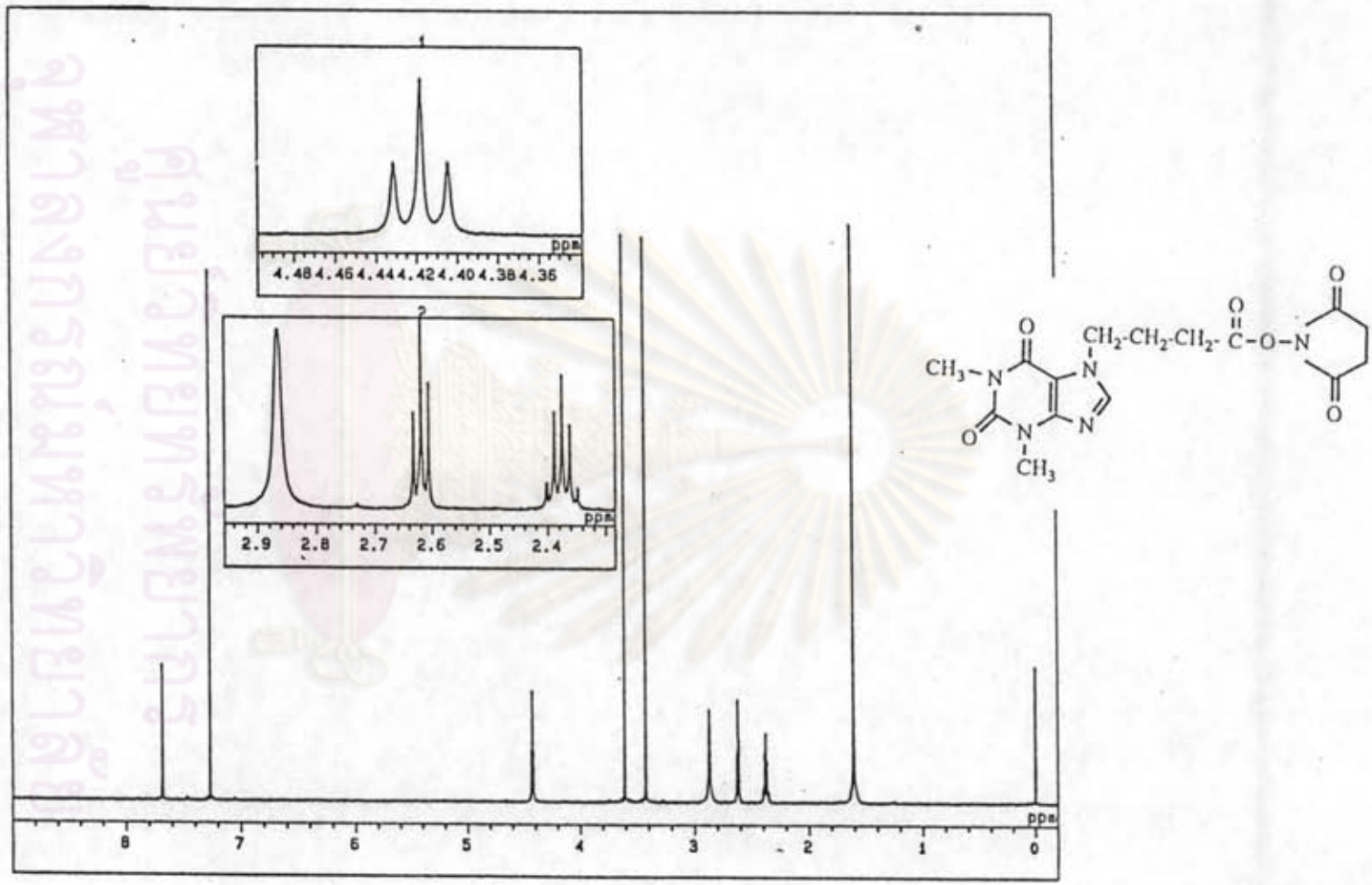
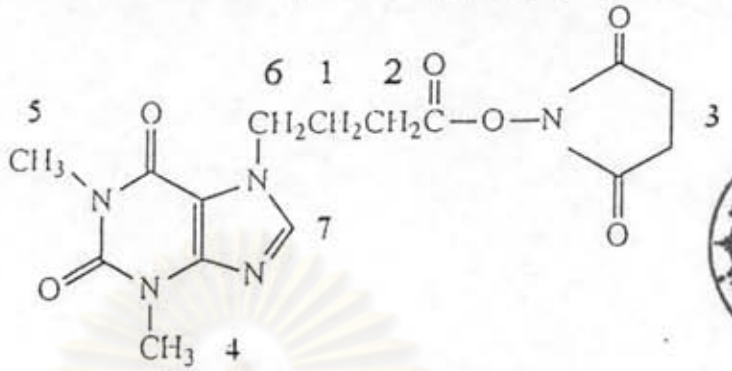


Figure 9 The ^{1}H-NMR spectrum of NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine in $CDCl_3$

Table 7 Assigment of ^{1}H-NMR Chemical shift of

NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine



POSITION	CHEMICAL SHIFT	MULTIPLICITY
1	2.36 - 2.38	MULTIPLET , 2H
2	2.60 - 2.63	TRIPLET , 2H
3	2.86	SINGLET , 4H
4	3.41	SINGLET , 3H
5	3.60	SINGLET , 3H
6	4.40 - 4.43	TRIPLET , 2H
7	7.68	SINGLET , H

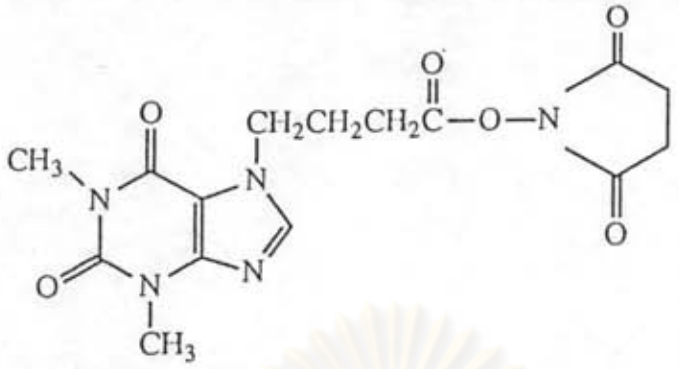
1.3 Conjugation of Theophylline to HRP

The chemical reaction for synthesis of HRP labeled theophylline was shown in Figure 10.

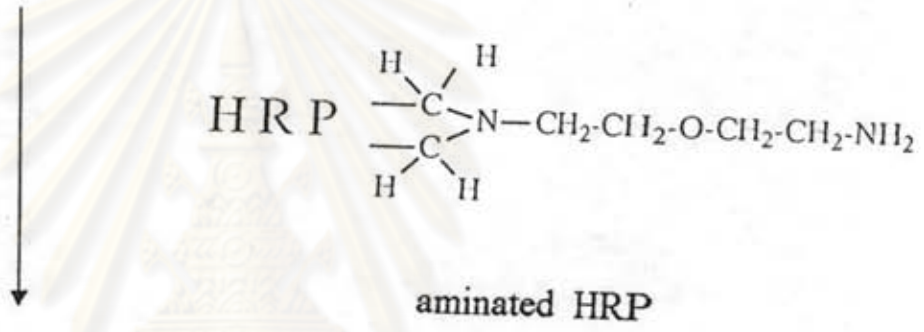
The obtained theophylline - HRP conjugate was brownish amorphous powder. The product was approximately 68.16 mg. The scanned ultraviolet spectrum of conjugated compound exhibited the maximum absorption of both HRP and theophylline at the wavelength of 403 and 269 nm., respectively as shown in Figure 11.

The molar extinction coefficient of aminated HRP was determined to be $4.336 * 10^4$, calculated from data in Table 8.

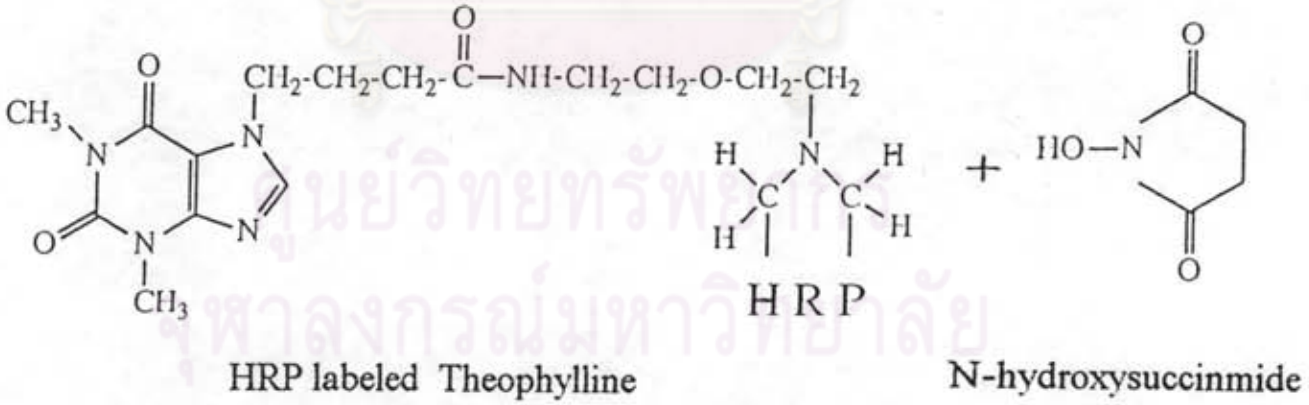
The molar extinction coefficient of NHS ester of theophylline was calculated from data Table 9 to be $1.045 * 10^4$



NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine



aminated HRP



HRP labeled Theophylline

N-hydroxysuccinmide

Figure 10 Chemical Reaction for Synthesis of HRP labeled Theophyliine

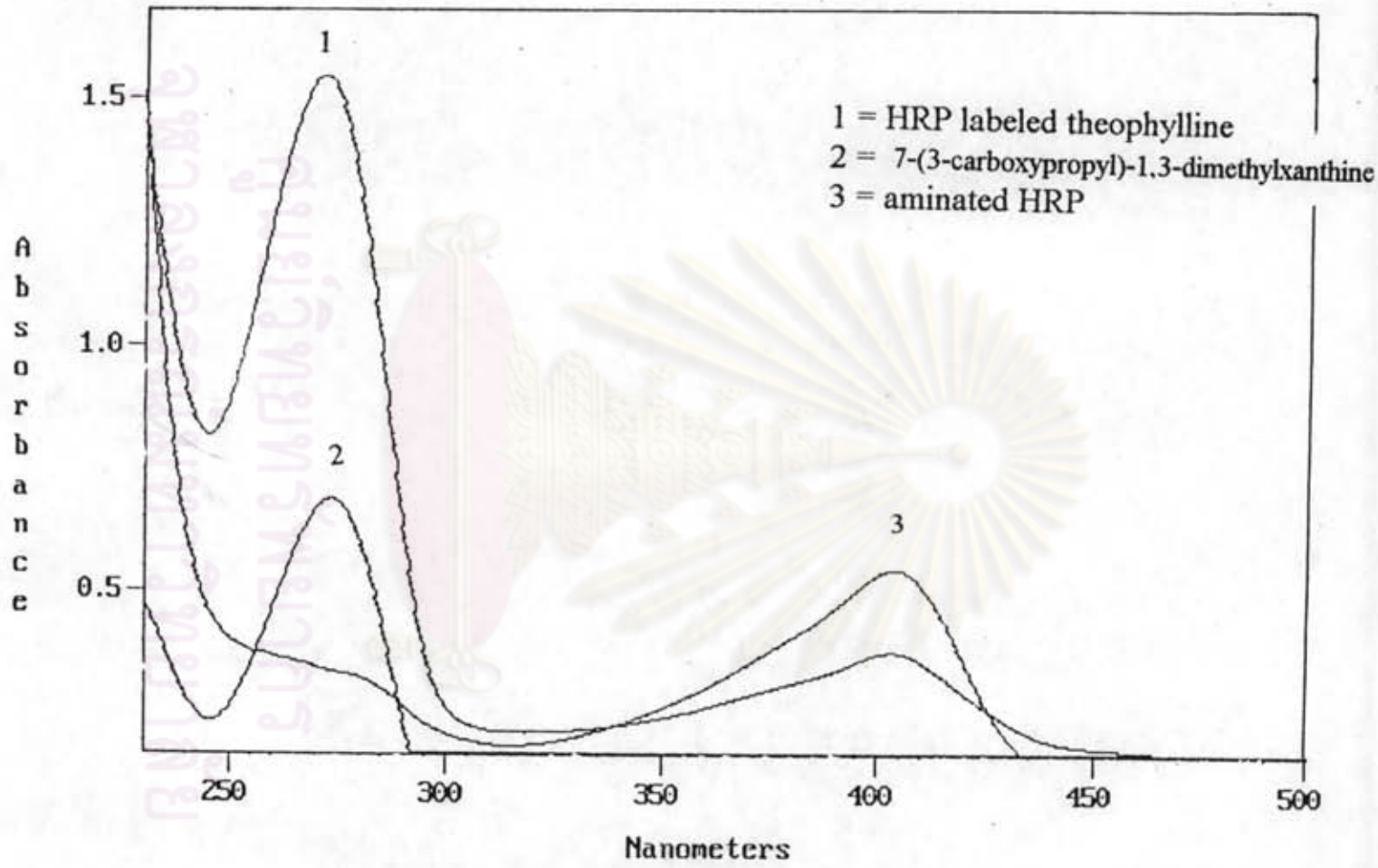


Figure 11 The UV spectrum of HRP labeled theophylline aqueous solution 0.2 mg /ml

Table 8 Ultraviolet Absorption Data of Aminated HRP

CONCENTRATION (* 10^{-8} M)	ABSORBANCE 403 nm	THE MOLAR EXTINCTION COEFFICIENT (E_{403} * 10^4)
1.25	0.542	4.336
1.25	0.538	4.304
1.25	0.546	4.368
	MEAN	4.336

Table 9 Ultraviolet Absorption Data of NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine

CONCENTRATION (mg /ml)	ABSORBANCE 269 nm	THE MOLAR EXTINCTION COEFFICIENT ($E_{269} * 10^4$)
0.020	0.838	1.044
0.020	0.840	1.046
0.020	0.841	1.047
	MEAN	1.045

The mole ratio of theophylline to HRP was then calculated from data in Table 10 to be approximately 11 theophylline molecules per one molecule of HRP. The results of this theophylline conjugate HRP was similar to that reported from Zuk et. al , 1985 which contained 10 - 12 theophylline molecules per molecule HRP. Therefore , according to these results it was confirmed that theophylline conjugate HRP was actually synthesized.

Table 10 Ultraviolet Absorption and Mole Ratio of Theophylline to HRP

CONCENTRATION (mg /ml)	ABSORBANCE 269 nm	ABSORBANCE 403 nm	MOLE RATIO OF THEOPHYLLINE TO HRP
0.100	0.688	0.269	10.6
0.100	0.701	0.274	10.6
0.100	0.692	0.271	10.7
		AVERAGE	10.6

2. Preparation of Antibody for Theophylline

2.1 Preparation of Immunogen

Immunogen A (7-(3-carboxypropyl)-1,3-dimethylxanthine-BSA conjugate)

The product was about 58.04 mg and its appearance was white fluffy powder.

The ultraviolet spectrum exhibited the maximum absorption of theophylline at 277 nm (Figure 12).

The spicific absorbance of theophylline in immunogen A was determined to be 34.68 , calculated from data in Table 11. The value of specific absorbance was used for determining mole ratio of theophylline to BSA in the conjugate. The ratio was 8 : 1 as shown in Table 12.

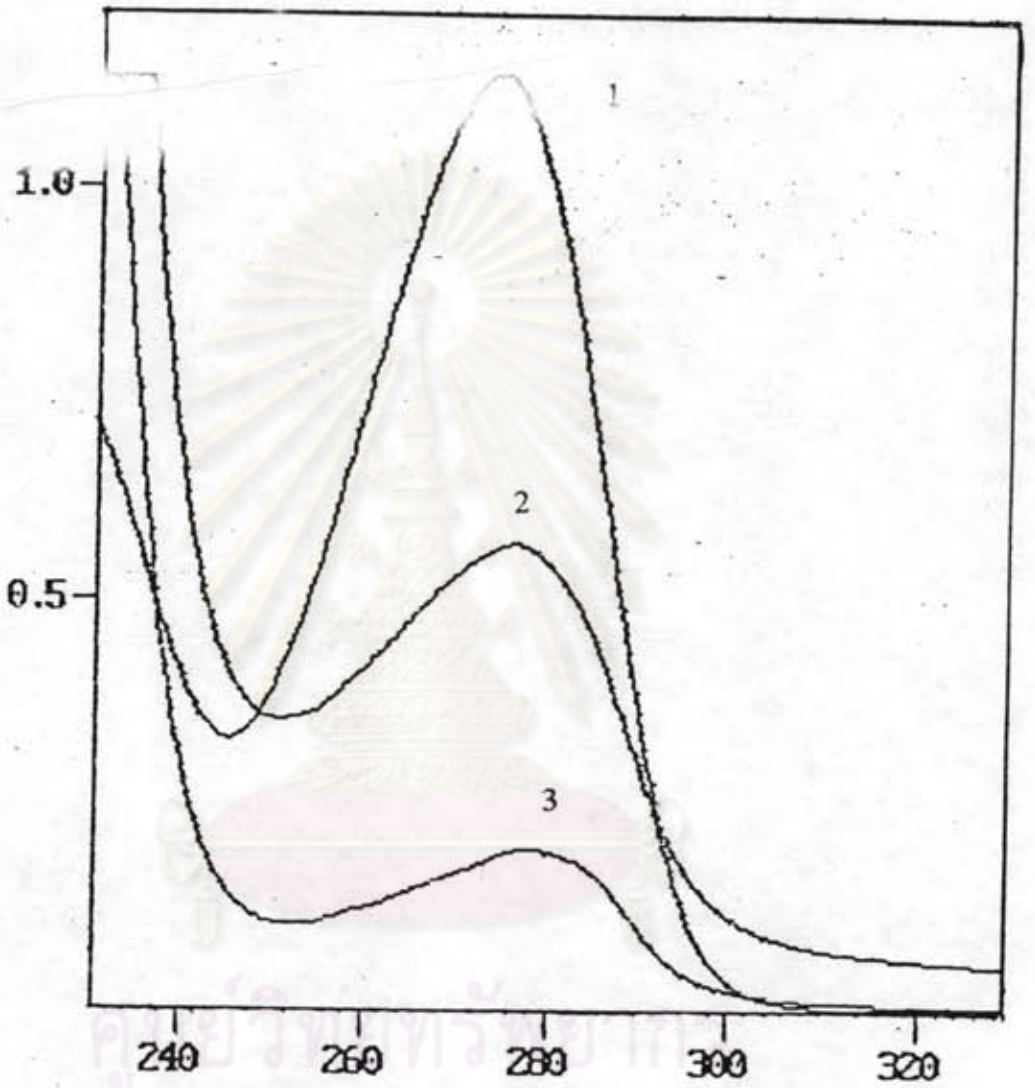


Figure 12 The UV spectrum of Immunogen A aqueous solution concentration 0.286 mg /ml

1 = 7-(3-carboxypropyl)-1,3-dimethylxanthine
2 = 7-(3-carboxypropyl)-1,3-dimethylxanthine-BSA
3 = BSA

Table 11 Ultraviolet Absorption Data of 7-(3-carboxypropyl)-1,3-dimethylxanthine

CONCENTRATION (mg /ml)	ABSORBANCE 275 nm	THE SPECIFIC ABSORBANCE (K)
0.029	1.006	34.69
0.029	0.996	34.34
0.029	1.015	35.00
	MEAN	34. 68

K = absorbance / (path length * conc. mg /ml)

Table 12 Determination of Theophylline - BSA
Mole ratio of Immunogen A

CONC. (mg/ml)	Abs 277 nm (conjugate)	Abs 277 nm (BSA)	Abs 277 nm (theophylline)	MOLE RATIO OF THEOPHYLLINE TO BSA
0.286	0.457	0.175	0.282	7.82
0.286	0.442	0.175	0.267	7.41
0.286	0.450	0.175	0.275	7.64
			AVERAGE	7.62

$$\text{mole ratio of theophylline to BSA} = \frac{\text{wt. of theophylline in conjugate / mw}}{\text{wt. of BSA in conjugate / mw}}$$

$$\text{wt. of theophylline in conjugate} = \frac{\text{absorbance of theophylline}}{\text{specific absorbance}}$$

Immunogen B (7-(3-carboxybutyl)-1,3dimethylxanthine-BSA conjugate)

7-(3-carboxybutyl)-1,3-dimethylxanthine

The product was colorless crystals and its ultraviolet spectrum exhibited the maximum absorption of theophylline at 274 nm (Figure 13).

The IR spectrum (KBr disk) as shown in Figure 14 and assigned to functional group of acid derivative shown in Table 13.

The ^{1}H-NMR spectrum (DMSO) in Figure 15, showed the following proton position in the structure at various chemical shifts and assigned proton of structure shown in Table 14.

According to IR and NMR spectra, the structure of 7- (3- carboxybutyl) -1,3- dimethylxanthine would be actually synthesized.

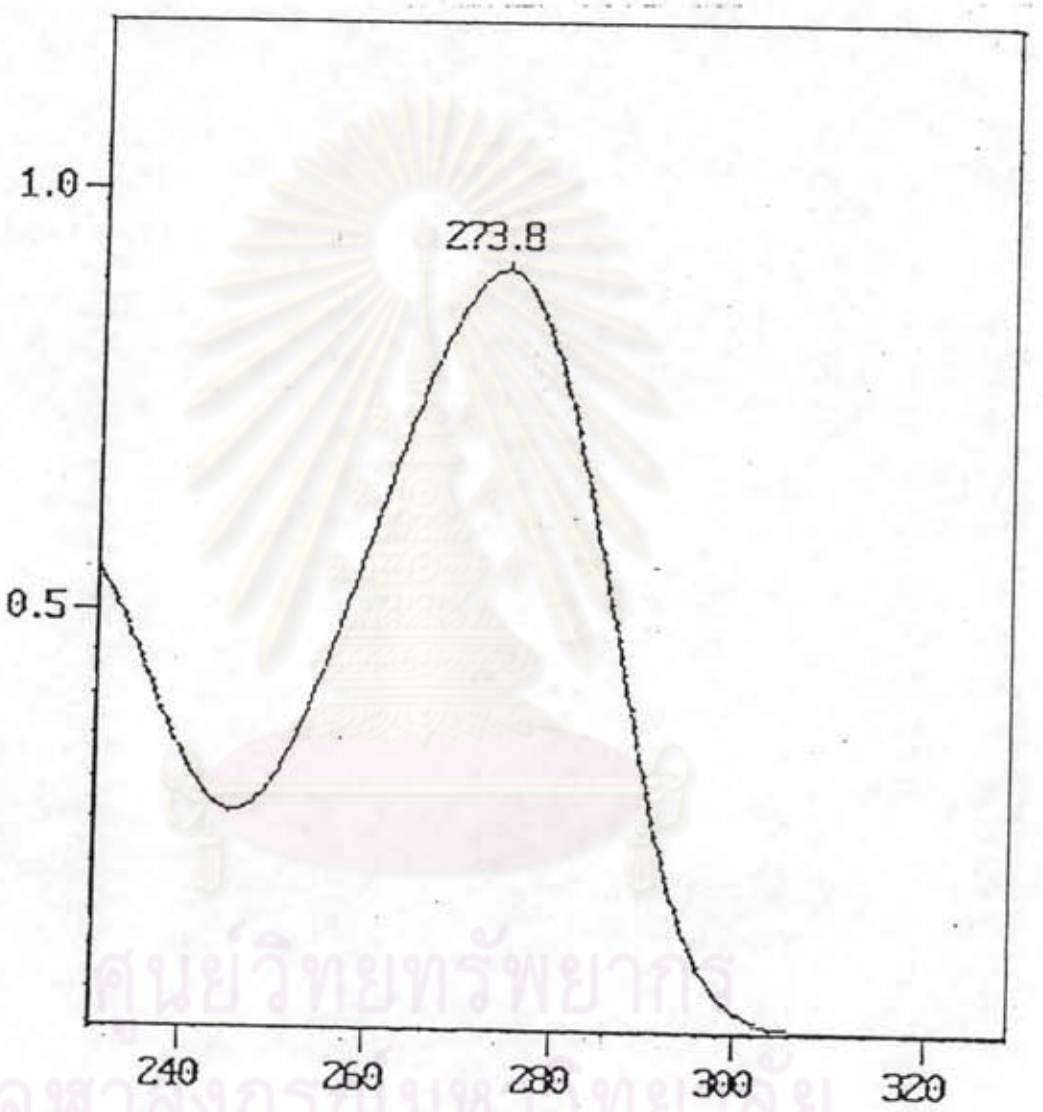


Figure 13 The UV spectrum of 7-(3- carboxybutyl) -1,3-dimethylxanthine aqueous solution concentration 0.03 mg /ml

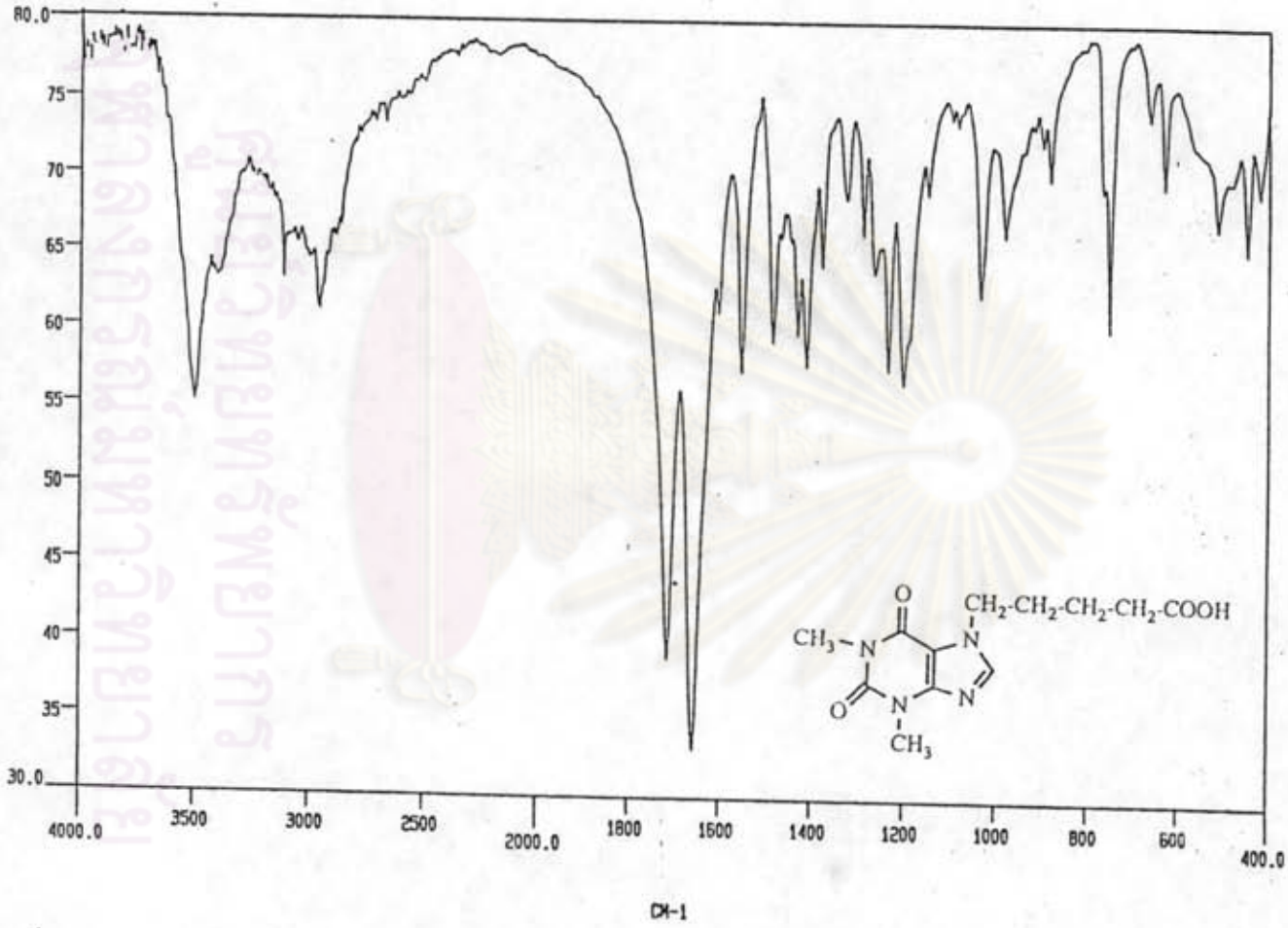


Figure 14 The IR spectrum of 7-(3-carboxybutyl)-1,3-dimethylxanthine

Table 13 Assignment of IR Spectrum of 7-(3-carboxybutyl)-1,3-dimethylxanthine

WAVENUMBERS (cm^{-1})	FUNCTIONAL GROUPS
3498	O - H STRETCHING
2850 - 3030	C - H STRETCHING
1656 , 1712	C = O STRETCHING
1395 - 1440	O - H BENDING
1210 - 1320	C - N STRETCHING

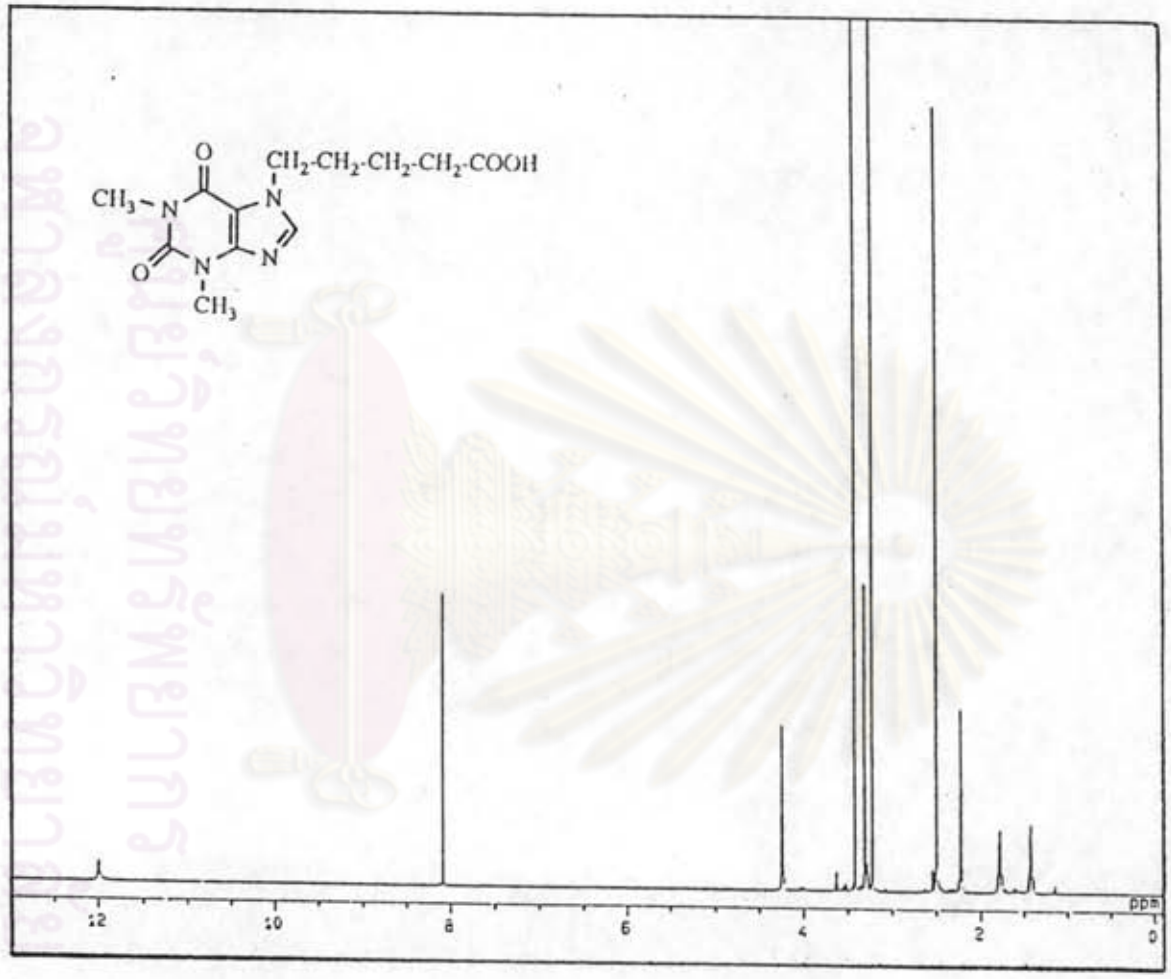
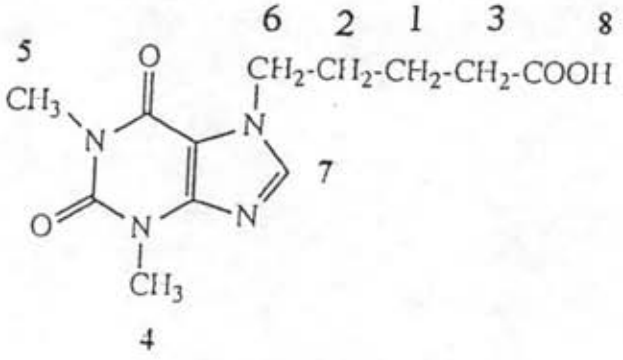


Figure 15 The ^{1}H-NMR spectrum of 7-(3-carboxybutyl)-1,3-dimethylxanthine in DMSO-d_6

Table 14 Assignment of ^{1}H-NMR chemical shift of **7-(3-carboxybutyl)-1,3-dimethylxanthine**

POSITION	CHEMICAL SHIFT	MULTIPLICITY
1	1.38 - 1.44	MULTIPLET , 2H
2	1.74 - 1.80	MULTIPLET , 2H
3	2.20 - 2.23	TRIPLET , 2H
4	3.21	SINGLET , 3H
5	3.40	SINGLET , 3H
6	4.22 - 4.24	TRIPLET , 2H
7	8.07	SINGLET , H
8	11.99	BROAD , H



For NHS ester of 7-(3-carboxybutyl) -1,3-dimethylxanthine , its appearance was white precipitate. The ultraviolet spectrum exhibited the maximum absorption of theophylline at 273 nm (Figure 16).

The IR spectrum of NHS ester of 7- (3- carboxybutyl) -1,3- dimethylxanthine (KBr disk) showed peaks at the following wavenumber ; see Figure 17 and assigned to functional group of structure shown in Table 15.

The ^{1}H-NMR spectrum ($CHCl_3$) as shown in Figure 18, the peaks at these chemical shifts indicated the following proton position shown in Table 16

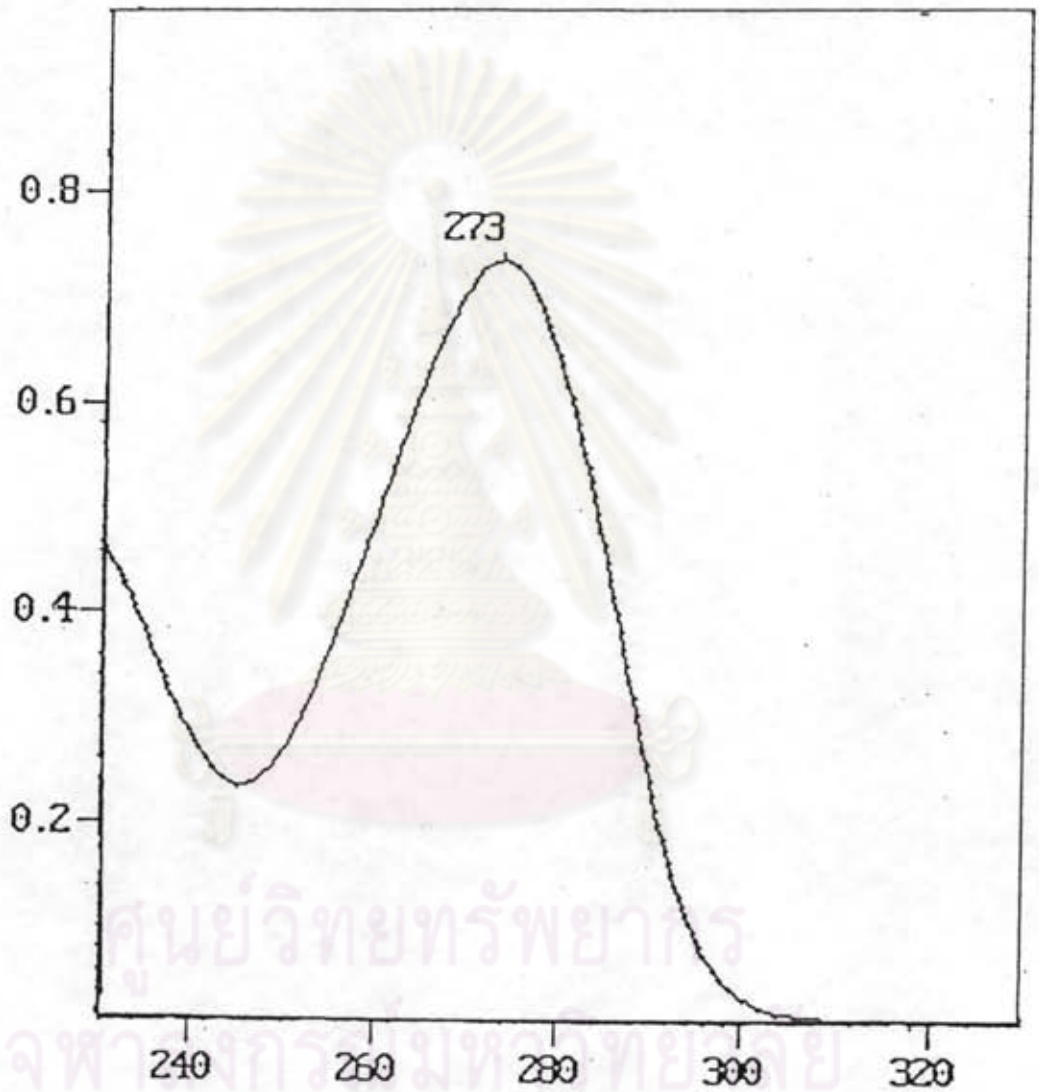


Figure 16 The UV spectrum NHS ester of 7-(3- carboxybutyl) -1,3-dimethylxanthine aqueous solution concentration 0.03 mg /ml

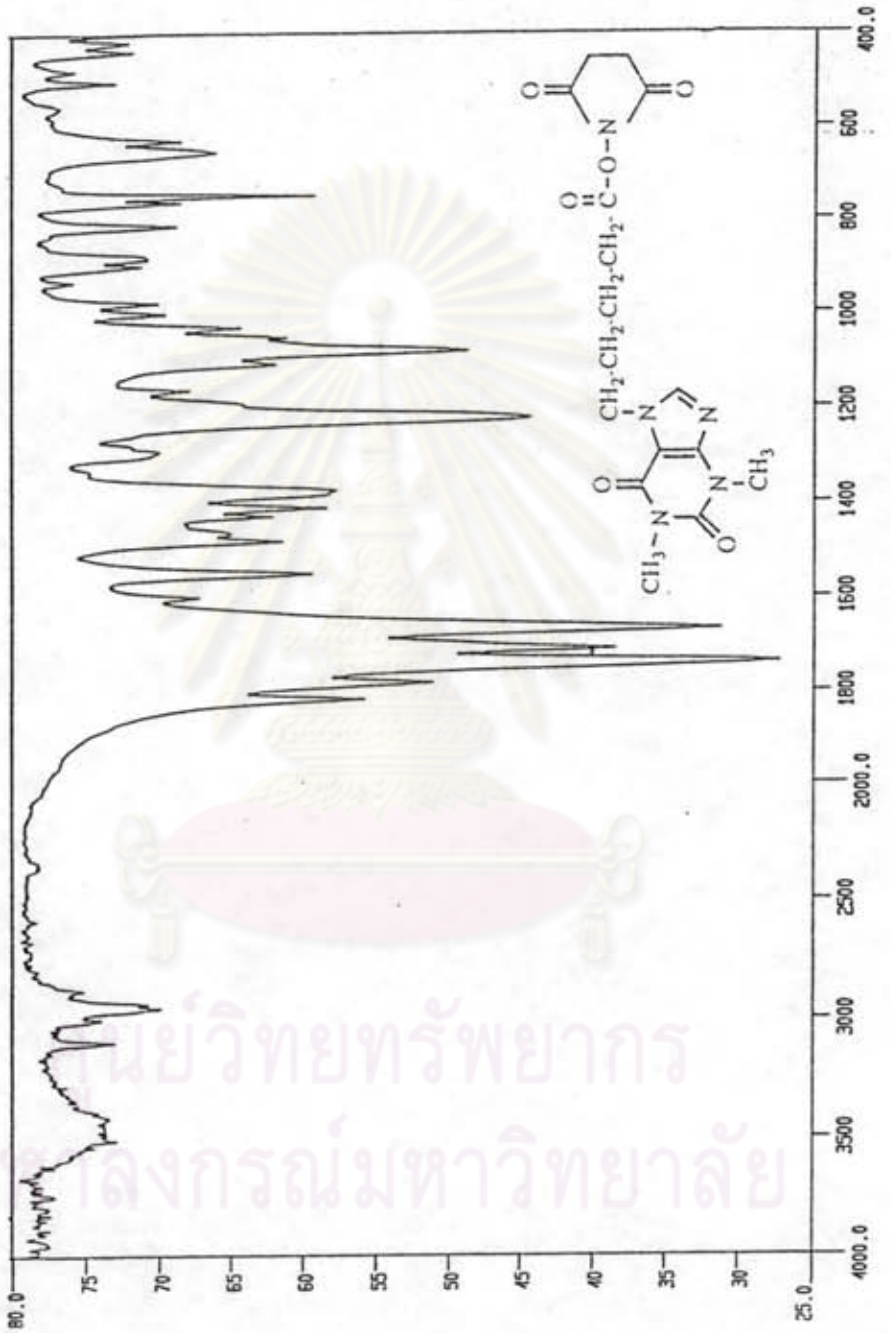


Figure 17 The IR spectrum of NHS ester of 7-(3-carboxybutyl)-1,3-dimethylxanthine

Table 15 Assignment of IR Spectrum of NHS ester of 7-(3-carboxybutyl)-1,3-dimethylxanthine

WAVENUMBERS (cm^{-1})	FUNCTIONAL GROUPS
1079 , 1219	C - O STRETCHING
1700 - 1800	C = O STRETCHING
2900 - 3100	C - H STRETCHING

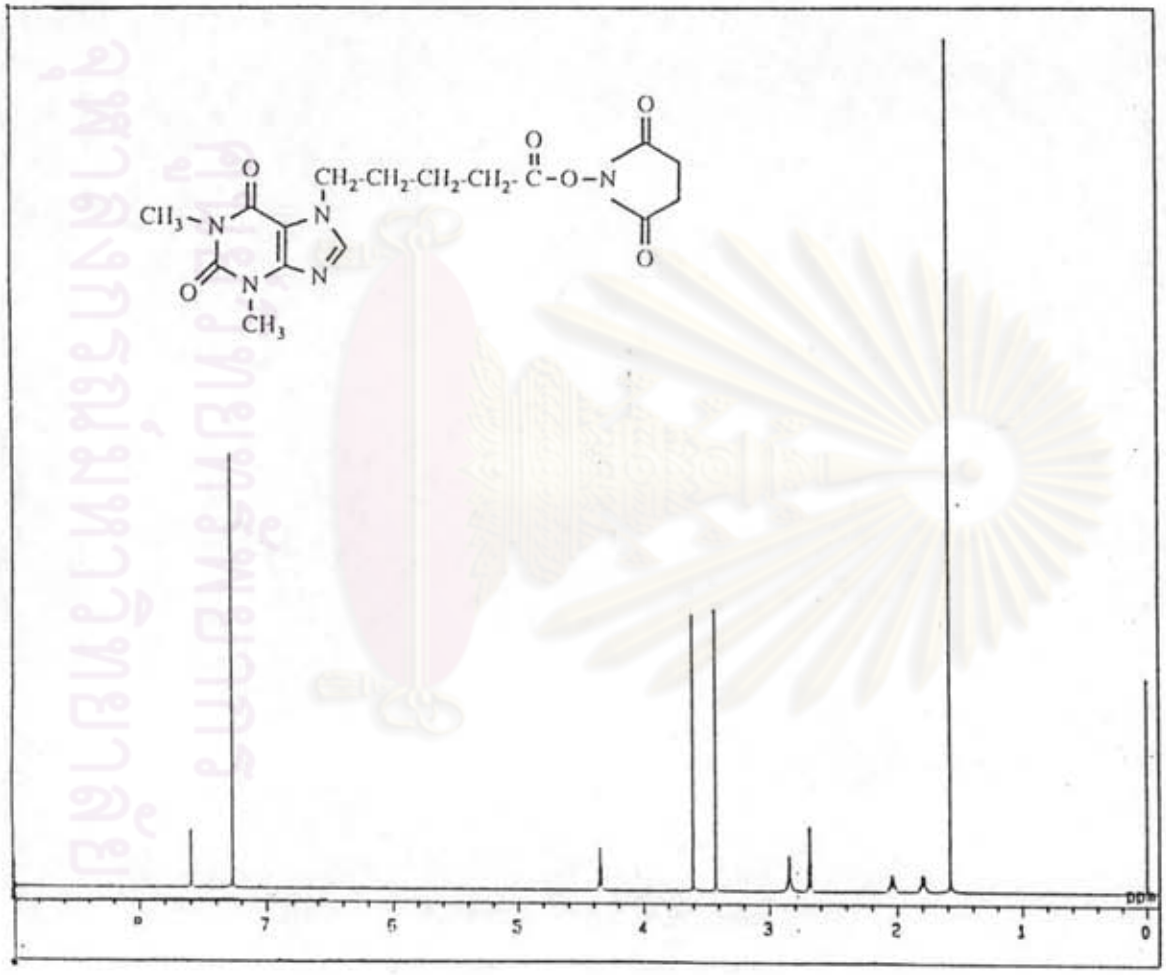
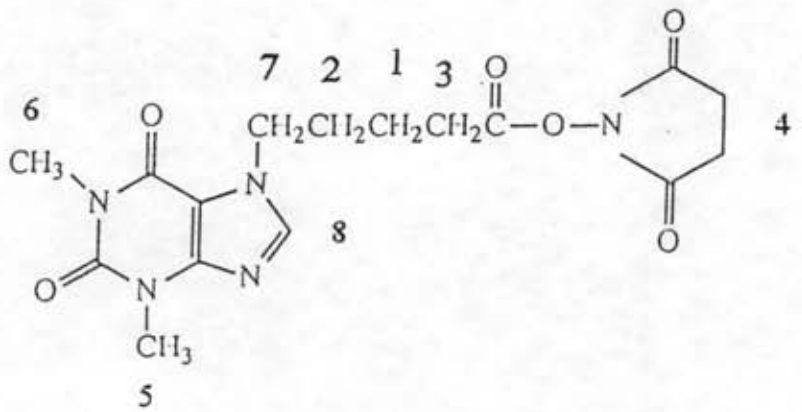


Figure 18 The ^{1}H-NMR spectrum of NHS ester of 7-(3-carboxybutyl)-1,3-dimethylxanthine in $CDCl_3$

Table 15 Assignment of ^{1}H-NMR chemical shift of NHS ester of 7-(3-carboxybutyl)-1,3-dimethylxanthine

POSITION	CHEMICAL SHIFT	MULTIPLICITY
1	1.75 - 1.81	MULTIPLET , 2H
2	2.00 - 2.06	MULTIPLET , 2H
3	2.66 - 2.69	TRIPLET , 2H
4	2.83	SINGLET , 4H
5	3.41	SINGLET , 3H
6	3.59	SINGLET , 3H
7	4.32 - 4.35	TRIPLET , 2H
8	7.58	SINGLET , H



The conjugated product of NHS ester of 7- (3-carboxybutyl) -1,3- dimethylxanthine to BSA as immunogen B was about 58.45 mg The product appeared as white fluffy powder and still retained the ultraviolet absorptivity of theophylline at 274 nm (Figure 19).

The specific absorbance (K) of theophylline in immunogen B was calculated to be 25.42 , according to the data from Table 17.

The calculated mole ratio of theophylline : BSA in immunogen B was 11.3 (Table 18).

The mole ratio of Theophylline to BSA in immunogen B was higher than in immunogen A as clearly shown in Table 12 & 18. This would possibly due to the NHS - ester method (for immunogen B) giving better yield than the carbodiimide method (for immunogen A). However both immunogens were used to induce antibody in rabbits.

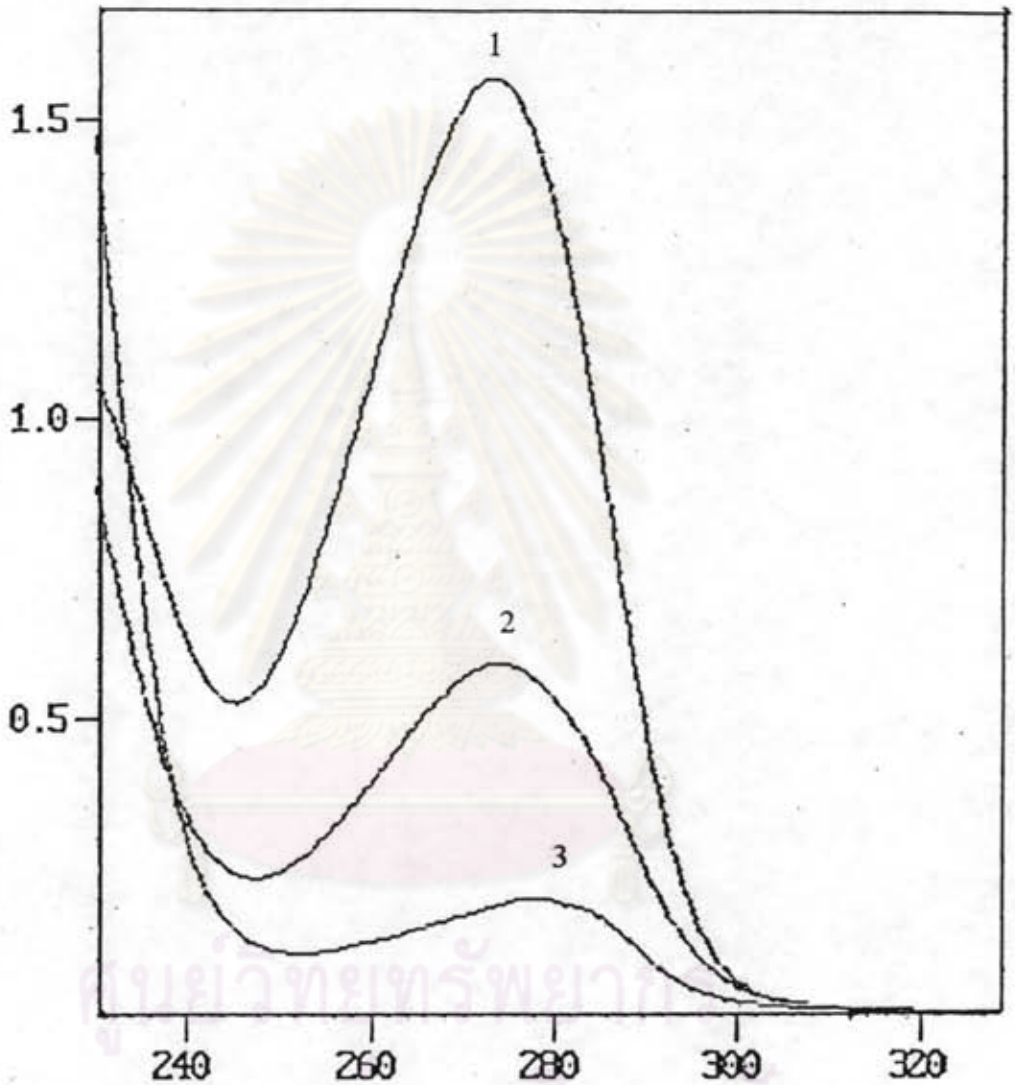


Figure 19 The UV spectrum of Immunogen B aqueous solution concentration 0.286 mg /ml

1 = 7-(3-carboxybutyl)-1,3-dimethylxanthine
2 = theophylline - BSA
3 = BSA

Table 17 Ultraviolet Absorption Data of NHS ester of 7-(3-carboxybutyl)-1,3-dimethylxanthine

CONCENTRATION (mg /ml)	ABSORBANCE 273 nm	THE SPECIFIC ABSORBANCE (K)
0.029	0.739	25.48
0.029	0.726	25.03
0.029	0.747	25.76
	MEAN	25.42

K = absorbance/ (path length * conc. mg./ml.)

Table 18 Determination of Theophylline - BSA
Mole ratio of Immunogen B

CONC (mg/ml)	Abs 277 nm (conjugate)	Abs 277 nm (BSA)	Abs 277 nm (theophylline)	MOLE RATIO OF THEOPHYLLINE TO BSA
0.286	0.599	0.186	0.413	11.3
0.286	0.608	0.186	0.422	11.6
0.286	0.587	0.186	0.401	11.0
			AVERAGE	11.3

$$\text{mole ratio of theophylline to BSA} = \frac{\text{wt. of theophylline in conjugate / mw}}{\text{wt. of BSA in conjugate / mw}}$$

$$\text{wt. of theophylline in conjugate} = \frac{\text{Absorbance of theophylline}}{\text{specific absorbance}}$$

2.2 Induction of anti - theophylline serum in rabbits

Eventhough , a variety of animal species can be used for raising antibody , such as horse , goat , rabbit , this study selected rabbit. Rabbit is considered to be most apprpriate animal in raising antibody for research or laboratory work (Porstmann and Kiessig , 1992 ; Kerr and Thrope , 1994). As ordinarily observed , the immunization schedule used in this study could induce antibody in rabbits with acceptable titer within 4 - 6 weeks. The use of complete Freund's adjuvant in the first dose and incomplete Freund's adjuvant in the second booster can greatly enhanced immune response by releasing the antigen slowly and to speed up the production of antiserum by stimulating the immunological response of animals.

2.3 Study the properties of rabbit anti - theophylline serum

2.3.1 Antibody Titer

As shown in Figure 20 (data also exhibited in appendix C), the titer of antibody produced from immunogen A and immunogen B were 1 : 15,000 and 1 : 65,000, respectively. These titer values imply that less amount of antiserum from antibody B can bind the same amount of HRP labeled theophylline compared to antibody A.

The result of HRP labeled theophylline binding to antibody C in Table 19 clearly indicated the least binding site of antibody C for HRP labeled theophylline comparing to antibody A and B. Since antibody C is commercial available for ready to be used, it may not need any more dilution before using. In the addition, antibody C was produced from immunogen that protein carrier conjugated to the position of C-8 on xanthine structure, while the conjugation of BSA to theophylline derivative was on the C-7 of xanthine. This site heterologous might be the retarding factor that dictated the less binding of HRP labeled theophylline to antibody C. Therefore, antibody C was then excluded from further study.

Figure 20 Antibody dilution curve of antibody A and B

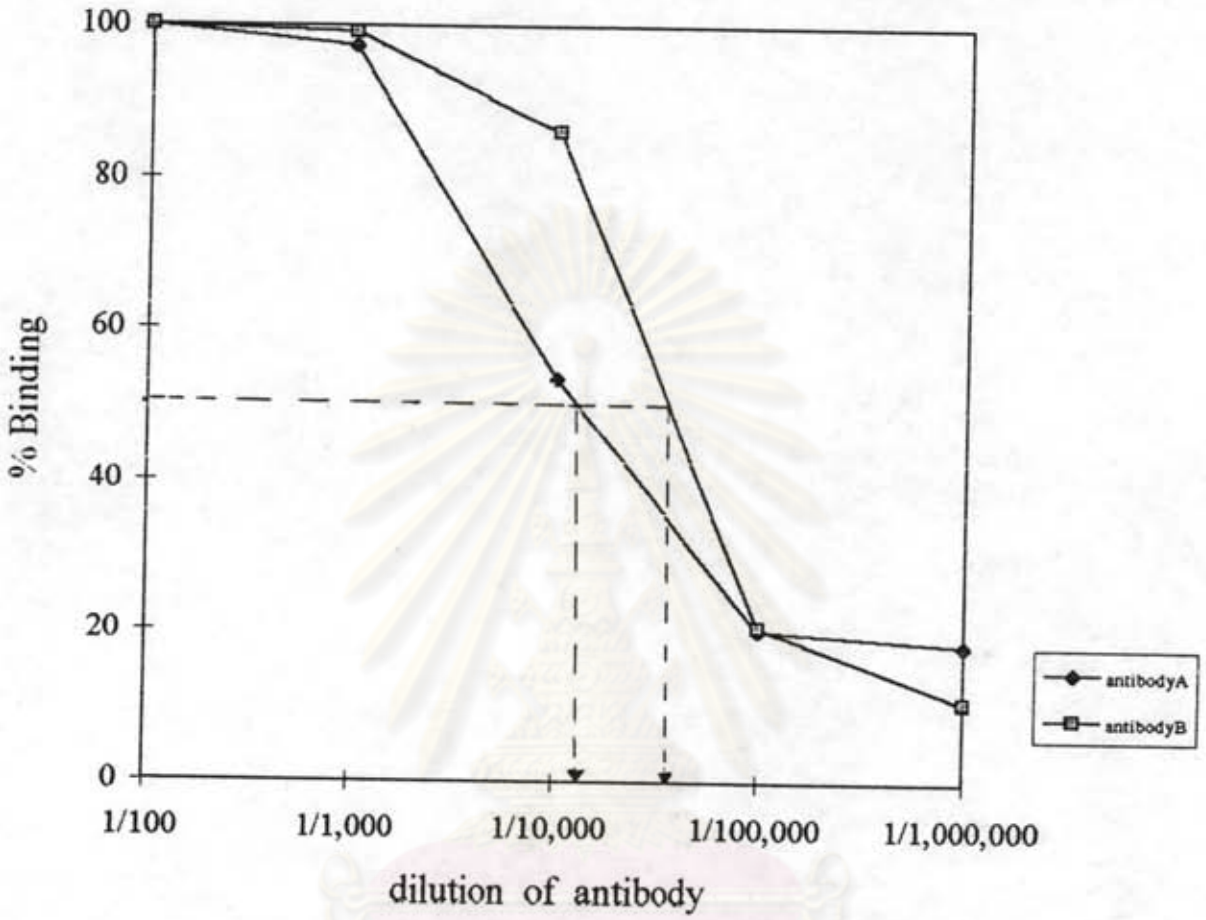


Table 19 The absorbance values of HRP labeled theophylline in binding to different dilution of antibody

antibody dilution	Absorbance at 492 nm		
	immunogen A	immunogen B	immunogen C
undiluted	-	-	0.580
1 : 10	-	-	0.434
1 : 100	1.434	2.220	0.240
1 : 1,000	1.391	2.102	0.231
1 : 10,000	0.764	1.909	0.185
1 : 100,000	0.289	0.460	0.186
1:1,000,000	0.262	0.238	-

Blank absorbance = 0.099

2.3.2 Capability of antibody in binding to HRP labeled theophylline

With fixed amount of HRP labeled theophylline , it' s clearly shown in Table 20 that antibody B was capable in binding to HRP labeled theophylline more than that from antibody A and C . The higher the absorbance observed , the higher the capability of that antibody binding to HRP labeled thophylline. From these results , it would be expected that using different immunogen in binding to the same HRP labeled theophylline required the different amount of HRP labeled theophylline. These results were similar to that reported from Weemen and Schuurs , 1975 ; Tsuji , 1980 ; Hosoda et. al., 1981 ; Kamaoka et. al., 1984 that antibody induced from immunogen with different derivative of hapten from enzyme labeled hapten could contain higher binding sites for enzyme labeled hapten than that antibody produced from immunogen with the same derivative of hapten to the preparation of enzyme labeled hapten.

For antibody C , it was marketed to be used for RIA , 100 mcl for one testing . In this study , although the same protein concentration of antibody A , B and C were

coated to the microplates , the capability of antibody C in binding to HRP labeled theophylline was lowest. This would also possibly due to the different of theophylline derivative used in immunogen and HRP labeled theophylline as already explained in 2.3.1 .

Table 20 Capability of antibody A , B and C in binding to HRP labeled theophylline

antibody	Absorbance value at 492 nm				
	1	2	3	4	MEAN
A	0.988	0.961	0.953	0.967	0.967 (SD=0.015)
B	1.813	2.081	1.947	1.970	1.953 (SD=0.110)
C	0.242	0.246	0.244	0.233	0.242 (SD=0.006)

Blank absorbance = 0.093

2.3.3 Specificity

The result from Table 21 indicated that with theophylline concentrations usually detected within the therapeutic range of theophylline , caffeine was able to be detected only when its concentration was as high as 748 and 290 mg/l for antibody A and B , respectively. As it was reported that peak caffeine concentrations in human serum after ingesting of 100 mg of caffeine , was 1.9 $\pm$ 0.5 mg/l (Grab and Reinstein , 1968 ; Cook et. al., 1976 ; Merriman et. al., 1978). It is therefore concluded that within the range of theophylline concentration detected , caffeine will not significantly interfere. In the other hand , antibody A and B are not specific for caffeine or no cross reaction of caffeine to theophylline.

Table 21 Cross Reactivity of anti - theophylline serum with caffeine

Drug	Mean of conc. of at drug giving 50 % competitive binding (mg/l) antibody A	Mean of conc. of at drug giving 50% competitive binding (mg/l) antibody B
Theophylline (n = 2)	15.2	15.0
Caffeine (n – 2)	748	290
% cross reactivity	2.03	5.16

cross reactivity of antibody C obtained from Sigma is 4%

3. Determination of HRP labeled Theophylline Properties in Enzyme Immunoassay of Theophylline

3.1 Determine the appropriate dilution of antibody for competitive reaction

The relationship between the absorbance and the theophylline concentration at different dilution antibody was shown in Figure 21 and 22 for antibody A and B , respectively. These relationship were not linear. While the absent theophylline , the absorbance could indicated that binding to HRP labeled theophylline in which the higher the antibody to bind to HRP labeled theophylline , the higher the observed absorbance. When theophylline concentration increased , competitive binding of theophylline with HRP labeled theophylline to the antibody site was not proportional to the amount of antibody. Thus these curve were not linear.

For constructing the logit - log curve provided to more linearity as shown in Figure 23 and 24 for antibody A and B , respectively and then the result from Table 22 indicated that the correlation coefficient (r) at the dilution 1 : 1,000 of antibody A and dilution 1 : 10,000 of antibody B provided the highest correlation coefficient. Therefore ,

the most appropriate dilution of each antibody represented curve from linear regression analysis as shown in Figure 25 and 26 for antibody A and B, respectively.

Figure 21 Dose response curve for Theophylline at different dilution of antibody A

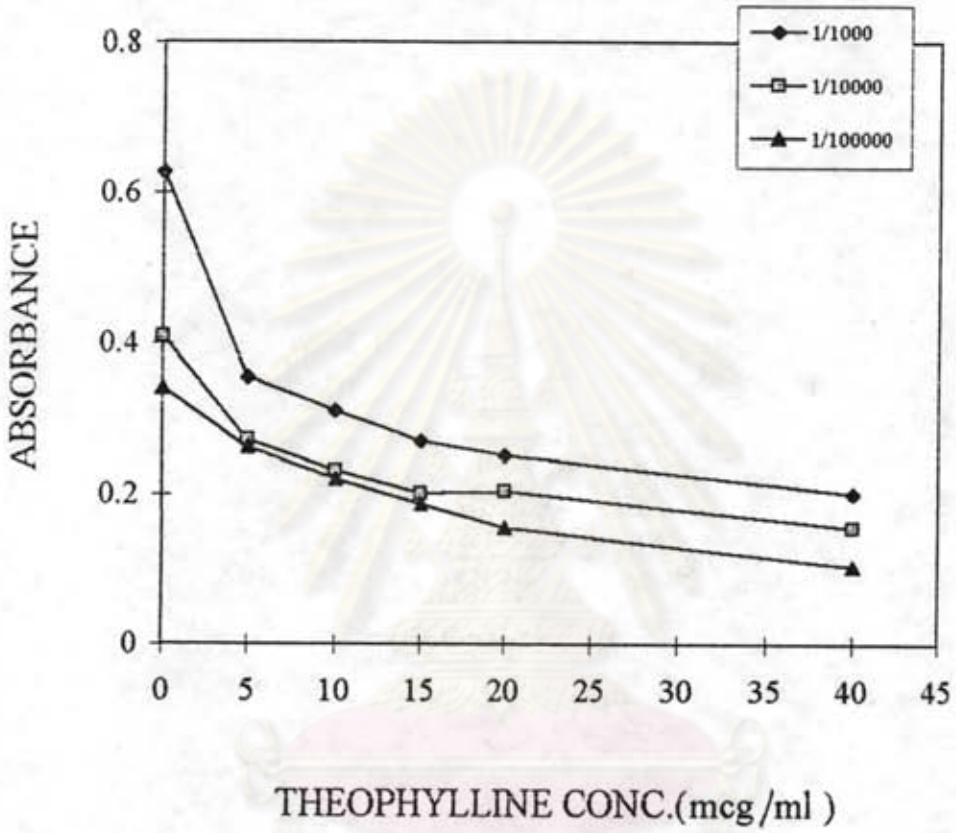


Figure 22 Dose response curve for Theophylline at different dilution of antibody B

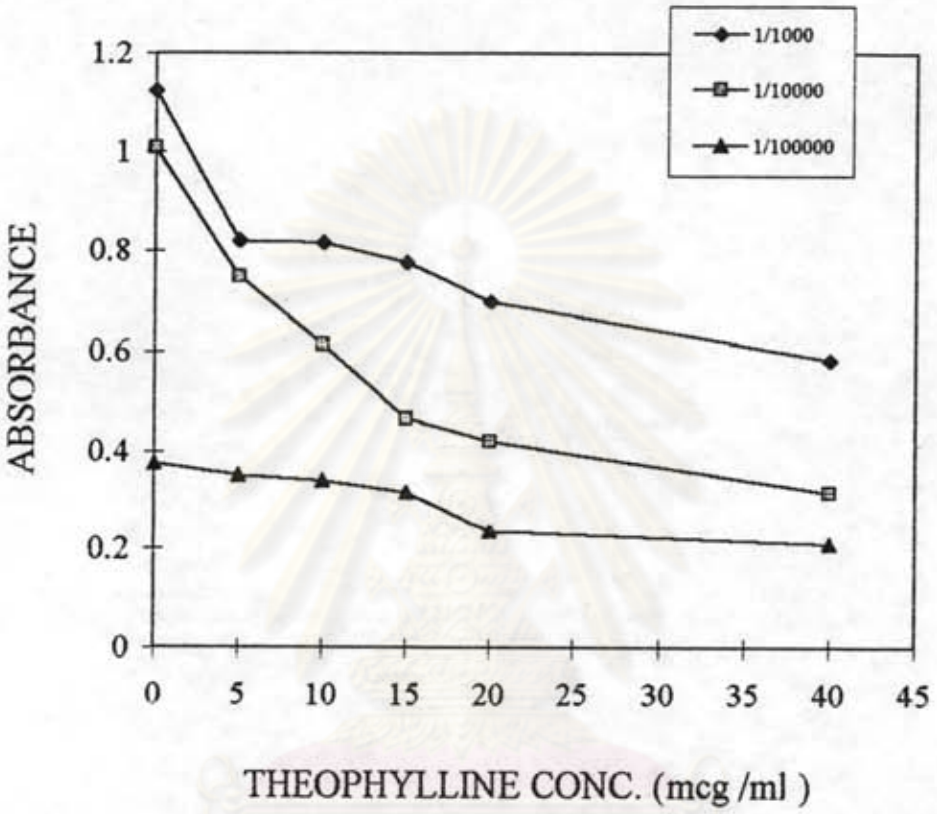


Figure 23 A logit-log plot of data from figure 21

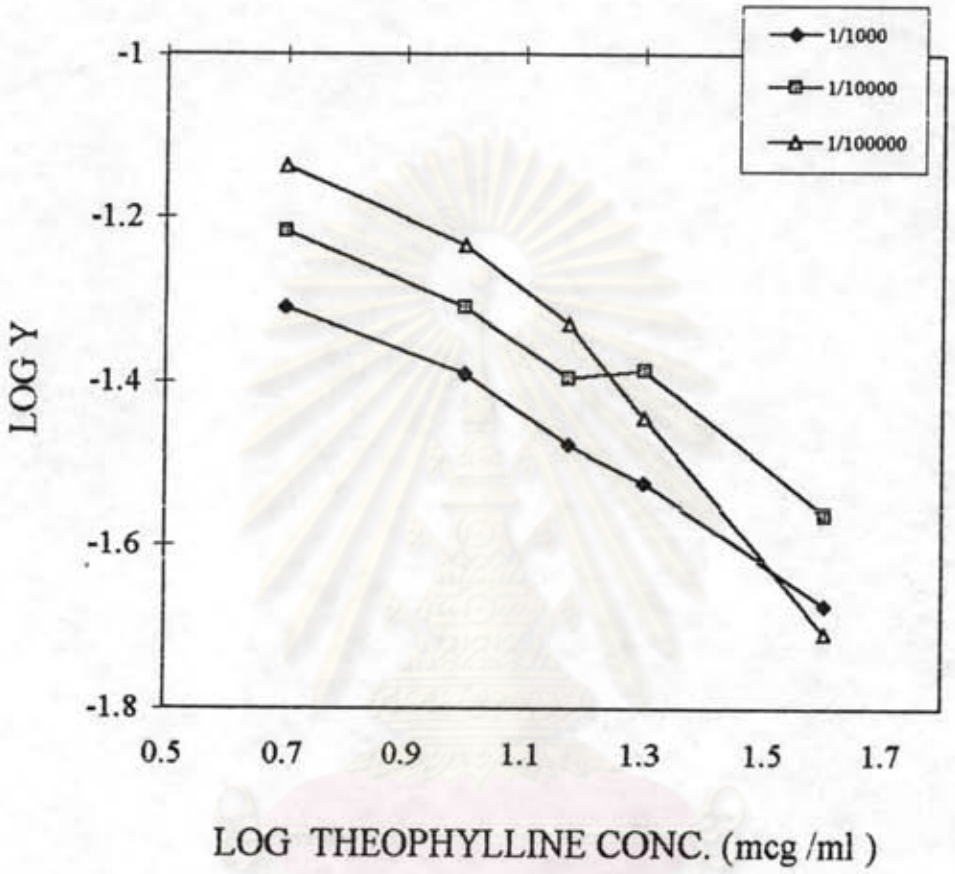


Figure 24 A logit-log plot of data from figure 22

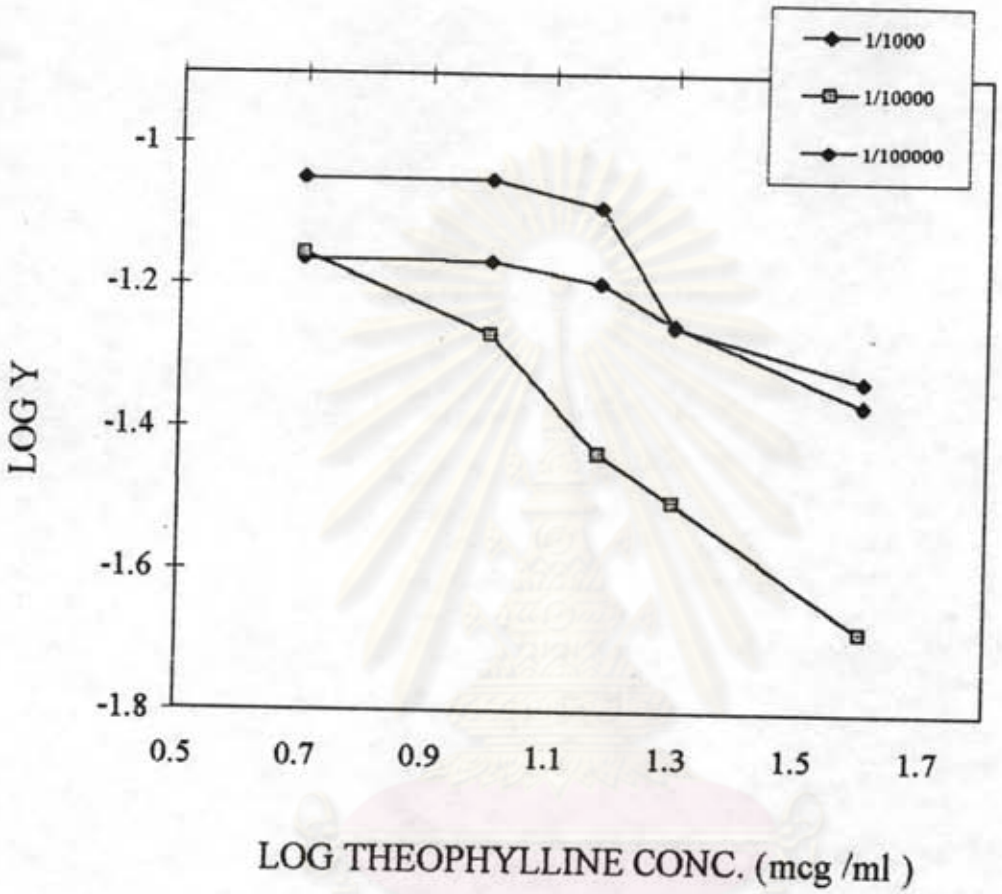


Table 22 The value of Correlation Coefficient from Linear Regression Analysis of each antibody dilution

Type of immunogen	dilution of antibody		
	1 : 1000	1 : 10,000	1 : 100,000
A	0.991	0.983	0.971
B	0.908	0.991	0.895

Figure 25 A represent curve from linear regression anlysis of antibody A at antibody dilution 1:1000

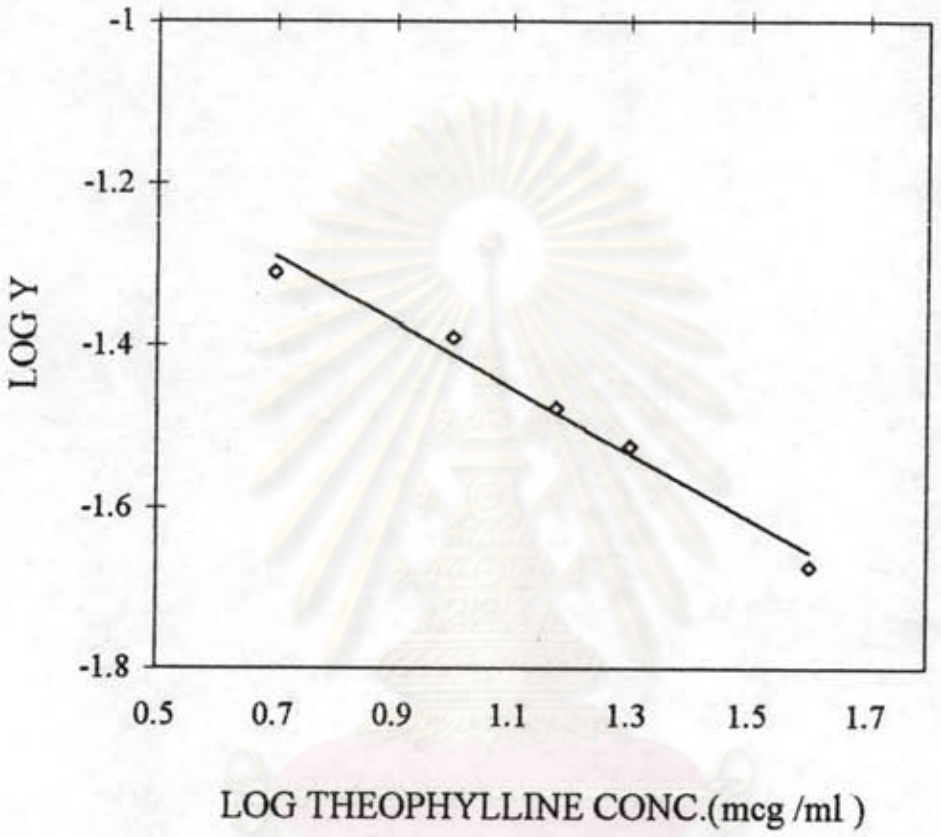
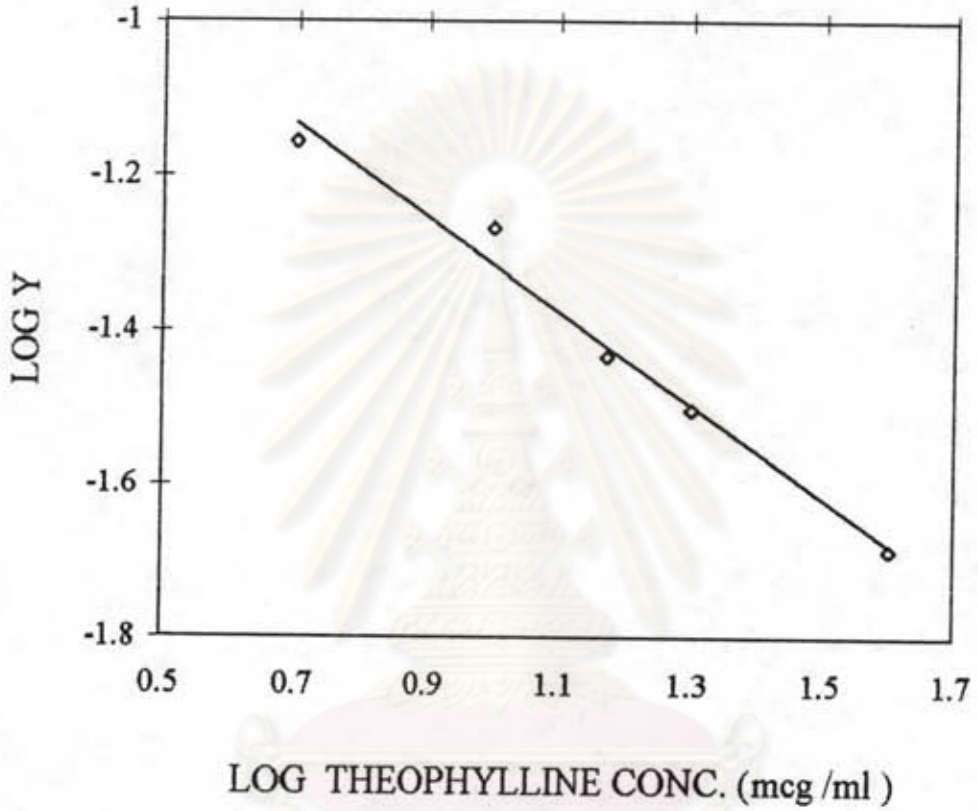


Figure 26 A represent curve from linear regression analysis of antibody B at antibody dilution 1 : 10,000



3.2 Determine the appropriate dilution of HRP labeled theophylline for competitive reaction

The relationship between the absorbance and the theophylline concentration at different dilution HRP labeled theophylline was shown in Figure 27 and 28 for antibody A and B , respectively. These relationship were not linear. At the same dilution of each antibody were coated to the microplates. When the HRP labeled theophylline at the high dilution (1 : 10,000) was used in competitive reaction , theophylline could bind to overall antibody sites. In the practically , The high dilution of HRP labeled theophylline was not appropriated because it could not detected to cover the usual theophylline concentration administration (0 - 40 mg/l). For at the dilution 1 : 1,000 and 1 : 5,000 of HRP labeled theophylline the observed absorbance was rather high which theophylline could be detected to cover the usual range of theophylline concentration.

For constructing the logit - log curve provided to more linearity as shown in Figure 29 and 30 for antibody A and B , respectively and then the result from Table 23 indicated that the correlation coefficient (r) at the dilution

1 : 5,000 of both antibody A and B of HRP labeled theophylline provided the highest correlation coefficient . Therefore , the most appropriate dilution of HRP labeled theophylline for competitive reaction represented curve from linear regression analysis as shown in Figure 31 and 32 for antibody A and B , respectively.

Figure 27 Dose response curve for Theophylline at different dilution of HRP labeled theophylline in the competition of antibody A

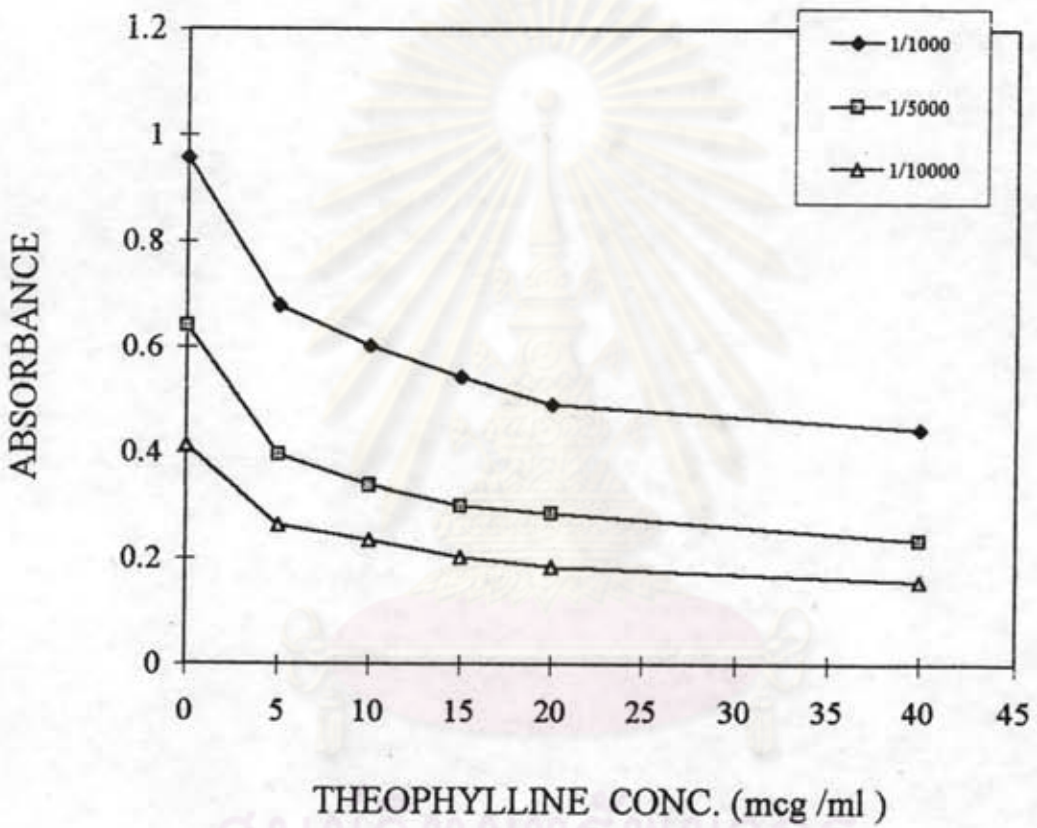


Figure 28 Dose response curve for theophylline at different dilution of HRP labeled theophylline in the competition of antibody B

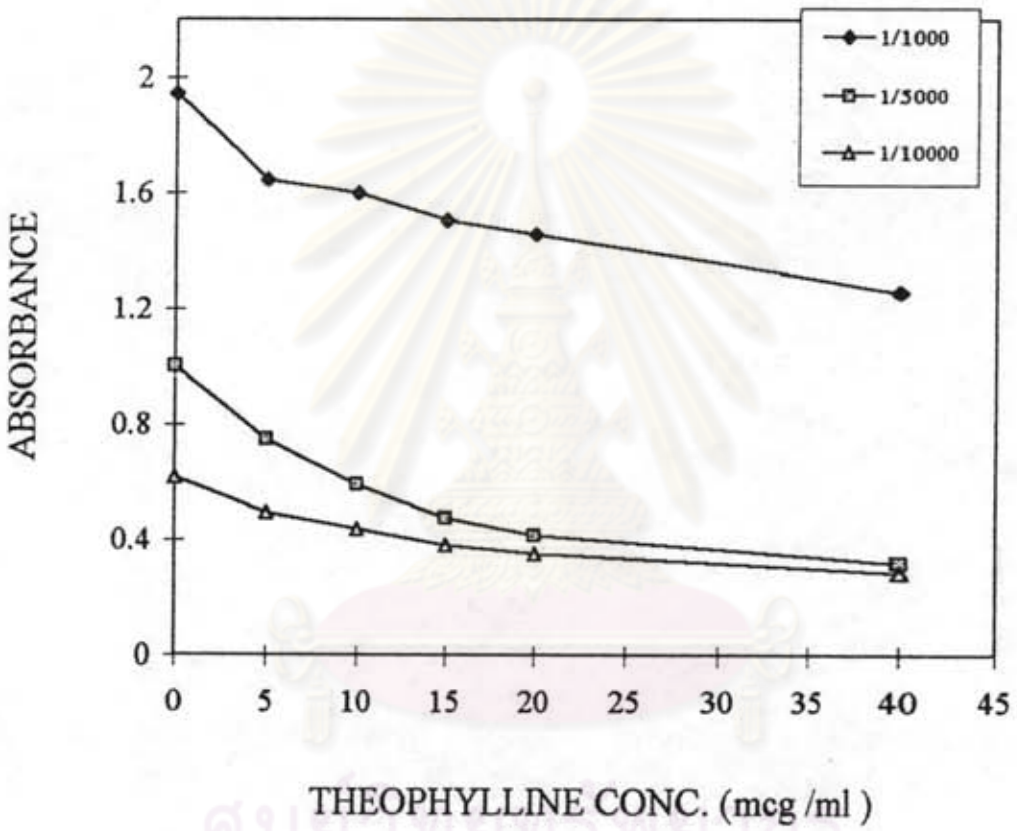


Figure 29 A logit-log plot of data from figure 27

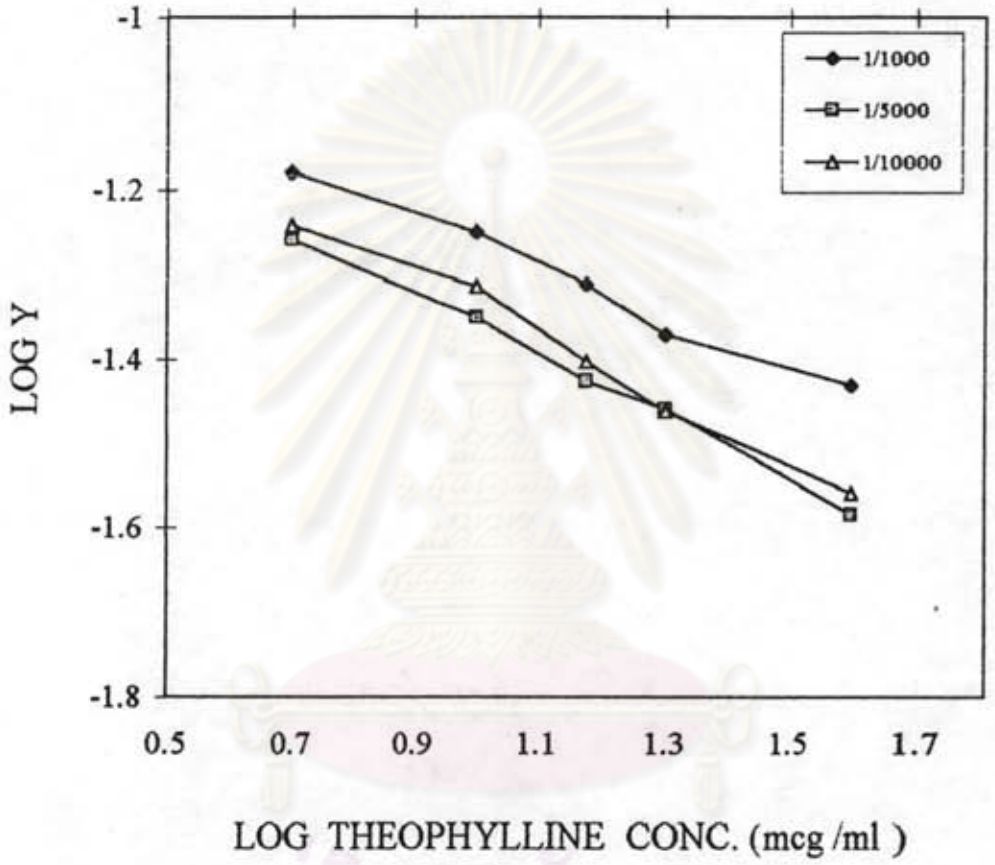


Figure 30 A logit-log plot of data from figure 28

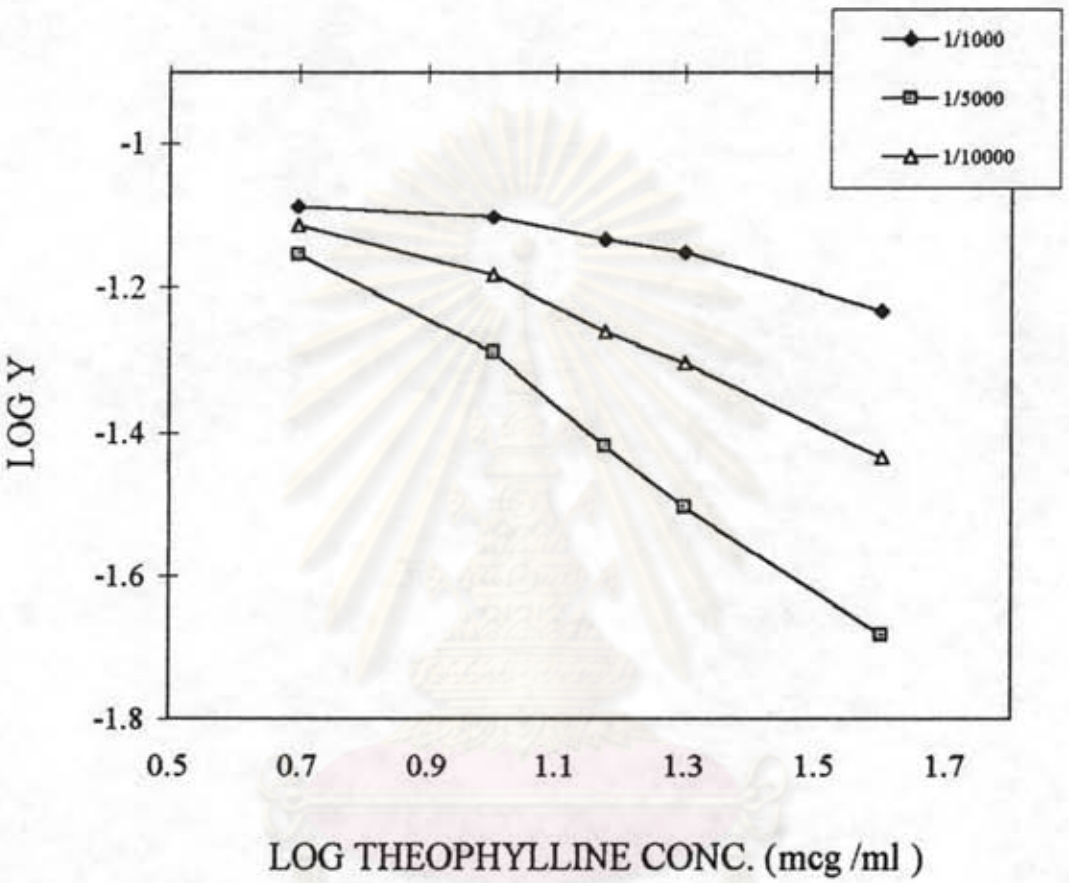


Table 23 The value of Correlation Coefficient from Linear Regression Analysis at the different dilution of HRP labeled theophylline

Type of immunogen	dilution of HRP labeled theophylline		
	1 : 1,000	1 : 5,000	1 : 10,000
A	0.991	0.998	0.992
B	0.948	0.996	0.979

Figure 31 A represent curve from linear regression analysis of antibody A at HRP labeld thephylline dilution 1:5000

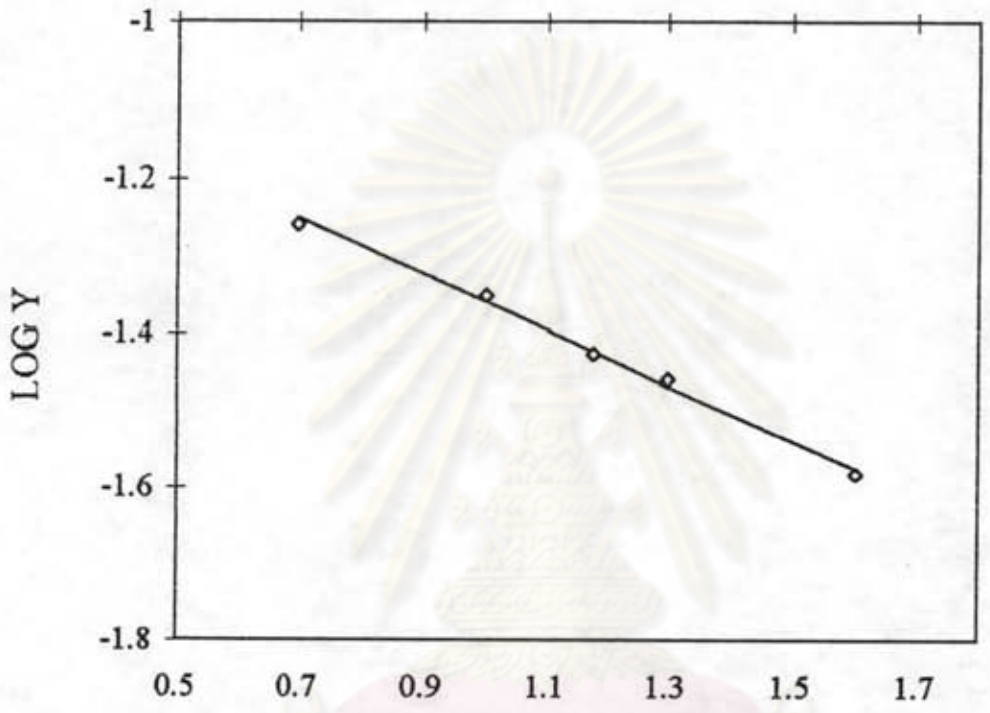
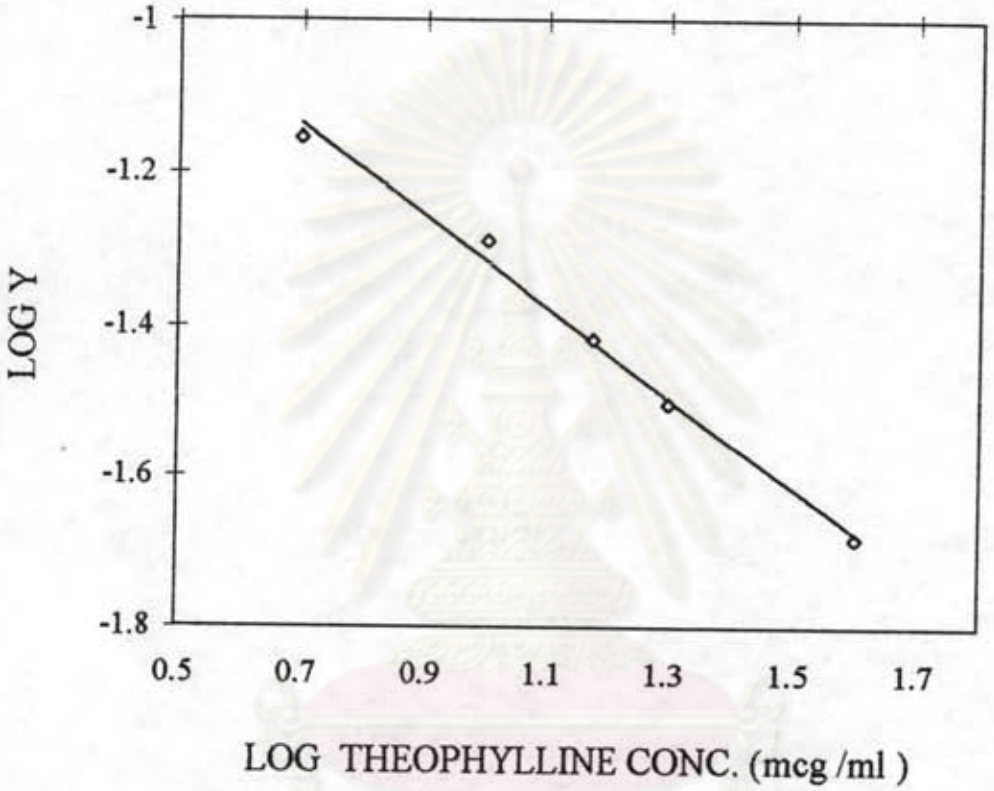


Figure 32 A represent curve from linear regression analysis of antibody B at HRP labeled theophylline dilution 1:5000



3.3 Study the competition between HRP labeled theophylline and theophylline in the sample

The relationship between the percentage of binding and the theophylline concentration of antibody A and B was shown in Figure 33 and 34 for antibody A and B , respectively. These relationship were not linear. While the absent theophylline , the percentage of binding could indicated that maximum binding of antibody to HRP labeled theophylline in which the theophylline concentration increased , the percentage of binding decreased was not proportional to the theophylline concentration. Thus these curve were not linear.

In general , standard curve was available to determine theophylline in sample for enzyme immunoassay provided the linear curve. Therefore , this study constructed the logit - log curve in which provided to more linearity and then the represented standard curve from linear regression analysis with the value of correlation coefficient to be 0.995 and 0.991 as shown in Figure 35 and 36 for antibody A and B , respectively.

Figure 33 Standard curve for theophylline of antibody A at HRP labeled theophylline 1 : 5,000 and antibody 1 : 1,000

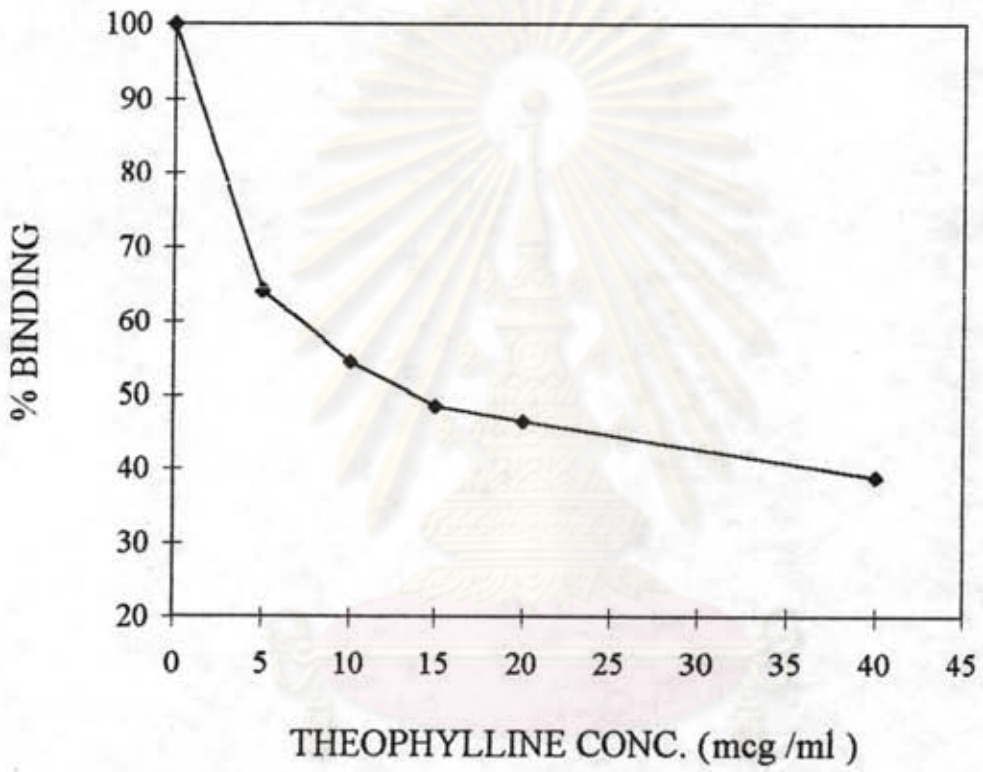


Figure 34 Standard curve for theophylline of antibody B at HRP labeled theophylline 1 : 5,000 and antibody 1 : 10,000

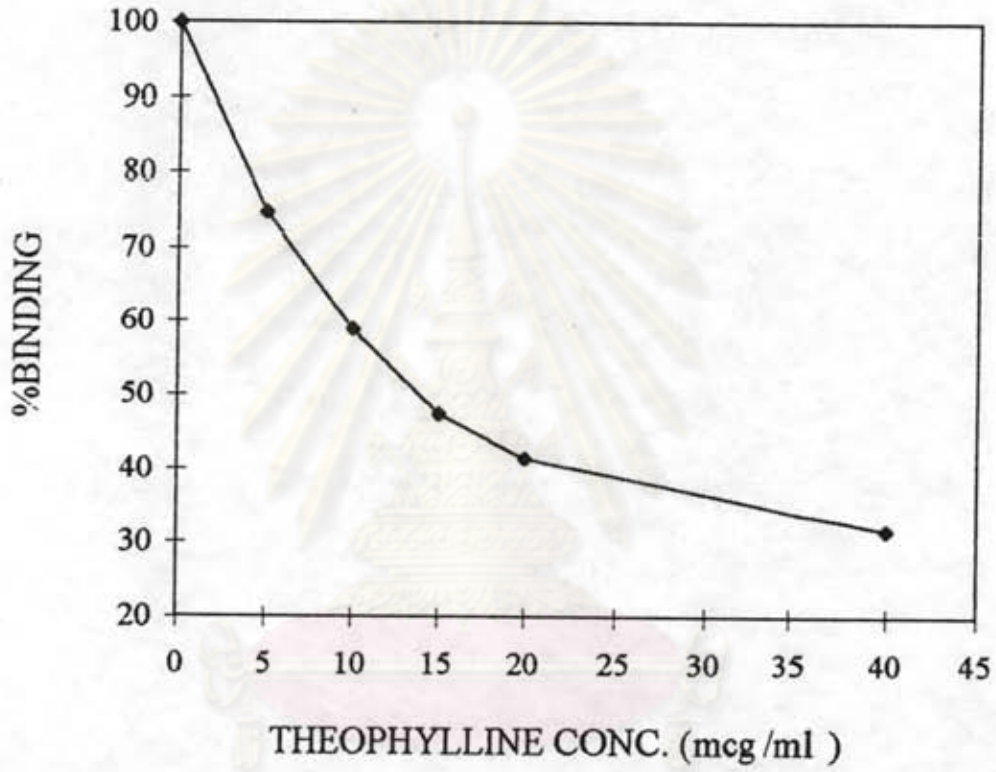


Figure 35 A logic plot of standard curve from linear regression analysis for theophylline of antibody A at HRP labeled theophylline 1:5,000 and antibody dilution 1:1,000

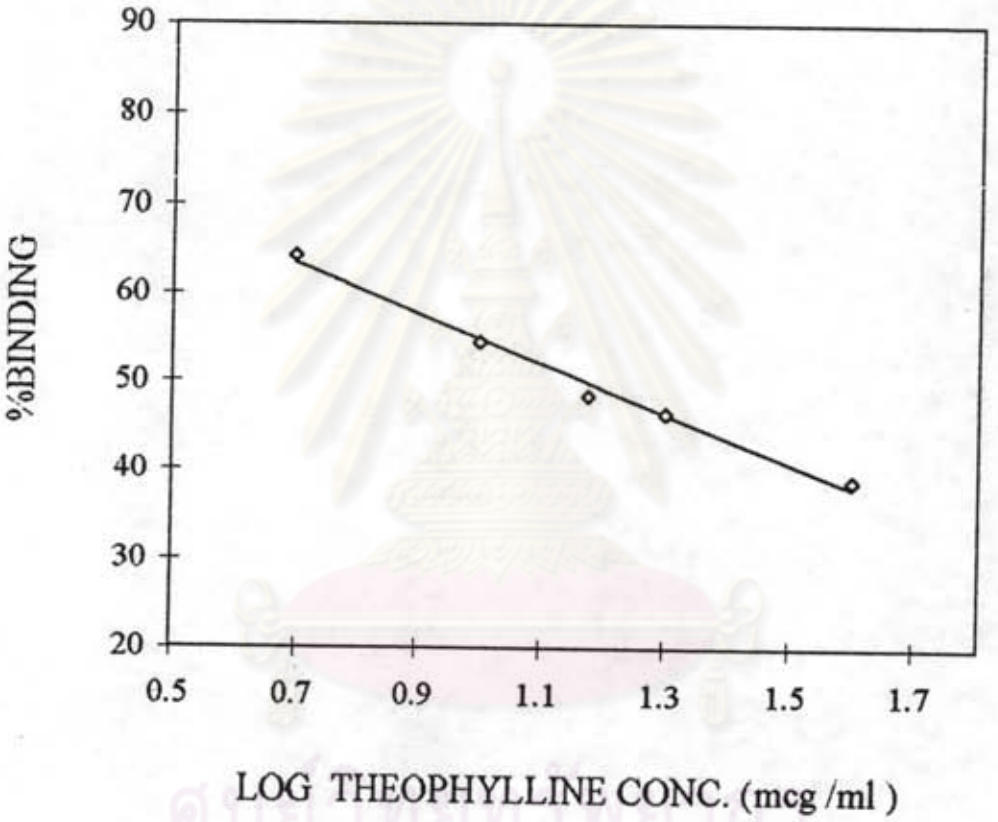
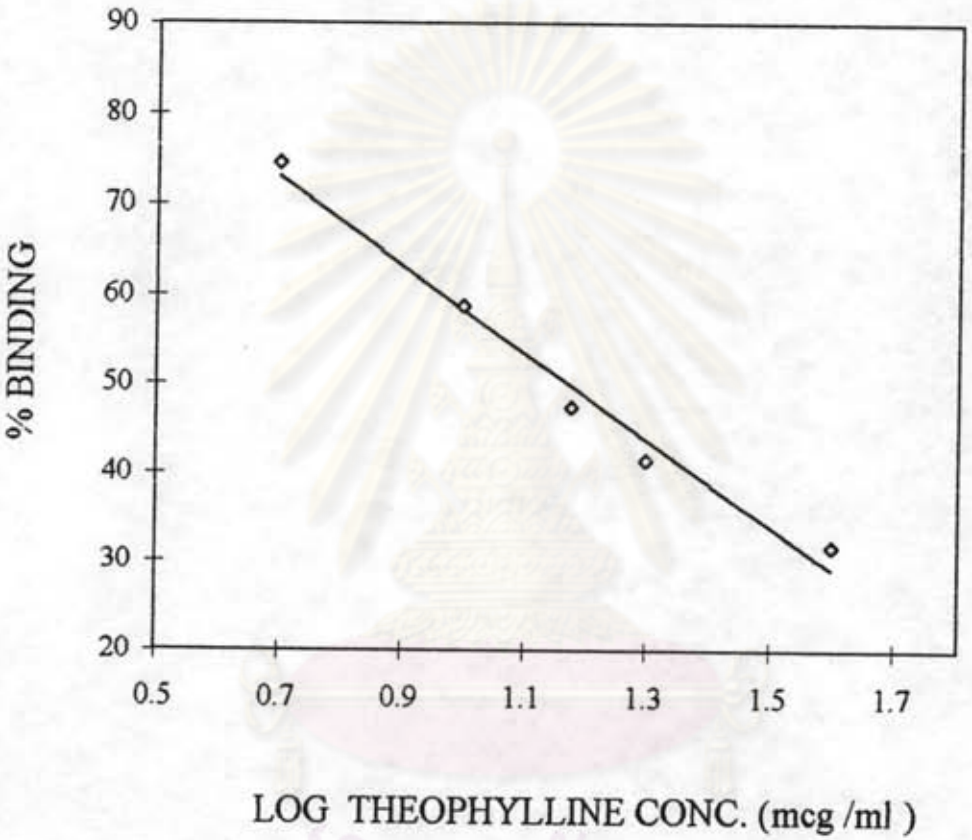


Figure 36 A logic plot of standard curve from linear regression anlysis for theophylline of antibody B at HRP labeled theophylline 1:5,000 and antibody dilution 1:10,000



The obtained standard curve indicated that the dilution of antibody and HRP labeled theophylline used in this study that covered the theophylline concentration range in sample.

The value of slope of each standard curve were 28.0 and 48.6 for antibody A and B, respectively. The result indicated that the sensitivity of antibody B was higher than antibody A . This would possibility due to the use of different theophylline derivative in the preparation of immunogen and HRP labeled theophylline. These results were similar to that reported by Tsuji , 1980 ; Weemen and Schuurs , 1975 ; Hosoda et. al. , 1981 Kamaoka et. al., 1984. Therefore , antibody B would possibly provided the higher sensitivity of the analytical result than antibody A . Nevertheless in this point, it was suggested that more study should be investigated.