CHAPTER II

METERIALS AND METHODS

Chemical compound and reagents

Theophylline and 2,2 - oxybis (ethylamine) were obtained from Aldrich Chemical Co. (Milwaukee, WIS, USA). Ethyl -4-bromobutylate , ethyl -5- bromovalerate , N- hydroxysuccinimide , Sodium borohydride , 1- ethyl 3-(3- dimethylaminopropyl) carbodiimide hydrochloride , horseradish peroxidase (type VI) , sodium metaperiodate ($NaIO_4$) , trinitrobenzenesulfonic acid , bovine serum albumin , and theophylline antiserum were received from Sigma Chemical Company (Montana , USA). O-phenylenediamine was from Zymed Laboratories , INC (San Fransico , USA). Complete Freund's adjuvant and Incomplete Freund's adjuvant were obtained from Difco (Detroit , Mich., USA) Sodium acetate , sodium bicarbonate , sodium chloride , sodium dihydrogenphosphate and disodium hydrogen phosphate were from E. Merck , Damstadt , Germany. Thimerosal is obtained from Keck's (San Fransico , USA) Tween 20 was from Merck - Schuchardt (USA).

All reagents were of analytical grade and used as received. They were all ordered from E. Merck, Damstadt, Germany. They were dimethylformamide, ethanol, ethylacetate, chloroform, methanol, hydrochloric acid, glacial acetic acid, sulfuric acid, hydrogenperoxide except glycerol was ordered from Farmitalia carlo erba.

Instrumentation

Instruments employed in this study were Nuclear Magnetic Resonance Spectrophotometer (Model JNM - 500, Joeol limited) Japan ; Infrared Spectrophotometer (Perkin - Elmer model FT - IR) ; Ultraviolet Spectrophotometer (Spectronic' 3000 Array, Milton Roy Co.) ; Buchi Capillary Melting point apparatus (Nach Dr. Tottoli, Buchi) Switzerland ; Analytical Balance (Satorious type, WRC 6001, Satorious - Werke GMBH) Germany ; pH - Meter (consort pH Meter P 307) Germany ; Vortex Mixer (Vortex - Genei, Scientific Industries Inc.) New York, USA ; Centrifuge (Labofuge, Heraeus - Christ GMBH) West Germany ; Micropipet (Socorex) USA ; Dura - Dry Freezed Dryer and Just - A - Tilt Shell Freezer ; Microplate reader (ELISA plate reader) ; Dialysis tubing (Spectra / Por$^{(R)}$).

METHOD

In order to succeed the main objective of this study, Three important works were got through. They were

1. To synthesize HRP labeled theophylline.

2. To prepare immunogen and antiserum to theophylline.

3. To determine competitive binding properties of synthetic HRP labeled theophylline.

1. Synthesis of HRP Labeled Theophylline.

1.1 To prepare HRP ready for conjugation.

HRP could be conjugated to hapten through its 2 - 4 amino groups per molecule (Tijssen and Kurstak , 1984 , Tijssen , 1985). To ensure enough amino groups in coupling to hapten , the aminated HRP was then prepared via the modified procedure of Wilson and Nakane (Wilson and Nakane ,1978).

Procedure

Slowly added 0.5 ml of freshy prepared 0.1 M sodium metaperiodate solution to 50.0 mg of HRP in 5.0 ml of 0.01 M sodium acetate , pH 4.5 . Allow the mixture to react in the dark for 30 minutes at room temperature. Then 0.4 ml of 1.0 M glycerol was added and stand for 30 minutes. Separate the oxidized enzyme from free periodate by dialyzed overnight at 4° c against 0.01 M sodium acetate at pH 4.5 . Then , slowly added to 1.0 ml of 1.0 M 2,2 - oxybis (ethylamine) , pH 9.5 , allowed to react for 3 hrs , at 4° c. A 0.5 ml of freshy prepared 1.0 M sodium borohydride was added , allowed to react for 2 hrs , at 4° c. Separate the aminated HRP from free amine by dialyzed overnight at 4° c against

0.1 M sodium carbonate, pH 9.5, at 4° c. The product was lyophilized and the number of amino groups attached to HRP was determined using ultraviolet spectrophotometer. Trinitrobenzenesulfonic acid (TNBS) was used as coloring agent and observing the absorption at 354 nm (Satake and Okuyama , 1960 ; Habee , 1966) as following :

Aminated HRP 4 mg/ml solution was added to the mixture of 1.0 ml of 4% sodium bicarbonate and 1.0 ml of 3 mcmol/ml trinitrobenzenesulfonic acid (TNBS). The solution was mixed and allowed to react in the dark at 4° c. After 2 hrs, the resulting orange - colored solution was added to 0.5 ml of 1 N hydrochloric acid and the absorbance was measured at 354 nm Vs. distilled water as a blank. The number of amino groups can be calculated from the molar extinction coefficient of amino groups in HRP.

1.2 To prepare theophylline ready for conjugation

Synthesis of theophylline derivatives

To conjugate theophylline with enzyme HRP , both compound have to be modified to exert the appropriate conjugation. As already mentioned , HRP was aminated to increase the amino group directed for conjugation. For theophylline , the carboxyl containing analogue was synthesized and this analogue can be directly conjugate to HRP. In this study , theophylline analogue in the form of butyric acid was synthesized according to the modifying method of Hu and Singh , 1980.

Procedure

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

A mixture of 1,3 - dimethylxanthine (theophylline) (1.802 gm , 10 mmole) , sodium carbonate (2.150 gm , 20.3 mmole) and ethyl 4- bromobutyrate (3.901 gm , 20 mmole) was stirred in dimethylformamide (20 ml) at room temperature . After 18 hrs , water was added and the product was extracted with chloroform. The combined chloroform solution was washed with

water and evaporated by rotary evaporator to yield a yellow is oily liquid. The oily liquid suspended in 10 % hydrochloric acid was heated at 80 - 90° c for 45 minutes and extracted two times with chloroform. The product was then formed in the aqueous layer which was recrystallized in water. The yield of product was 0.7568 gm (42%) , mp. 176 - 177° c

IR (Figure 5)	2572 - 3512 cm^{-1} (O-H stretching)
(Kbr disk)	1657 , 1699 cm^{-1} (C = O , acid)
	1395 - 1440 cm^{-1} (O - H bending)
	1260 - 1320 cm^{-1} (C - N stretching)

^{1}H-NMR (Figure 6)	1.99 - 2.05 ppm. (m , 2H)
(DMSO - d_6)	2.18 - 2.21 ppm. (t , 2H)
	3.23 ppm. (s , 3H)
	3.42 ppm. (s , 3H)
	4.25 - 4.28 ppm. (t , 2H)
	8.05 ppm. (s, 1H)

In this study , N-hydroxysuccinimide (NHS) ester method was selected to prepare HRP labeled theophylline. Active site of this method was N-hydroxysuccinimide ester. Therefore , N-hydroxysuccinimide ester of 7-(3-carboxypropyl)-1,3- dimethylxanthine was synthesized by a modification of peptide synthesis of L. Wackerle , 1979.

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

The 7-(3-carboxypropyl)-1,3-dimethylxanthine (0.4256 gm , 1.6 mmole) in dimethylformamide 15 ml was reacted to 1- ethyl -3- (3-dimethylaminopropyl) carbodiimide hydrochloride (0.4608 gm , 2.4 mmole) and N - hydroxysuccinimide (0.2760 gm , 2.4 mmole) at 0° c . The resulting solution was stirred at 4° c for 40 hrs., the product was filtered and washed with methanol and dried in vacuo. The overall yield was 0.235 gm (40%) , mp. 215 - 216° c. The structure of product obtained was determined using IR and NMR techniques. Its UV absorption spectrum was also determined before coupling with HRP.

IR (Figure 8)	2900 - 3150 cm^{-1} (C - H stretching)
(Kbr disk)	1700 - 1800 cm^{-1} (C = O)
	1084 , 1223 cm^{-1} (C - O , ester)

^{1}H-NMR (Figure 9)	2.36 - 2.38 ppm. (m , 2H)
($CDCl_3$)	2.60 - 2.63 ppm. (t , 2H)
	2.86 ppm. (s , 4H)
	3.41 ppm. (s , 3H)
	3.60 ppm. (s , 3H)
	4.40 - 4.43 ppm. (t , 2H)
	7.68 ppm. (s , 1H)

1.3 Conjugation of theophylline to HRP

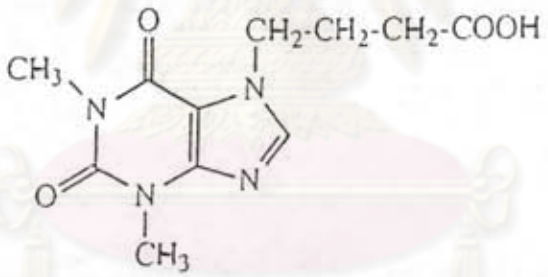
The synthesized NHS ester of 7-(3-carboxypropyl)-1,3-dimethylxanthine and the aminated HRP were conjugated according to the following procedure;

Added 0.0125 gm/ml solution of NHS ester of 7-(3-carboxypropyl)-1,3- dimethylxanthine in dimethylformamide, in 100 mcl aliquouts, to 50 mg/5ml of the stirred solution of aminated HRP in of sodium carbonate, pH 9.5. After the last addition, the mixture was gently stirred at 4° c for 16 hrs. Then dialyzed overnight against cold phosphate buffer saline, pH 7.0. The residue was finally freezed - dried and the mole ratio of theophylline conjugate to HRP was then determined via ultraviolet spectrophotometer. The mole ratio of theophylline conjugated to HRP was determined from the measuring absorbance at 403 and 269 nm for HRP and theophylline, respectively.

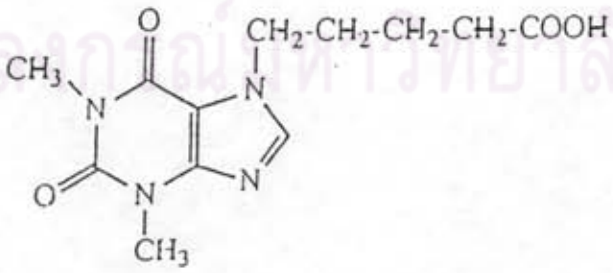
2. Preparation of Antibody for Theophylline

2.1 Preparation of immunogen

Immunogen of theophylline for inducing antibody in rabbit was prepared by conjugating theophylline derivative (act as hapten) to BSA (bovine serum albumin) which act as protein carrier. In this study two theophylline derivatives were individually conjugated to BSA in which two immunogens with the chain length of derivative on theophylline differentiated by one carbon atom as shown below were used.



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



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

Procedure for immunogen A preparation

7- (3-carboxypropyl) -1,3- dimethylxanthine 40.0 mg and 1- Ethyl- 3 (3- dimethylaminopropyl) carbodiimide hydrochloride 40.0 mg were mixed in 1.0 ml pyridine : water (1:1 by volume) and then added dropwise to a stirred 30 mg/ml of bovine serum albumin aqueous solution. After being stirred at 4° c for 45 minutes , the mixture was dialyzed successively at 4° c for 3 day against distilled water. The residue was lyophilized and kept for further used.

Procedure for immunogen B preparation

7-(3-carboxybutyl)-1,3-dimethylxanthine was synthesized and identified under the same procedure as 7-(3-carboxypropyl)-1,3- dimeyhlxanthine except the reacting reagent was ethyl -5-bromovalerate. The yield of product was 0.7206 gm (40%) , mp. 108 - 110° c

IR (Figure 14)	: 3498 cm^{-1}	(O - H stretching)
(KB r disk)	2850 - 3030 cm^{-1}	(C - H stretching)
	1656 , 1712 cm^{-1}	(C = O , acid)
	1395 - 1440 cm^{-1}	(O - H bending)
	1210 -1320 cm^{-1}	(C - N stretching)

^{1}H-NMR (Figure 15)	1.38 - 1.44 ppm (m , 2H)
(DMSO-d_6)	1.74 - 1.80 ppm (m , 2H)
	2.20 - 2.33 ppm (t , 2H)
	3.21 ppm (s , 3H)
	3.40 ppm (s , 3H)
	4.22 - 4.24 ppm (t , 2H)
	8.07 ppm (s , 1H)
	11.99 ppm (broad , 1H)

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

1- Ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (0.4608 gm , 2.4 mmole) and N- hydroxysuccinimide (0.2760 gm , 2.4 mmole) were added to a solution of 7-(3-carboxybutyl)-1,3-dimethylxanthine (0.4480 gm , 1.6 mmole) in dimethylformamide (15 ml) , the mixture was reacted according to the same procedure as that used to synthesis NHS ester of 7-(3-carboxyproyl)-1,3- dimethylxanthine. The yield was 0.2240 gm (38%) , mp. 164 - 165° c

IR (Figure 17)	2900 - 3100 cm^{-1} (C - H stretching)
(Kbr disk)	1700 - 1800 cm^{-1} (C=O , ester)
	1079 , 1219 cm^{-1} (C - O stretching)

^{1}H-NMR (Figure 18)	1.75 - 1.81 ppm (m , 2H)
(DMSO-d_6)	2.00 - 2.06 ppm (m , 2H)
	2.66 - 2.69 ppm (t , 2H)
	2.83 ppm (s , 4H)
	3.41 ppm (s , 3H)
	3.59 ppm (s , 3H)
	4.32 - 4.35 ppm (t , 2H)
	7.58 ppm (s , 1H)

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

The obtained NHS ester of 7-(3-carboxybutyl)-1,3-dimethylxanthine 0.0226 gm was dissolved in 1.0 ml DMF. This solution was added slowly to a stirred solution of the BSA 17.5 mg/ml of 0.1 M sodium carbonate , pH 9.5. The mixture was gently stirred at 4° c for 16 hrs. then , dialyzed overnight against cold phosphate buffer saline pH 7.0. The residue was then lyophilyzed.

The number of theophylline molecule linked to BSA for both immunogens were determined via ultraviolet

spectrophotometric method. The absorbance of the theophylline molecules in the theophylline conjugate BSA was calculated by substracting the absorbance of BSA molecules in the theophylline conjugate BSA from the absorbance of theophylline conjugate BSA at a maximum absorbance of this conjugate and calculated via the specific absorbance of theophylline.

2.2 Induction of Anti - theophylline serum in Rabbits

Procedure

Immunogen A and B, the amount of each as shown in Table 1 was dissolved in sterile normal saline solution and emulsified with an equal volume of incomplete Freund's adjuvants except for initial injection which carried out with complete Freund's adjuvants. Freund' s adjuvants is a pale yellow clear solution composed of mineral oil and *M. butyricum*. This solution is called complete Freund's adjuvants, if *M.butyricum* is not added into the mixture it is called incomplete Freund's adjuvants.

Female New - Zealand white rabbits weight ranged 2.0 - 3.0 kg were used in this study. The rabbit was immunized subcutaneously with 1.0 ml of immunogen emulsion at the dose of 0.5 mg/ml and 1.0 mg/ml for immunogen A and B, respectively. The second booster dose was injected after one or one and a half month of the first dose. Blood samples were collected from the marginal ear vein of rabbit every week and just before the next injection. The blood collected in sterile glass tube was left clotting and the clear serum was separated

after centrifugation at 3,000 rpm for 15 minutes and kept frozen at -20° c for subsequent testing of antibody activity. The blood sample collection was discontinued whenever the antibody specific to theophylline could not be detected in the serum. This took up to 3 months. The anti - theophylline serum to immunogen A (antibody A) and to immunogen B (antibody B) obtained were used to determine antibody titer , capability of antibody in binding to HRP labeled theophylline and specificity via microplate method.

Presently , antibody to theophylline is marketed by Sigma Chemical company. This antiserum was induced from theophylline -8- KLH. In this study , the antiserum of Sigma (antibody C) was also used to compare for its titer , capability of antibody in binding to HRP labeled theophylline and specificity with the antiserum induced in the laboratory.

Table 1 Immunization Schedule

	IMMUNOGEN A*		IMMUNOGEN B*
DAY	concentration (mg/ml)	DAY	concentration (mg/ml)
0	0.5	0	1.0
14	0.5	14	1.0
28	0.5	28	1.0
63	1.0	42	1.5
98	1.0	54	1.5

* Immunogen was mixed with equal volume of complete Freund's adjuvant in the first dose and incomplete Freund' s adjuvant in in the following doses.

2.3 Study the properties of rabbit anti - theophylline serum

Rabbit anti - theophylline serum was characterized for antibody titer, the capability of antibody in binding to HRP labeled theophylline and the specificity.

2.3.1 Antibody Titer

The titer of antibody is expressed as the dilution of antibody which bind to HRP labeled theophylline 50% . In this study, antibody titer was determined via microplate method, as described below :

Procedure

A ninetysix - well microplate was coated with each of antibody specific to immunogen A, B and C in six different dilutions of antibody (1 : 10 - 1 : 1,000,000) 100 mcl per well , 3 wells for each antibody dilution. The microplate was kept refrigerated at 4° c overnight and washed three times with phosphate buffer saline containing 0.05% tween 20 (PBS- T).

One hundred microliters of 3% w/v bovine serum albumin in PBS-T pH 7.4 was added in each well. The plate was incubated at 37° c for 1 hr and washed three times with PBS-T. Then 100 mcl of 1,000 - fold dilution of HRP labeled theophylline was added to each well. The mixture was incubated at 37° c for 2 hrs , the unbound HRP labeled theophylline was washed three times. One hundred microliters of (1.08 mg/ml) the o - phenylenediamine - hydrogenperoxide substrate solution was added to each well of the plate. Within 15 min in the dark , the orange color of HRP - substrate complex was formed at room temperature. A 50 mcl of 4 N sulfuric acid was added to terminate the enzyme reaction. The absorbance of this solution was determined at the wavelength of 492 nm with microplate reader. The percentage of HRP labeled theophylline bound to antibody was calculated from the obtained absorbance from the following relationship ;

$$\% \text{ Binding} = \frac{\text{absorbance at different dilution of antibody} * 100}{\text{absorbance at maximum dilution of antibody}}$$

Plot antibody dilution against % binding , this antibody dilution curve was used to determine the titer value of antibody.

2.3.2 Capability of antibody in binding to HRP labeled theophylline

Since three different antibodies were used in this study which based on three different immunogens, the capability of each antibody in binding to same HRP in HRP labeled theophylline would be varied. Therefore, this study was set up to compare binding capability of antibody A, B and C to the same HRP labeled theophylline for determining the appropriate dilution of HRP labeled theophylline in competitive reaction.

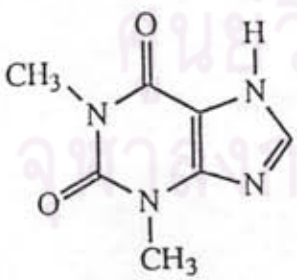
Procedure

Each 100 mcl of antibody A and B at the dilution 1: 1,000 and 100 mcl of antibody C at the dilution 1: 100 were coated to each well of the microplates, 4 wells for each antibody dilution, using the same procedure as those aforementioned for titer determination. One hundred microliters of 1:1,000 dilution of HRP labeled theophylline was added to each well in the coated microplates. After the incubation of the microplates at 37° c for 2 hrs, the unbound HRP labeled theophylline was washed three times with PBS-T. The absorbance

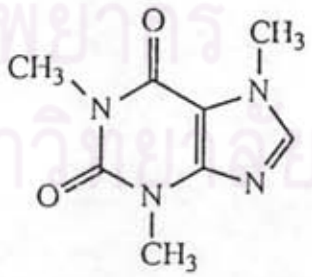
related to amount of HRP labeled theophylline bound to each antibody was measured at wavelength of 492 nm and then the capacity of binding of each antibody was compared. The higher the absorbance observed, the higher the capability of that antibody binding to HRP labeled theophylline.

2.3.3 Specificity

To assess the specificity of antibody, the experiment was set up to determine whether any compound can also bind to antibody and compete with HRP labeled theophylline. Theoretically, compounds with identical structure or functional group as immunogen could compete with the labeled hapten for binding to antibody. Caffeine was used as cross - reacting compound for this study because of the similarity in the structure as shown :



Theophylline



Caffeine

In the addition , refreshments , other drug compounds , sweets , usually contain caffeine. Therefore , in this study included the investigation of cross- reacting of caffeine to antibody A and B.

Procedure

Antibody A and B of 1,000- fold dilution were coated in each well of the plate. The microplates was kept and prepared under the same procedure as that described for antibody titer determination except that 100 mcl of standard caffeine in the concentration range of 0 , 0.5 , 1.0 , 2.0 g/l as well as 100 mcl of HRP labeled theophylline in the dilution 1 : 5,000 were simultaneously added to the well duplicatedly. In addition , theophylline in the concentration range of 0 , 5.0 , 10.0 , 15.0 , 20.0 , 40.0 mg/l as well as 100 mcl of HRP labeled theophylline in the dilution 1:5,000 were simultaneously added to the well duplicatedly. After this step , the method would follow the same procedure as mentioned in titer determination. The amount of caffeine and theophylline required to compete with HRP labeled theophylline 50% for the same binding sites of antibody A and B were determined. The percentage of cross reactivity was calculated from the following equation :

$$\% \text{ cross reactivity} = \frac{\text{mg of theophylline/l at 50\% competition}}{\text{mg of caffeine/l at 50 \% competition}} * 100$$

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

Theoretically, any hapten labeled compound appropriate to be used in enzyme immunoassay method have to exert the property of label compound as well as the property of hapten to compete with sample for the same antibody. HRP activity in HRP labeled theophylline has already been proven after the compound was synthesized (1.3). Therefore, the property of HRP labeled theophylline competing with theophylline in sample for the binding site on antibody molecule was studied to ensure that this labeled compound would be able to used for developing the method of theophylline enzyme immunoassay in the future.

3.1 Determine the appropriate dilution of antibody for competitive reaction

Procedure

Antibody A and B in their different dilutions 1: 1,000, 1: 10,000 and 1: 100,000, were individually coated to each well of the microplate , 3 wells for each antibody dilution and washed after incubation, according to the same procedure

as those described for titer determination. The competition was study between standard theophylline in the concentration range 0 - 40 mg/l and HRP labeled theophylline in the dilution 1 : 5,000 by subsequently addition. One hundred microliters of standard theophylline and HRP labeled theophylline were added to each coated well. The competition reaction was proceeded under 37° c for 2 hrs. After the free HRP labeled theophylline was washed three times with PBS-T , substrate solution of the o - phenylenediamine - hydrogenperoxide in the volume 100 mcl were added, the orange color of HRP - substrate complex was proceeded as already mentioned. The absorbance of this complex was determined at wavelength of 492 nm with microplate reader. The measured absorbance were plotted against theophylline concentration at different dilution of antibody. The logit - log plot was constructed by plotting the log theophylline concentration on the x - axis and the log Y on the y - axis. The log y was calculated from following equation ; (Standefer and Saunders , 1978)

$$\text{Log } Y = \frac{\ln B / B_0}{(1 - B / B_0)}$$

B = absorbance of different conc of theophylline

B_0 = absorbance of theophylline conc 0 mg/l

This logit - log curve was also constructed in order to determine the most appropriate antibody dilution used for competitive reaction. The values of correlation coefficient (r) from linear regression analysis of each antibody dilution were compared in which the highest correlation coefficient indicate appropriate antibody dilutions used for this study.

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

In the development of the new theophylline enzyme immunoassay , both the appropriate dilution of antibody and HRP labeled theophylline were required for competitive reaction. From 3.1 the most appropriate dilution of antibody were determined by fixing the amount of HRP labeled theophylline at the dilution 1 : 5,000. Therefore , this study was set up to confirm that the dilution of antibody and HRP labeled theophylline were the most appropriate dilution for competitive reaction.

Procedure

The most appropriate dilution of each antibody from 3.1 were coated in each well of the plate , 3 wells for each antibody , using the same procedure as those aforementioned for titer determination. The competition was studied between standard theophylline in the concentration range 0 - 40 mg/l and HRP labeled theophylline in the different dilutions 1 : 1,000 , 1 : 5,000 and 1 : 10,000 by subsequently

addition. One hundred microliters of standard theophylline and HRP labeled theophylline were added to each coated well. The competitive reaction was proceeded under 37° c for 2 hrs. After this step , the method of determination would follow that mentioned in 3.1 except that the measured absorbance were plotted against theophylline concentrations at the different dilutions of HRP labeled theophylline.

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

This part of study was set up to confirm that the most appropriate dilutions of antibody A and B and dilution of HRP labeled theophylline obtained from 3.1 and 3.2 were used to study the competition between HRP labeled theophylline and theophylline which leading to construct the possible standard curve.

Procedure

Following the same procedure as those described for appropriate dilution of antibody determination but use the most appropriate dilution of antibody and HRP labeled theophylline obtained from 3.1 and 3.2, respectively. The competition was studied between standard theophylline in the concentration range 0 - 40 mg/l and HRP labeled theophylline in the appropriate dilution from 3.2 by subsequently addition. After this step, the method of determination would follow that mention in 3.1 except that the measured absorbance was calculated to the percentage binding by following equation :

$$\% \text{ Binding} = B/B_o * 100$$

B = absorbance of different conc of theophylline

B_0 = absorbance of theophylline conc 0 mg/l

The standard curve was constructed by plotting the percentage binding against theophylline concentrations. The values of correlation coefficient (r) from linear regression analysis and the value of slope of each standard curve were compared in order to determine possibility of using HRP labeled theophylline in theophylline enzyme immunoassay.