ความจำเพาะของการตรวจวัดแอมเฟตามีน และ/หรือเมทแอมเฟตามีน ทางอิมมูโนวิทยา ด้วยหลักการเฮเทอโรโลกัสคอมบิเนชัน

นางสาววลัยลักษณ์ เมธาภัทร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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SELECTIVE IMMUNO-DETECTION OF AMPHETAMINE AND/OR METHAMPHETAMINE BY PRINCIPLE OF HETEROLOGOUS COMBINATIONS

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for the Degree of Master of Pharmacy in Pharmaceutical Chemistry

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การศึกษาความจำเพาะของการวิเคราะห์แอมเฟตามีนและเมทแอมเฟตามีน ทำโดยการสังเคราะห์อนุพันธ์แอมเฟตามีน 2 อนุพันธ์ และ อนุพันธ์เมทแอมเฟตามีน 2 อนุพันธ์ คือ N-(3-aminopropyl)amphetamine (ก) , N-(4-aminobutyl)amphetamine (ข) , N-(3-aminopropyl) methamphetamine (ค) และ N-(4-aminobutyl)methamphetamine (ง) เพื่อใช้เตรียมเป็นอิมมูโนเจนและสารติดฉลากด้วยเอนไซม์เปอร์ออกซิเดส โดยศึกษาปฏิกิริยาการแย่งที่ระหว่างแอมเฟตามีนและเมทแอมเฟตามีน กับสารติดฉลากด้วยเอนไซม์ในการจับกับแอนติบอดี โดยอาศัยหลักการของเฮเทอโรโลกัส จากการศึกษาพบว่า การใช้แอนติซีรัมและสารติดฉลากจากอนุพันธ์ ค สามารถตรวจวัดแอมเฟตามีนและเมทแอมเฟตามีนได้ โดยมีความไวของการวิเคราะห์เท่ากับ 34 ต่อ log ของความเข้มข้น และสามารถเกิดปฏิกิริยาข้ามได้กับ อีเฟดรีน ซูโดอีเฟดรีน และฟีนิลโพรพาโนลามีน ด้วยค่า relative reactivity เท่ากับ 0.27 , 0.14 และ 0.14 ตามลำดับ สำหรับคอมบิเนชันจากแอนติซีรัมของอนุพันธ์ ค และสารติดฉลากจากอนุพันธ์ ก สามารถตรวจวัดแอมเฟตามีน เมทแอมเฟตามีนและอีเฟดรีนได้ โดยมีความไวของการวิเคราะห์เท่ากับ 25 ต่อ log ของความเข้มข้น และจะเกิดปฏิกิริยาข้ามกับ ซูโดอีเฟดรีนและฟีนิลโพรพาโนลามีน ด้วยค่า relative reactivity เพียง 0.05 และ 0.10 ตามลำดับ ดังนั้นด้วยหลักการของเฮเทอโรโลยี คอมบิเนชันทั้ง 2 ลักษณะ จึงมีความจำเพาะเพียงพอในการตรวจวัดแอมเฟตามีนและเมทแอมเฟตามีน ด้วยโพลีโคลนอลแอนติบอดี อย่างไรก็ตามควรได้รับการพัฒนาเป็นวิธีวิเคราะห์ทางอิมมูโนแอสเสย์ ก่อนที่จะนำไปใช้ในการตรวจวัดการใช้ยาในทางที่ผิดต่อไป

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The application of heterology principle for immuno-detection of amphetamine and methamphetamine was studied. N-(3-aminopropyl)amphetamine (A) , N-(4-aminobutyl)amphetamine (B) , N-(3-aminopropyl)methamphetamine (C) and N-(4-aminobutyl)methamphetamine (D) were synthesized for preparing immunogen and labeling with peroxidase enzyme. The reaction that amphetamine and methamphetamine could compete with HRP-labeled in binding to antibody by heterologous pattern was determined. Amphetamine and methamphetamine could be selectively determined using the combinations of antiserum and enzyme-labeled from (C) with the sensitivity of competitive binding equal to 34 per log concentration. Ephedrine, pseudoephedrine and phenylpropanolamine could cross-reacted with amphetamine in the relative reactivity of 0.27, 0.14 and 0.14, respectively. Selective determination of amphetamine, methamphetamine and ephedrine was resulted from the combinations of antiserum from (C) and enzyme-labeled from (A). The sensitivity of competitive binding equal to 25 per log concentration. Pseudoephedrine and phenylpropanolamine could cross-reacted with methamphetamine in the relative reactivity of 0.05 and 0.10, respectively. These two types of combinations were selective for both amphetamine and methamphetamine. Therefore, amphetamine and methamphetamine could be successfully determined using polyclonal antibody and heterology study. The further immunoassay development was suggested before utilizing in drug-abused test in Thailand.

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LIST OF ABBREVIATIONS

°C	centigrade degree
%	percent
A	absorbance
a	specific absorptivity
BSA	bovine serum albumin
$CDCl_3$	chloroform-d
cm	centimeter
ELISA	enzyme linked-immunosorbent assay
g	gram
^{1}H-NMR	proton nuclear magnetic resonance
Hz	hertz
HRP	horseradish peroxidase enzyme
IR	infrared spectrophotometry
J	coupling constant
kg	kilogram
L	litre
M	molar
mg	milligram
ml	millilitre
MS	mass spectrophotometry
N	normality
nm	nanometer
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline with tween 20
ppm	part per million
r	correlation coefficient

UV	ultraviolet
µg	microgram
µl	microlitre
v/v	volume by volume
w/v	weight by volume

CHAPTER I

INTRODUCTION

Immunoassay is the indirect analytical method involving antigen-antibody reaction, competitive reaction and specific reaction that depend upon the labeled on antigen or antibody. It is the label that is analyzed and related to sample or hapten.

Practically, two types of antibodies are used in the immunoassay. They are monoclonal antibody and polyclonal antibody. Monoclonal antibody is derived from single antibody-producing cells to form 'hybridoma' clones (Kohler and Milstein, 1975; Tijssen, 1985 ; Wild, 1994). Therefore, monoclonal antibody is specific to a single antigen determinant of immunogen. But the process for monoclonal antibody production is rather complicate and time consuming. In contrast, polyclonal antibody can bind to various antigens that are similar in chemical structures to immunogen (Tijssen, 1985 ; Catty and Raykundalia, 1988 ; Wild, 1994). The production of polyclonal antibody is less complicate and less costly. Nontheless, main problem of polyclonal antibody is its non-specificity in binding to other related substances. To overcome this shortcoming the principle of heterologous combinations were proposed (Van Weemen and Schuurs, 1975, 1976). By this principle, it was claimed that both specificity and sensitivity of the method could be raisable (Hosada et al., 1980, 1983).

Heterologous combination is the principle dealing with the difference in chemical structures of immunogen and enzyme-labeled compound that used in immuno-analysis. Three types of heterology have been reported (Van Weemen and Schuurs, 1975 ; Piran, Riordan, and Silbert, 1990 ; Khosravi, and Papanastasiou-Diamandi, 1993). They are bridge, hapten and site heterology.

Bridge heterologous combination is the combination that the different cross-linkers of the haptens are used in immunization and labeling enzyme. For example, the immunoassay of 11-deoxycortisol that antibody was induced from 4-(2-carboxymethylthio)-11-deoxycortisol and enzyme was labeled on 4-(2-carboxyethylthio)-11-deoxycortisol (Hosada, Kabayashi, and Nambara, 1983).

Hapten heterologous combination is the combination that the different but related haptens are used for inducing antibody and labeling enzyme. The example of hapten heterologous combinations was the immunoassay of thyroxine that antibody was induced from triiodothyronine (T_3) and enzyme was labeled on diiodothyronine(T_2). This study reported the reducing of specificity in hapten heterologous combinations (Piran et al., 1990).

Site heterologous combination is the combination that the haptens with the different position of the same linkage used in immunization and enzyme labeled. The use of estradiol-17-succinyl as the immunogen and estradiol-11-succinyl for labeling with enzyme for estradiol immunoassay was one of the examples. The data show that site heterology is effective in increasing the sensitivity of the assays (Van Weemen and Schuurs, 1975).

The combination of heterology, in which two or three of the heterology types are combined, is also possible. Bridge and hapten heterologous combinations are the combination that the haptens for labeling enzyme and for inducing antibody not only different in the cross-linkers on the structure of compound but also different in the type of hapten used. The immunoassay of estradiol that used 11α-OH-estradiol 11-hemisuccinate as immunogen and enzyme was labeled on 11α-OH-estrone 11-hemiglutarate (Van Weemen and Schuurs, 1975) was reported.

Bridge and site heterologous combinations are the combination that the haptens for labeling enzyme and for inducing antibody are different in the cross-linkers and the position on the structure of compound. The example of bridge and site heterologous combinations are the immunoassay of theophylline that antibody was induced from 7-(3-carboxypropyl)-1,3-dimethylxanthine and enzyme labeled on 8-(4-carboxybutyl)-1,3-dimethylxanthine (Suthasinee Pitchayawasin, 1997).

These principles have also been applied to many other enzyme immunoassays for steroid hormones and various drugs with the use of polyclonal antibodies and different types of heterology (Van Weemen and Schuurs, 1972, 1975 ; Hosada et al., 1981 ; Hosada et al., 1985, 1986). No absolute conclusion could be made in the pattern of heterology for any individual reaction. The appropriate heterology for any immunoassay can only be obtained from the experiment for each compound (Hosada et al., 1980, 1983a, 1983b).

Recently, theophylline immunoassay has been successfully developed using bridge heterologous combinations. This developed method was very specific to theophylline without any cross-reaction from caffeine (Suthasinee Pitchayasin, 1997). Therefore, it is interesting to apply heterology principle for immuno-analyzing of other drug compounds.

Amphetamine and methamphetamine are the focus of interest because of their wide-spread abuse. Several techniques of immunoassay for amphetamine and/or methamphetamine have been reported such as enzyme multiplied immunoassay, radioimmunoassay, polarization fluoroimmunoassay and latex agglutination inhibition reaction test. (Cheng et al., 1973 ; Colbert, Gallacher, and Mainwaring-Burton, 1985 ; Ruangyuttikarn and Moody, 1988 ; Aoki, Hirose, and Kuroiwa, 1990 ; Mongkolsirichaikul et al., 1994). No any report using heterologous immuno-

detection of amphetamine and methamphetamine was shown. Hence, the study of heterologous combinations using polyclonal antibody in enzyme immunoassay of amphetamine and methamphetamine detection could be meaningful.

The objective of the study

To investigate various patterns of immuno-detection for amphetamine and methamphetamine using principle of heterologous combinations.

The significance of the study

To establish the heterologous patterns for amphetamine and methamphetamine that would be valuable for immunoassay development.

CHAPTER II

EXPERIMENTS

Material and Method

Chemical compounds and reagents

All chemical compounds and reagents were of analytical grade and used as received.

Ammonia solution (Merck , Germany)

Ammonium sulfate (Merck , Germany)

d-Amphetamine sulfate (Sigma , USA , under government control)

Bovine serum albumin (Sigma , USA)

N-(3-bromopropyl)phthalimide (Fluka Chemie AG , Germany)

N-(4-bromobutyl)phthalimide (Fluka Chemie AG , Germany)

Chloroform (Merck , Germany)

Chloroform-d (Sigma , USA)

Citric acid (Farmitalia Carlo Erba , Germany)

Complete Freund 's adjuvant (Life Technologies , USA)

l-Ephedrine hydrochloride (Under government control)

Ethanol , absolute (Merck ,Germany)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (Sigma, USA)

Glacial acetic acid (Merck , Germany)

Horseradish peroxidase ($RZ \geq 3$) (Amresco , USA)

80% Hydrazine hydrate (Merck , Germany)

Hydrochloric acid (Merck , Germany)

35% Hydrogen peroxide (Merck , Germany)

Incomplete Freund's adjuvant (Life Technologies , USA)

d-Methamphetamine hydrochloride (Sigma , USA , under government control)

Methanol (Merck , Germany)

Normal saline 0.9% (General Hospital Products Public , Thailand)

O-Phenylenediamine (Zymed Laboratories , USA)

dl-Phenylpropanolamine (Sigma ,USA)

Potassium chloride (Merck , Germany)

Potassium dihydrogen phosphate (Sigma , USA)

d-Pseudoephedrine hydrochloride (Sigma , USA)

Sodium acetate (Merck ,Germany)

Sodium bicarbonate (Merck , Germany)

Sodium borohydride (Sigma , USA)

Sodium carbonate (Merck , Germany)

Sodium chloride (Merck , Germany)

Sodium dihydrogen phosphate (Merck , Germany)

Sodium hydroxide (Merck , Germany)

di-Sodium hydrogen phosphate (Merck , Germany)

Sodium metaperiodate (Sigma , USA)

Sulfuric acid (Merck , Germany)

Tween 20 (Merck , Germany)

p-Xylene (Fluka Chemie AG , Germany)

Apparatus

Analytical Balance (METTLER® TOLEDO , AG 245 , Switzerland)

Centrifuge (Hettich , EBA 12 , Germany)

Dialysis tubing (Spectra/Por , USA)

Infrared Spectrophotometer (Perkin-Elmer model FT-IR , USA)

Immuno-plate (NUNC , Denmark)

Microplate reader (ELISA plate reader Bio-Rad model 3550 , Bio-Rad Laboratories., USP)

Micropipet (Socorex , USP)

Nuclear Magnetic Resonance Spectrophotometer (Model dpx 300 , BRUKER , Germany)

pH-meter (Consort pH meter model C231 , Belgium)

Ultraviolet Spectrophotometer (Spectronic 3000 array , Milton Roy Co. , USA)

Vacuum Concentrator (Heto model , CT 110 , Denmark.)

Vortex (Genei Scientific Industries Inc. , New York , USP)

Experimental Animals

Female New-Zealand white rabbits weighing about 1.5 – 2.5 kg obtained from the National Swine Research and Training Center, Kamphangsean Campus, Kasetsart University.

Methods

This study composed of many experimental steps as described in the following.

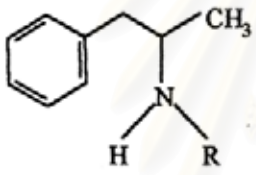
1. Chemical synthesis of amphetamine and methamphetamine derivatives.
2. Preparation of HRP-labeled amphetamine and methamphetamine derivatives.
3. Induction of antibody against amphetamine and methamphetamine in rabbits.
4. Determination of proper dilution of HRP-labeled hapten and antibody used for the competitive reaction.
5. Heterologous combinations for amphetamine and methamphetamine detection.
6. Cross-reaction determination.

1. Chemical synthesis of amphetamine and methamphetamine derivatives.

(Cheng et al., 1973 ; Mongkolsirichaikul, et al., 1993)

The derivatives of amphetamine and methamphetamine were synthesized as described in Figure 1. They were N-(3-aminopropyl)amphetamine (3-APA), N-(4-aminobutyl)amphetamine (4-ABA), N-(3-aminopropyl)methamphetamine (3-APM) and N-(4-aminobutyl)methamphetamine (4-ABM).

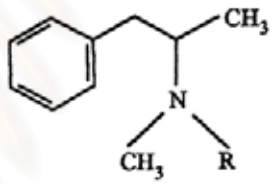
Derivatives of amphetamine



R = $(CH_2)_3NH_2$; 3-APA

R = $(CH_2)_4NH_2$; 4-ABA

Derivatives of methamphetamine



R = $(CH_2)_3NH_2$; 3-APM

R = $(CH_2)_4NH_2$; 4-ABM

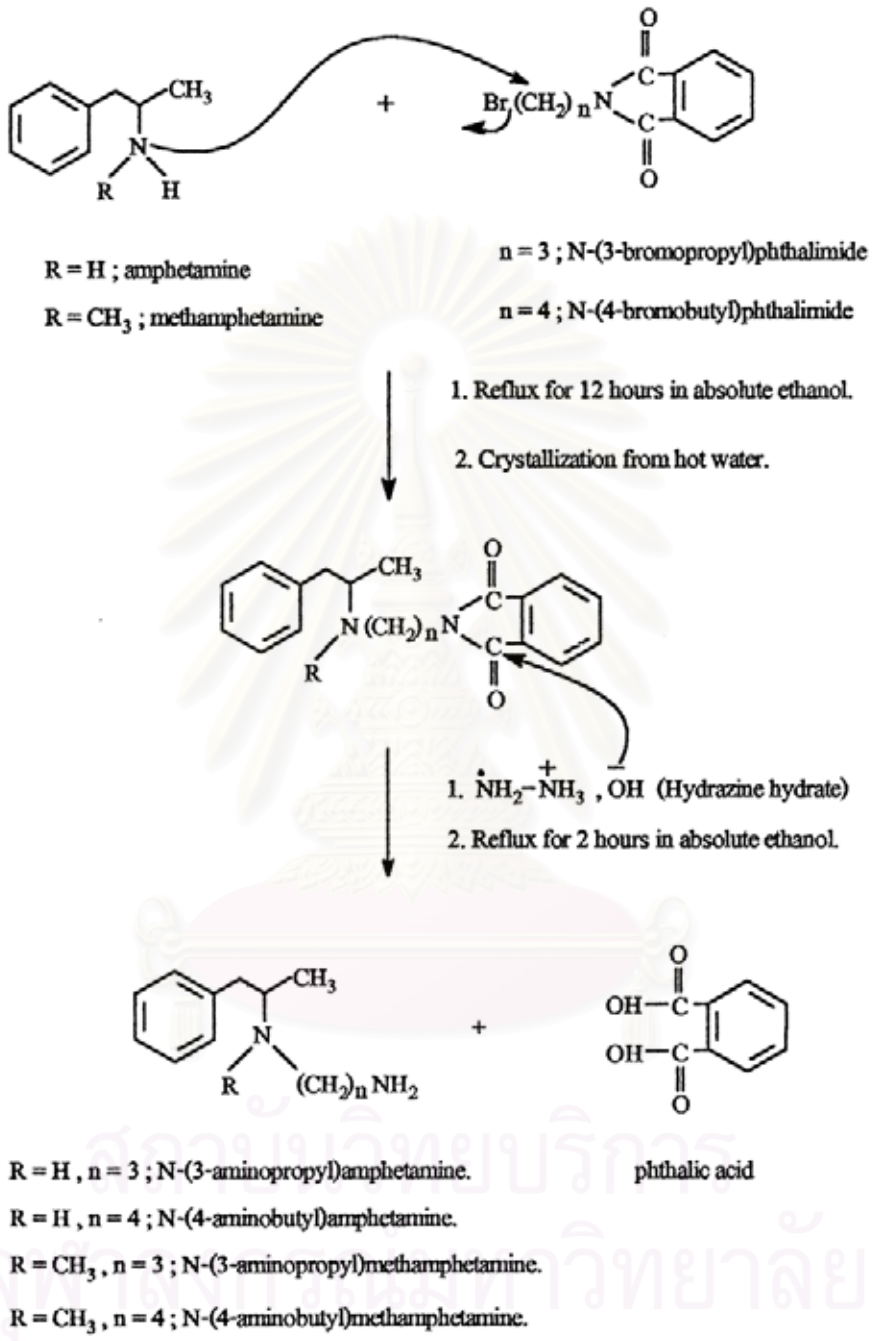


Figure 1 The synthesis reaction for amphetamine and methamphetamine derivatives.

1.1 Chemical synthesis of N-(3-aminopropyl)amphetamine (3-APA) and N-(4-aminobutyl)amphetamine (4-ABA).

Preparation of amphetamine free base

Amphetamine sulfate 37.0 g (0.1 mole) was dissolved in 50 ml of 70% ethanol and 42.0 ml (0.3 mole) of triethylamine was added. The mixture was stirred overnight at room temperature and extracted with three portions of 50 ml chloroform. The combined chloroform extracts were washed with two portions of 30 ml water. The combined organic phase was dried over anhydrous sodium sulfate and the solvent was evaporated. The pale yellow liquid of amphetamine free base was obtained.

Preparation of N-(3-aminopropyl)amphetamine (3-APA) and N-(4-aminobutyl)amphetamine (4-ABA).

To produced 3-APA or 4-ABA, the 23.0 g (0.17 mole) of amphetamine free base was refluxed with N-(3-bromopropyl)phthalimide 54.7 g (0.2 mole) or N-(4-bromobutyl)phthalimide 57.6 g (0.2 mole), respectively, in 100 ml absolute ethanol for 12 hours. The solvent was evaporated and the residue was dissolved and crystallized from hot water. A 25.0 ml (0.5 mole) in 100 ml absolute ethanol of 80% hydrazine hydrate was added and refluxed for 2 hours. Ethanol was removed before acidified to pH 3.0 with 1 N hydrochloric acid, the resulting phthalic acid precipitate was removed by filtration. The filtrate was adjusted to pH 10.0 with 5 N sodium hydroxide and extracted with three portions of 50 ml chloroform. The combined extracts were evaporated to yield the yellow liquid. The purification was performed by column chromatographic technique, using silica gel 60 as stationary phase and methanol : ammonia (100 : 1.5) as eluent solvent. The final products of N-(3-aminopropyl) amphetamine and N-(4-aminobutyl)amphetamine obtained, were pale yellow liquid.

that were confirmed using ultraviolet (UV), infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrophotometric (MS) techniques.

1.2 Chemical synthesis of N-(3-aminopropyl)methamphetamine (3-APM) and N-(4-aminobutyl)methamphetamine (4-ABM).

Preparation of methamphetamine free base

The above procedure was repeated for the preparation of methamphetamine free base. The pale yellow liquid of methamphetamine free base was obtained.

Preparation of N-(3-aminopropyl)methamphetamine (3-APM) and N-(4-aminobutyl)methamphetamine (4-ABM)

The above procedure for the preparation of 3-APA and 4-ABA was repeated for the preparation of 3-APM and 4-ABM. The final products obtained were pale yellow liquid. That would be N-(3-aminopropyl)methamphetamine and N-(4-aminobutyl)methamphetamine. They were confirmed using ultraviolet (UV), infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrophotometric (MS) techniques.

2. Preparation of horseradish peroxidase (HRP) – labeled amphetamine and methamphetamine derivatives.

The derivatives of amphetamine and methamphetamine were labeled with HRP according to the method of Wilson and Nakane (1978). The reaction of conjugation was depicted in Figure 2.

Procedure for 3-APA-HRP formation.

To a solution of 10.0 g (0.25 mmole) HRP in 50 ml of 0.01 M sodium acetate pH 4.5, 10.0 ml of freshly prepared 1.0 M sodium metaperiodate was slowly added and stirred gently in the dark for 30 minutes at room temperature. After which 10.0 ml of 10.0 M glycerol was added and allowed the reaction to proceed for 30 minutes. The mixture was dialyzed overnight at 4 °C against 0.01 M sodium acetate buffer pH 4.5 to remove the excess periodate. The dialysate was slowly added into the solution of 3-APA 0.24 mg/ml of 0.5 M sodium carbonate buffer pH 9.5 The reaction was proceeded for 4 hours at 4 °C . A 10.0 ml of freshly prepared 10.0 M sodium borohydride was added and allowed the reaction for 2 hours at 4 °C before dialyzing the mixture overnight at 4 °C against water. The precipitate, if any, was removed by centrifugation. The final clear solution was lyophilized to obtain 3-APA-HRP that were stored at -48 °C for further use. The activity of HRP in the conjugate was also determined.

For the preparation of 4-ABA-HRP, 3-APM-HRP and 4-ABM-HRP, the aforementioned procedure was followed.

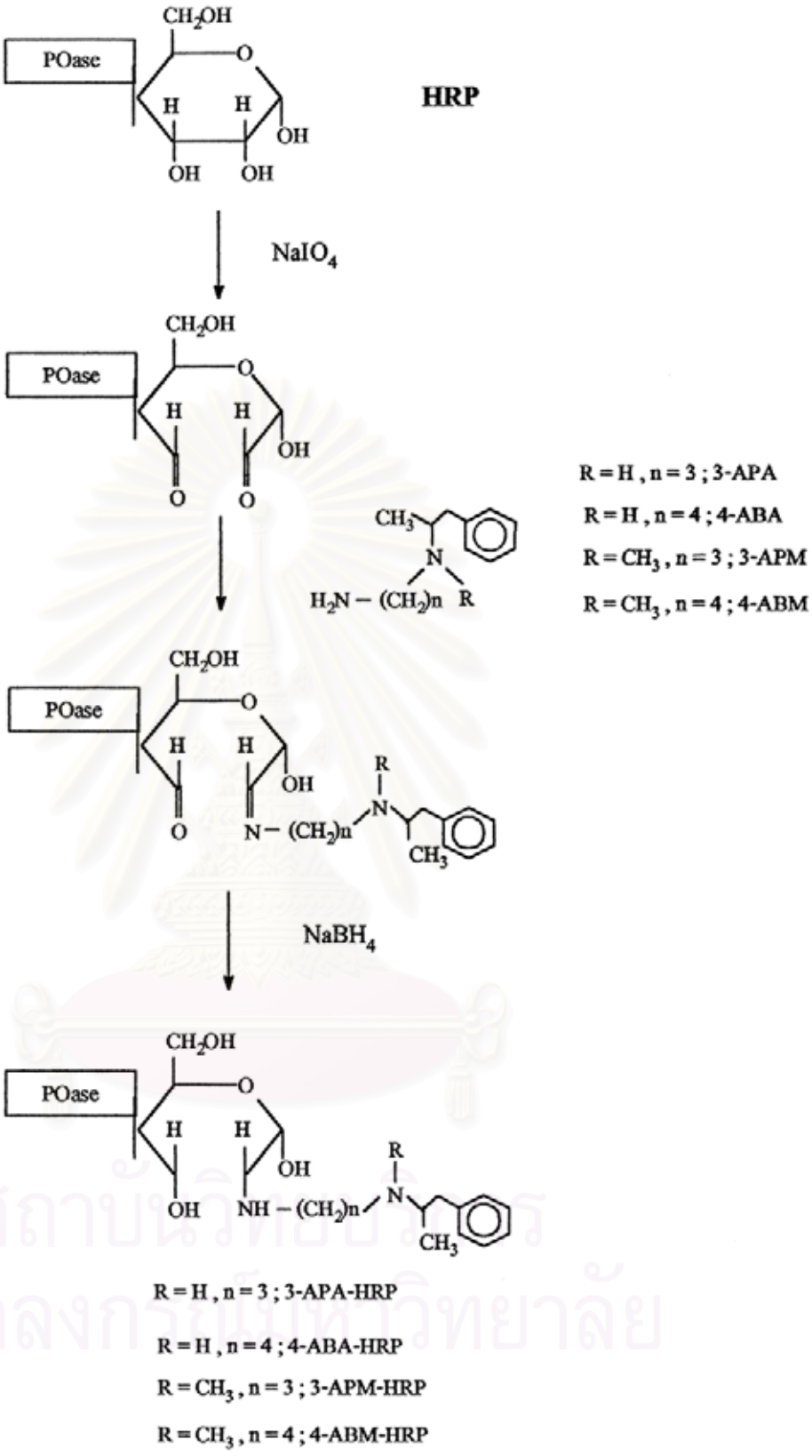


Figure 2 The chemical reaction of HRP-labeled amphetamine and methamphetamine derivatives

3. Induction of antibody against amphetamine and methamphetamine in rabbits.

3.1 Preparation of immunogens

(Cheng et al., 1973 ; Mongkolsirichaikul et al., 1993)

The derivatives of amphetamine and methamphetamine are haptens that couldn't induce antibody. They must be conjugated with macromolecule to become immunogen. Therefore, the derivatives of amphetamine and methamphetamine were individually conjugated to BSA according to the reaction displayed in Figure 3.

Procedure

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) 0.096 g (0.50 mmole) and BSA (0.001-0.005 mmole) were added respectively into a aqueous solution of N-(3-aminopropyl)amphetamine (0.50 mmole in double distilled water) with continuous stirring for 3.5 hours at room temperature (25 °C). The reaction mixture was dialyzed at 4 °C against double distilled water to remove excess derivatives. The conjugated protein of 3-APA-BSA was then lyophilized in which the white fluffy powder was obtained. The product was stored at -48 °C for further use.

For 4-ABA-BSA, 3-APM-BSA and 4-ABM-BSA, this same procedure was also followed.

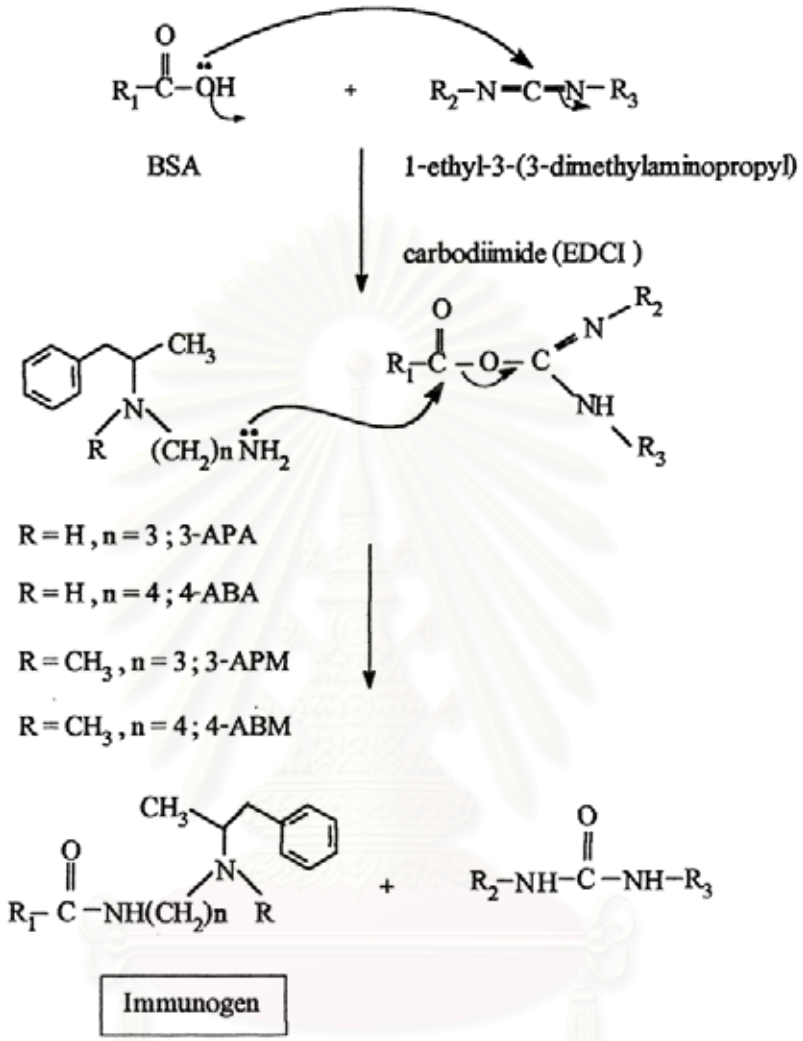


Figure 3 The chemical reaction of amphetamine and methamphetamine derivatives – BSA

3.2 Induction of antibody in rabbits.

A variety of animal species can be used for raising antibody, such as horse, goat, pig, rat, rabbit (Catty and Raykundalia, 1988 ; Porstmann and Kiessig, 1992 ; Kemney, 1992). Rabbit, goat and sheep are usually for polyclonal antibody induction (Tijssen, 1985). In this study New Zealand white rabbits was selected as the animal for raising antibody.

Preparation of immunogen emulsion

For the first immunization, immunogen emulsion was prepared by vigorously mixing 2.0 mg of the immunogen in 1 ml of sterile normal saline solution with an equal volume of complete Freund's adjuvant. For the subsequent immunization, incomplete Freund's adjuvant was insteadly used.

Immunization of rabbits

Two female rabbits, each weighing 2.0 – 3.0 kg, were immunized subcutaneously with 1.0 ml of immunogen emulsion described above through the fold of skin at the shoulder blade according to the specified time table. Whole blood was collected weekly from the marginal ear vein and separated for serum. The immunoglobulin of each serum was obtained from the precipitation under ice-cold saturated ammonium sulfate. The titer value of this partial purified antiserum was determined.

Determination of antibody's titer

The titer of antiserum is defined as the dilution at which 50% of HRP-labeled hapten bind to the antibody (Yalow and Berson , 1964 : Ciabatton, 1987).

Procedure

The serial dilutions of antibody, from 1 : 10 to 1 : 10^7 , were prepared from the partial purified antiserum 1.0 mg/ml in 0.05 M sodium carbonate buffer pH 9.6 . The well of microtiter plate was coated with 100 µl of serially diluted antibody, 3 well for each antibody dilution and incubated overnight at 4 °C . After, the plate was three times washing with PBS-T pH 7.4 , a 100 µl of 2% BSA in PBS pH 7.4 was added and incubated at 37° C for 1 hour. The plate was washed three times with PBS-T pH 7.4 , and then 100 µl of HRP-labeled hapten (3-APA-HRP, 4-ABA-HRP, 3-APM-HRP or 4-ABM-HRP) was added and incubated at 37° C for 2 hours. The plate was again washed before adding a 100 µl of freshly prepared OPD substrate solution (dissolve OPD tablet in 12.0 ml of citrate-phosphate buffer pH 5.0 , containing 12 µl of 35% hydrogen peroxide) to each well. The enzyme-substrate reaction was proceeded for 20 minutes in the dark at room temperature. The reaction was stopped by adding 50 µl of 4 N sulfuric acid. The absorbance of the final solution was determined at 490 nm using microplate reader. The percentage of enzyme binding to antiserum was calculated and the titer of antibody was determined from the plot of %binding against the dilution of antibody.

$$\% \text{ Binding} = \frac{\text{Absorbance of each diluted antiserum}}{\text{Maximum absorbance of diluted antiserum}} \times 100$$

Table 1 Immunization table

3-APA-BSA		4-ABA-BSA		3-APM-BSA		4-ABM-BSA	
Day	Concentration (mg/ml)	Day	Concentration (mg/ml)	Day	Concentration (mg/ml)	Day	Concentration (mg/ml)
0	1.5	0	2.0	0	1.0	0	1.0
14	1.5	14	2.0	13	1.0	13	1.0
28	2.0	28	3.0	38	1.5	38	1.5
49	3.0	42	3.0	52	1.5	52	1.5
				66	1.5	66	1.5

4. Determination of proper dilution of HRP-labeled hapten and antibody used for the competitive reaction.

The proper dilution of HRP-labeled hapten and antibodies for producing competitive reaction of amphetamine or methamphetamine was determined using microtiter plate. The %binding was calculated and plotted against the log concentration of amphetamine or methamphetamine. From the regression analysis of the %binding and log of standard concentration, the value of correlation coefficient (r) was determined. The value of r informed the appropriate dilution of enzyme-labeled hapten and antibody in competitive reaction.

The combinations of 3-APA-HRP and 3-APA-Ab as well as the combinations of 4-ABA-HRP and 4-ABA-Ab were determined in competitive reaction for amphetamine. The competitive reactions for methamphetamine were determined with the combinations of 3-APM-HRP and 3-APM-Ab as well as the combinations of 4-ABM-HRP and 4-ABM-Ab.

Procedure

The serial dilution of HRP-labeled hapten in PBS pH 7.4 and antibody in 0.05 M sodium carbonate buffer pH 9.6 were prepared. The microtiter plate technique as already described in section 3 was followed. The aqueous solutions of amphetamine and the solution of methamphetamine in the concentration range of 0 – 5,000 ng/ml were used in the competitive reactions.

The logic plot of %binding and log of amphetamine or methamphetamine concentration was determined. From the logic plot, the combinations that showed the scatter data or no competitive response would be defined as no response (NR). The

regression analysis was used for those with competitive response such that the value of correlation coefficient (r) of each logic plot was determined. The highest value of correlation coefficient (r) of any competitive reaction would be chosen indicated the appropriateness of the system (Standefer and Saunders, 1978). Thus, the reaction with the highest value of r was used for the heterologous combinations.

5. Heterologous combinations for amphetamine and methamphetamine detection

The proper dilution of enzyme-labeled hapten and antibodies from the competitive reaction in section 4 was used in heterology studies. The possible combinations of all enzyme-labeled hapten and antibodies for competitive reaction were shown in Table 2 .

Procedure

Two or three dilutions of antisera and one or two dilutions of HRP-labeled hapten were used in competitive reaction. Amphetamine and methamphetamine aqueous solution in the concentration range of 0-5,000 ng/ml were analyzed for each combination. The procedure as already described in section 3 was followed.

Interpretation of competitive-data

The logic-plot was done for each competitive data in which the regression analysis was determined. The highest value of r for each heterologous combination would be selected as the representative competitive reaction of that heterology for further cross-reaction determination.

Table 2 Possible combinations between antiserum and enzyme-labeled hapten.

HRP-labeled hapten	Antiserum			
	3-APA-Ab	4-ABA-Ab	3-APM-Ab	4-ABM-Ab
3-APA-HRP	I*	II*	III*	IV*
4-ABA-HRP	II	I	IV	III
3-APM-HRP	III	IV	I	II
4-ABM-HRP	IV	III	II	I

* I = Homologous combinations.

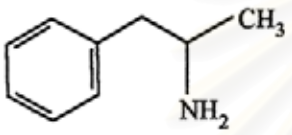
II = Bridge heterologous combinations.

III = Hapten heterologous combinations.

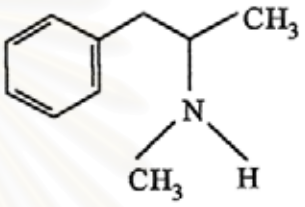
IV = Bridge and hapten heterologous combinations.

6. Cross-reaction determination

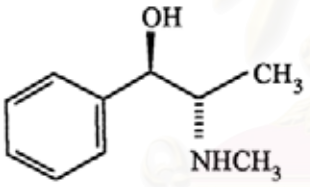
The representative heterologous combination from section 5 was confirmed for the selectivity of the competitive reaction in determining amphetamine or methamphetamine or other aromatic amine compounds. Ephedrine, pseudoephedrine and phenylpropanolamine were the amine compound used for this cross-reaction study. The similarity of chemical structure of all these compounds were shown.



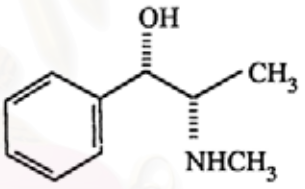
d - Amphetamine



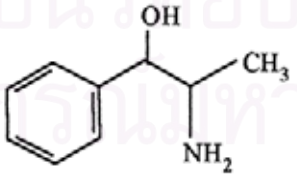
d - Methamphetamine



l - Ephedrine



d - Pseudoephedrine



dl - Phenylpropanolamine

Procedure

At the proper dilution of representative heterologous combinations for amphetamine, methamphetamine, ephedrine, pseudoephedrine and phenylpropanolamine were analyzed comparing to 1,000 ng/ml of amphetamine. The larger the concentration of these compounds used in competitive reaction, the smaller the cross-reaction was.

For representative heterologous combinations of methamphetamine, amphetamine, ephedrine, pseudoehedrine and phenylpropanolamine were also determined and compared with 1,000 ng/ml of methamphetamine in the same manner.

CHAPTER III

RESULT AND DISCUSSION

1. Chemical synthesis of amphetamine and methamphetamine derivatives.

1.1 Chemical synthesis of amphetamine derivatives

Both, N-(3-aminopropyl)amphetamine (3-APA) and N-(4-aminobutyl) amphetamine (4-ABA), synthesized were pale yellow liquid. They were confirmed by their UV, IR, NMR and MS results to be identically to those reported from Mongkolsirichaikul et al., 1993. The UV, IR NMR and MS informations were displayed in Appendix A.

1.2 Chemical synthesis of methamphetamine derivatives.

The derivatives of methamphetamine, N-(3-aminopropyl)methamphetamine (3-APM) and N-(4-aminobutyl)methamphetamine (4-ABM), were also pale yellow liquid similar to amphetamine derivatives. They were also confirmed by their UV. IR, NMR and MS results (Choi et al., 1994). The detailed information of UV, IR, NMR and MS were in Appendix B.

2. Preparation of HRP-labeled amphetamine and methamphetamine derivatives.

The products of 3-APA-HRP, 4-ABA-HRP, 3-APM-HRP, and 4-ABM-HRP were brownish amorphous powder. The aqueous solution of these compounds in PBS pH 7.4 showed the maximum absorption of derivatives at 257 and enzyme HRP at 402 nm, respectively. This confirmed the existance of both derivative and enzyme as shown in Figure 4 was the UV spectra for 3-APA-HRP, the UV spectra for 4-ABA-HRP, 3-APM-HRP and 4-ABM-HRP were in the similar pattern. The activity of HRP in every enzyme-labeled amphetamine and methamphetamine derivative was proven to be remained. Therefore, these enzyme-labeled compound were ready to be used.

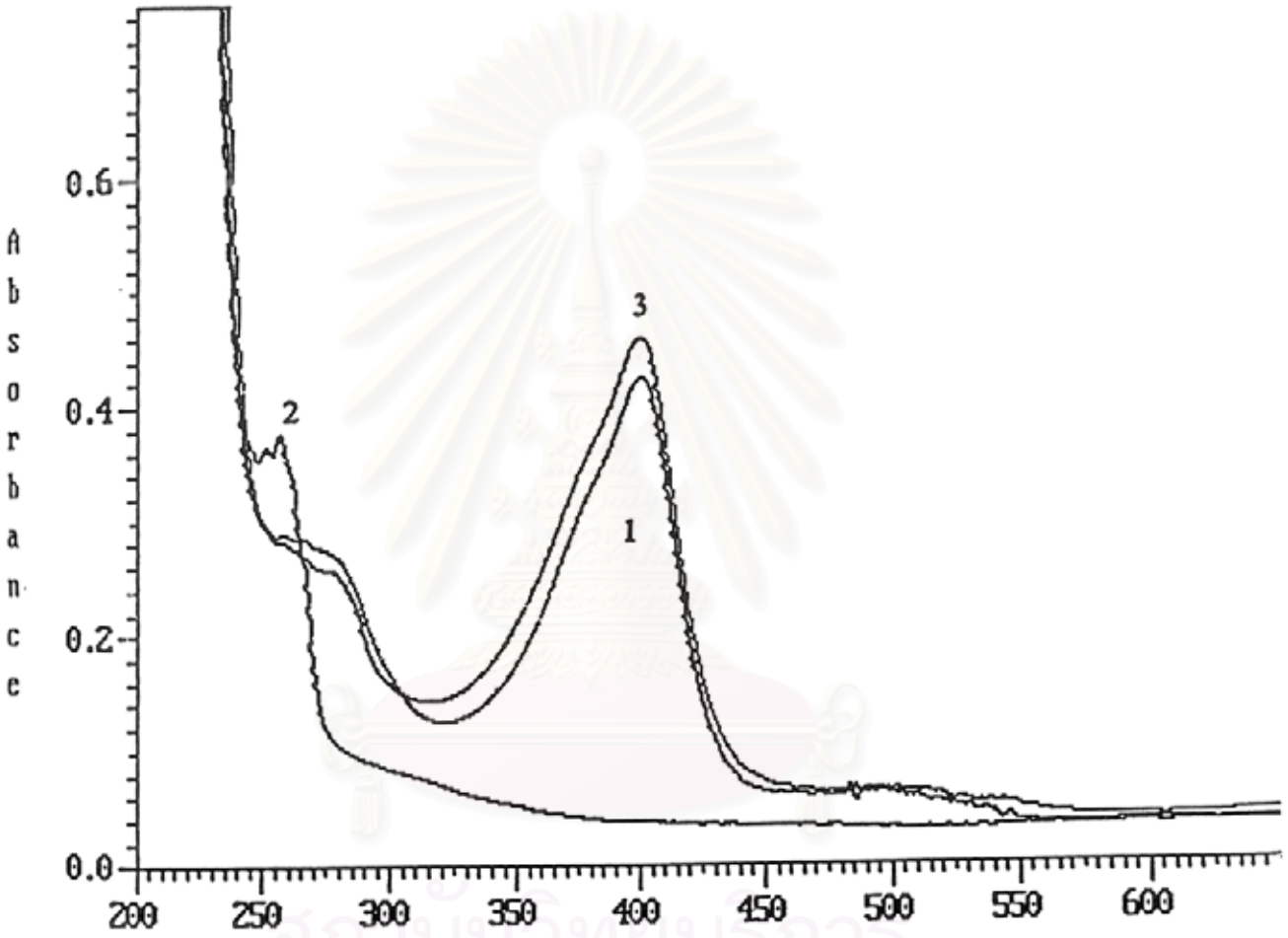


Figure 4 The UV spectra of aqueous solution of 3-APA-HRP, 3-APA and HRP-aldehyde.

1 = 3-APA-HRP 0.50 mg/ml (maximum absorbance at 402 nm)

2 = 3-APA 0.40 mg/ml (maximum absorbance at 257 nm)

3 = HRP-aldehyde 0.25 mg/ml (maximum absorbance at 402 nm)

3. Induction of antibody against amphetamine and methamphetamine in rabbits.

All four immunogens prepared, 3-APA-BSA, 4-ABA-BSA, 3-APM-BSA and 4-ABM-BSA, were white fluffy powder. Their aqueous solution exhibited the maximum absorption of derivative at 257 and BSA at 280 nm. The ultraviolet spectra of 3-APA-BSA, 3-APA and BSA in water were shown in Figure 5. This indicated that the existance of both derivative and BSA in the immunogen. The ultraviolet spectra of 4-ABA-BSA, 3-APM-BSA and 4-ABM-BSA were also similar to 3-APA-BSA. Therefore, these compounds were suitable to be immunogen.

These immunogens could induced antibody in rabbits with the titer value ranged between 1 : 2,000 – 1 : 4,000 as shown in Table 3 and Figure 6.

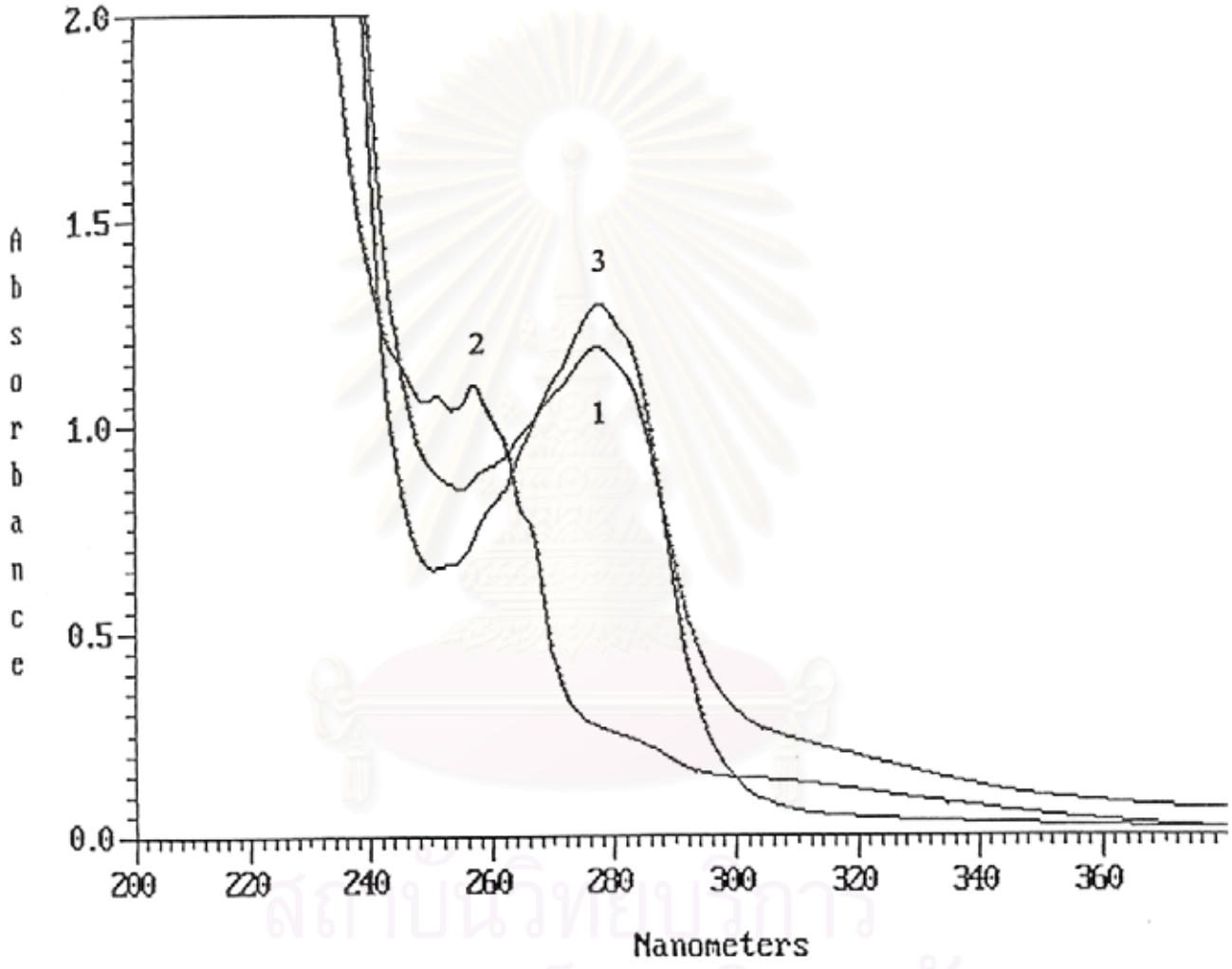


Figure 5 The UV spectra of aqueous solution of 3-APA-BSA , 3-APA and BSA

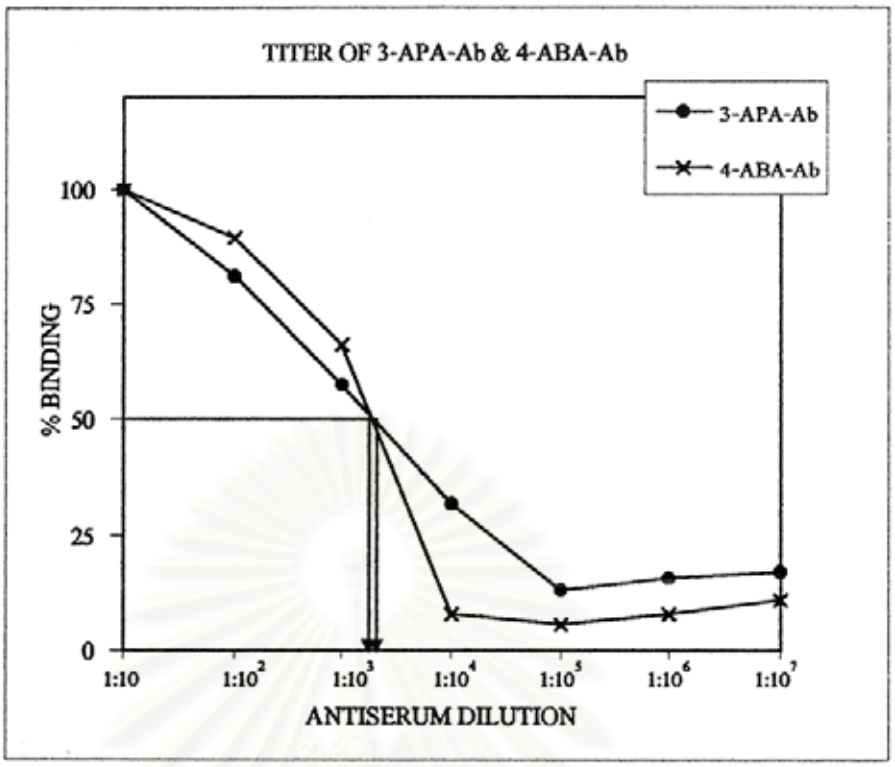
1 = 3-APA-BSA 1.80 mg/ml (maximum absorbance at 280 nm)

2 = 3-APA 0.80 mg/ml (maximum absorbance at 257 nm)

3 = BSA 1.80 mg/ml (maximum absorbance at 280 nm)

Table 3 The titer of antibodies induced in rabbits.

Immunogen	Titer
3-APA-BSA	1 : 3,000
4-ABA-BSA	1 : 3,500
3-APM-BSA	1 : 2,000
4-ABM-BSA	1 : 4,000



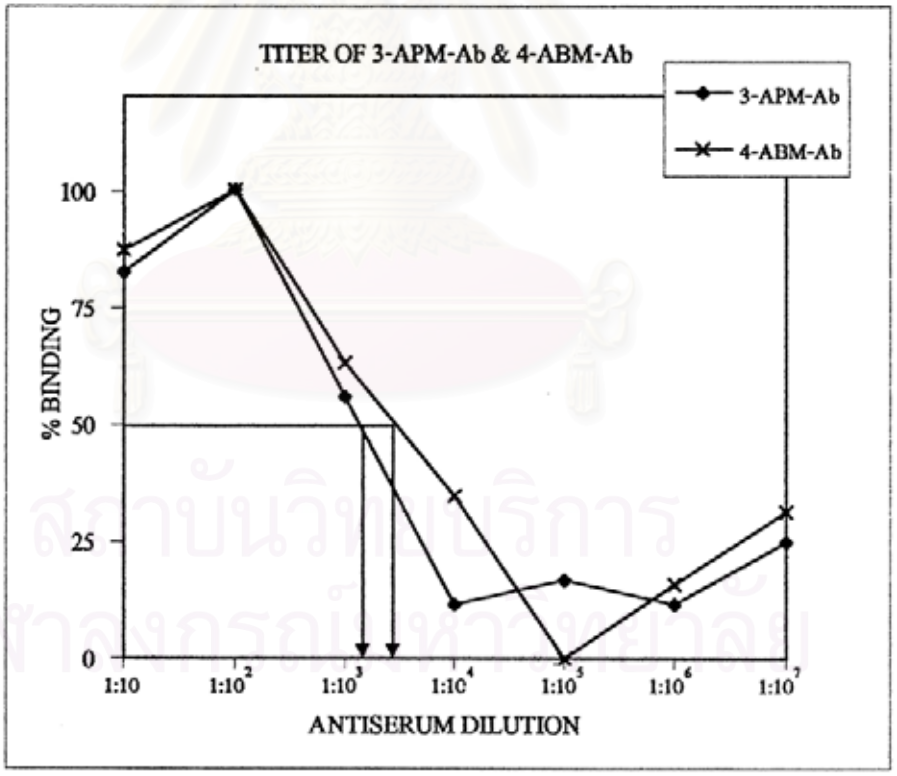


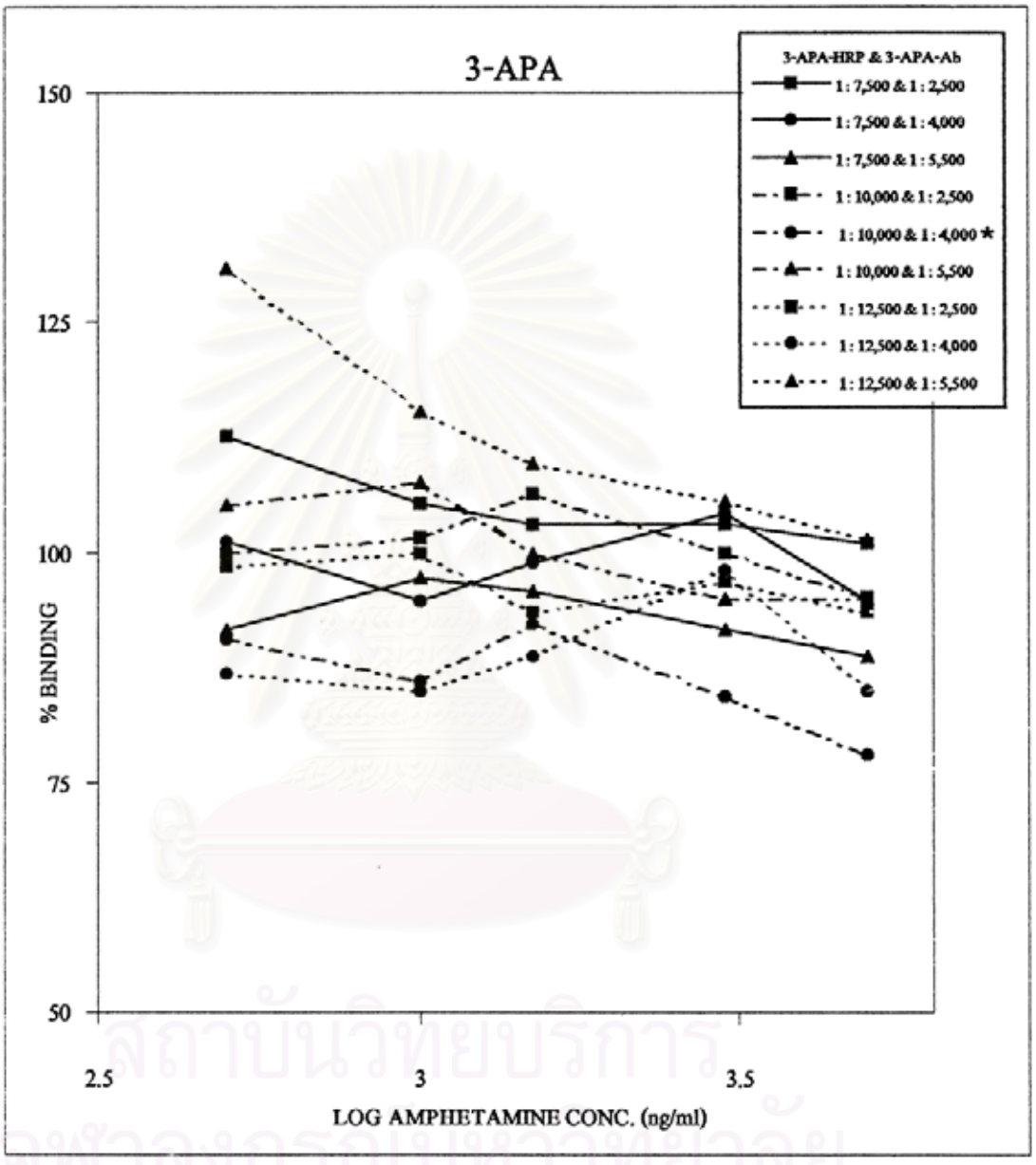
Figure 6 The relationship between %binding and dilution of antibody for titer determination.

4. Determination of proper dilution of HRP-labeled hapten and antibody used for the competitive reaction.

Competitive reaction for amphetamine detection.

A logic plot of the percentage of binding against log amphetamine concentration at different dilutions of 3-APA-HRP and 3-APA-Ab were depicted in Figure 7. The dilutions of 3-APA-HRP (1 : 10,000) and 3-APA-Ab (1 : 4,000) were the better proportion for amphetamine detection with the r value of 0.7801 as indicated in Table 4.

For 4-ABA-HRP and 4-ABA-Ab, the logic plot in Figure 8 showed the scatter response for almost every dilution used. Only the dilution of 4-ABA-HRP 1 : 12,500 and 4-ABA-Ab 1 : 3,500 as tabulated in Table 5 that showed the competitive response for amphetamine with the r value of 0.8266 .



* the selected combinations.

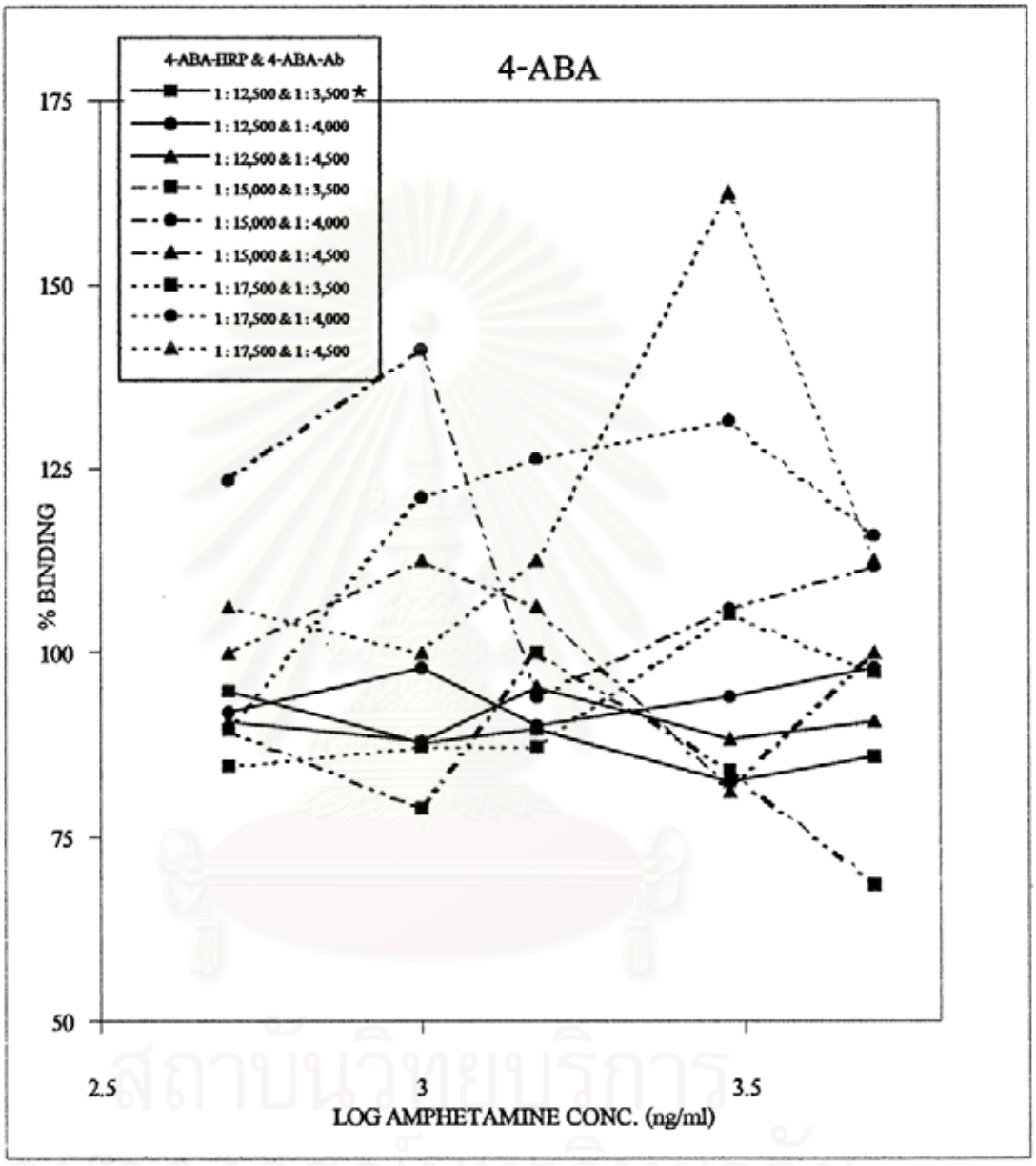
Figure 7 Dilution determination for 3-APA-HRP and 3-APA-Ab .

Table 4 Proper dilution of 3-APA-HRP and 3-APA-Ab for competitive reaction of amphetamine.

Dilution of 3-APA-HRP	Dilution of 3-APA-Ab	Correlation coefficient (r)
1 : 7,500	1 : 2,500	NR
	1 : 4,000	NR
	1 : 5,500	0.5038
1 : 10,000	1 : 2,500	NR
	1 : 4,000	0.7801*
	1 : 5,500	NR
1 : 12,500	1 : 2,500	0.6588
	1 : 4,000	0.2805
	1 : 5,500	NR

* the proper dilution for the competitive reaction.

NR no competitive response.



* the selected combinations.

Figure 8 Dilution determination for 4-ABA-HRP and 4-ABA-Ab .

Table 5 Proper dilution of 4-ABA-HRP and 4-ABA-Ab for competitive reaction of amphetamine

Dilution of 4-ABA-HRP	Dilution of 4-ABA-Ab	Correlation coefficient (r)
1 : 12,500	1 : 3,500	0.8266*
	1 : 4,000	NR
	1 : 4,500	NR
1 : 15,000	1 : 3,500	NR
	1 : 4,000	NR
	1 : 4,500	NR
1 : 17,500	1 : 3,500	NR
	1 : 4,000	NR
	1 : 4,500	NR

* the proper dilution for the competitive reaction.

NR no competitive response.

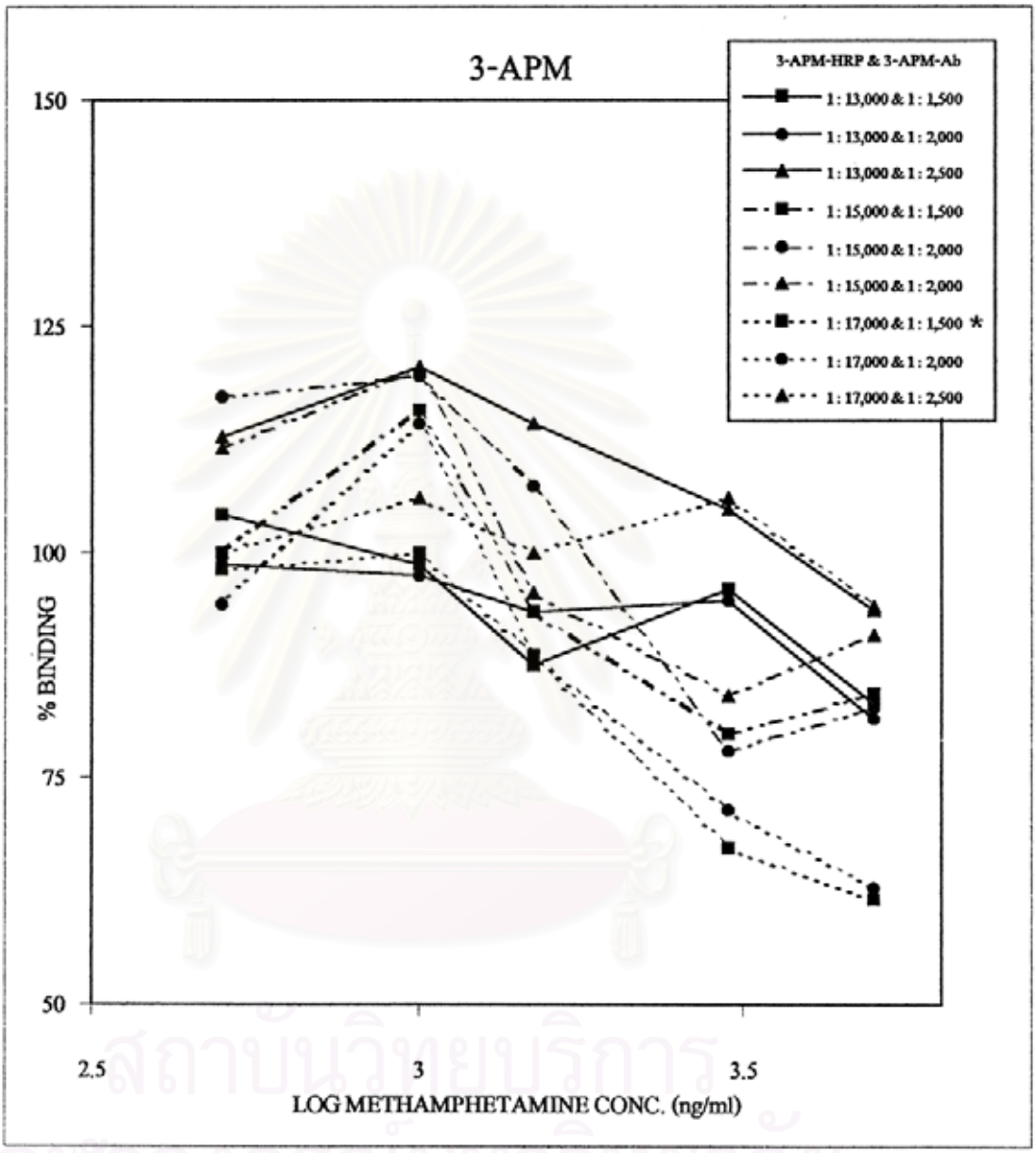
Competitive reaction for methamphetamine detection.

A logic plot of the percentage of binding against log methamphetamine concentration at different dilutions of 3-APM-HRP and 3-APM-Ab, and 4-ABM-HRP and 4-ABM-Ab were showed in Figure 9 and Figure 10, respectively.

For 3-APM, only two competitive reaction could be observed. But the dilutions of 3-APM-HRP of 1 : 17,000 and 3-APM-Ab of 1 : 1,500 were the better combination with the higher r value of 0.9406 (Table 6).

Only two competitive reaction were also displayed for 4-ABM. The better couple with the higher r value of 0.9398 was 4-ABM-HRP (1 : 10,000) and 4-ABM-Ab (1 : 3,000).

Therefore the overall suitable dilution for competitive reaction of all four derivatives for amphetamine and methamphetamine were summarized in Table 8. These dilution values would be further used in heterology determination.



* the selected combinations.

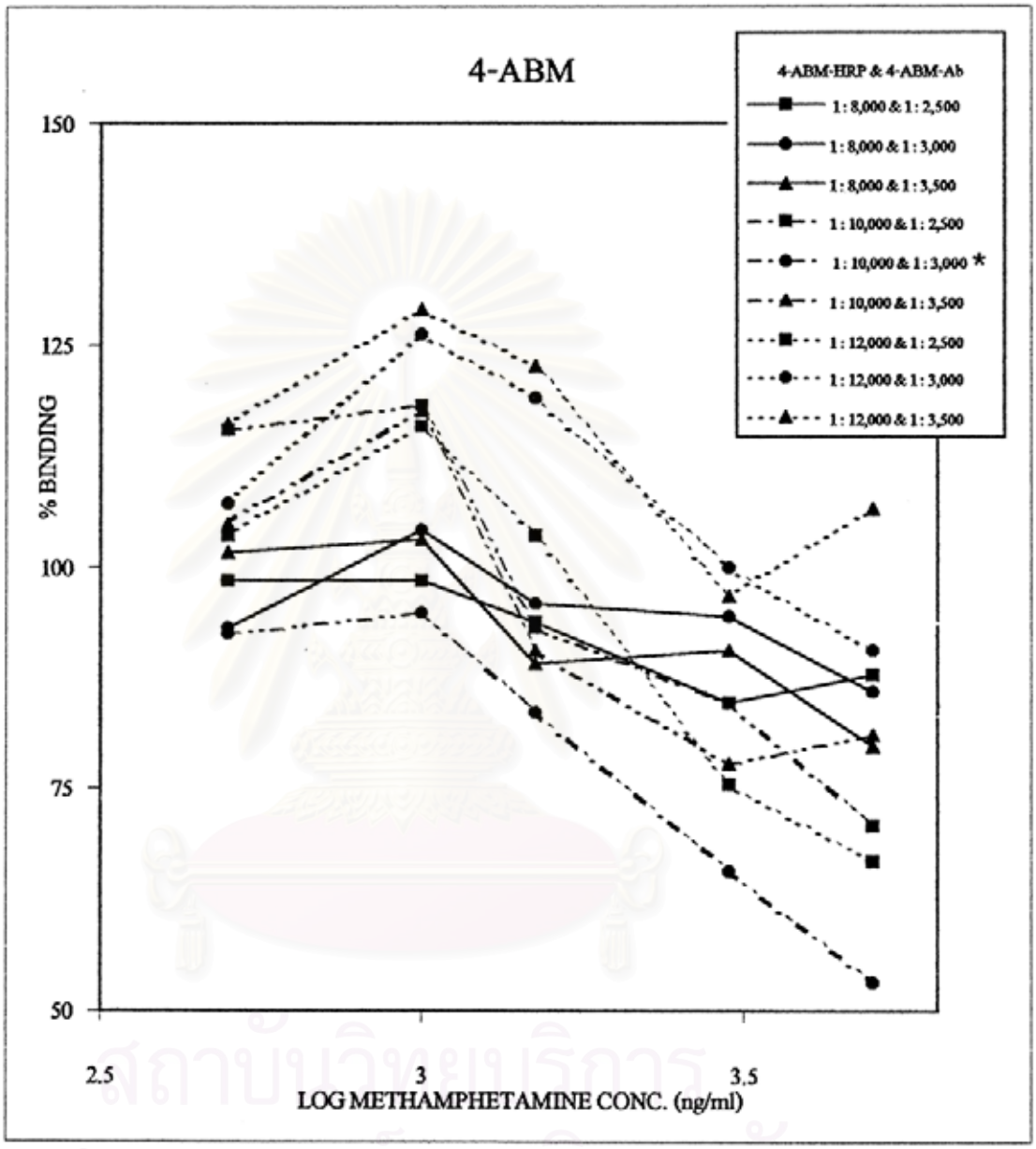
Figure 9 Dilution determination for 3-APM-HRP and 3-APM-Ab .

Table 6 Proper dilution of 3-APM-HRP and 3-APM-Ab for competitive reaction of methamphetamine.

Dilution of 3-APM-HRP	Dilution of 3-APM-Ab	Correlation coefficient (r)
1 : 13,000	1 : 1,500	NR
	1 : 2,000	0.8404
	1 : 2,500	NR
1 : 15,000	1 : 1,500	NR
	1 : 2,000	NR
	1 : 2,500	NR
1 : 17,000	1 : 1,500	0.9406*
	1 : 2,000	NR
	1 : 2,500	NR

* the proper dilution for the competitive reaction.

NR no competitive response.



* the selected combinations.

Figure 10 Dilution determination for 4-ABM-HRP and 4-ABM-Ab .

Table 7 Proper dilution of 4-ABM-HRP and 4-ABM-Ab for competitive reaction of methamphetamine.

Dilution of 4-ABM-HRP	Dilution of 4-ABM-Ab	Correlation coefficient (r)
1 : 8,000	1 : 2,500	0.8911
	1 : 3,000	NR
	1 : 3,500	NR
1 : 10,000	1 : 2,500	NR
	1 : 3,000	0.9398*
	1 : 3,500	NR
1 : 12,000	1 : 2,500	NR
	1 : 3,000	NR
	1 : 3,500	NR

* the proper dilution for the competitive reaction.

NR no competitive response.

Table 8 Summarized the proper dilution for the competitive reactions.

Derivatives	Dilution of HRP-labeled hapten	Dilution of antibodies
3-APA	1 : 10,000	1 : 4,000
4-ABA	1 : 12,500	1 : 3,500
3-APM	1 : 17,000	1 : 1,500
4-ABM	1 : 10,000	1 : 3,000

5. Heterologous combinations for amphetamine and methamphetamine detections.

5.1 Homologous study.

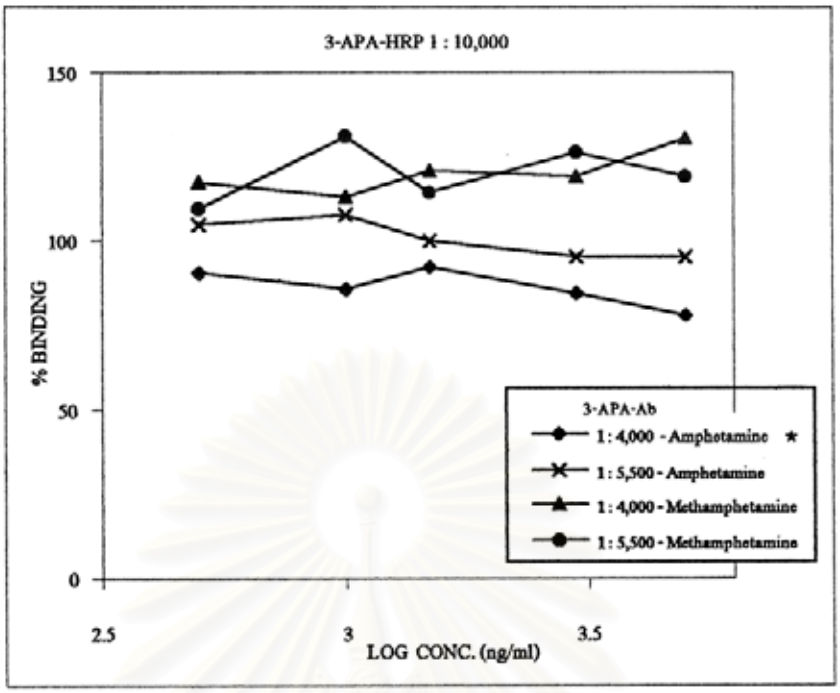
Homologous combinations between 3-APA-HRP and 3-APA-Ab.

From Figure 11(A), amphetamine could be detected by utilizing 3-APA-HRP 1 : 10,000 and 3-APA-Ab 1 : 4,000 with the r value of 0.7801. In the contrary, with this same proportion, methamphetamine couldn't be determined (Table 9). However, the competition of amphetamine with 3-APA-HRP was not quit good. Only 11% binding change per log concentration of amphetamine. This would possible be that 3-APA-HRP was strongly bound to 3-APA-Ab such that amphetamine was hardly to compete with.

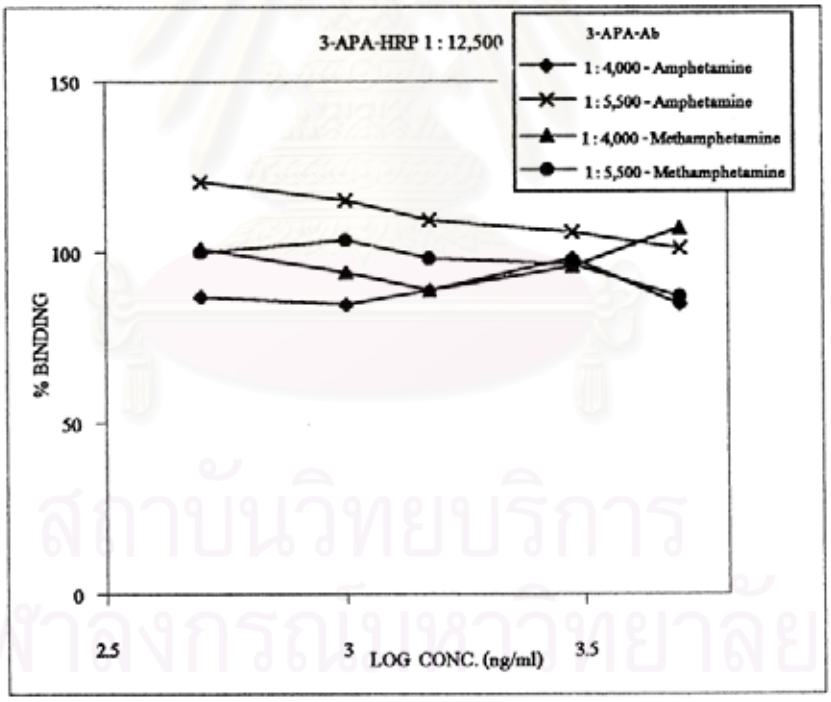
Homologous combinations between 4-ABA-HRP and 4-ABA-Ab.

The combinations of 4-ABA-HRP 1 : 12,500 and 4-ABA-Ab 1 : 3,500 was the better competitive reaction for amphetamine as shown in Figure 12(A), with the r value of 0.8266 (Table 10). Although, the competitive reaction could be observed but the sensitivity of competition was very low as indicated from the slope value of 9.498. Therefore, it might be able to conclude that homologous combinations for amphetamine or methamphetamine detection from either 3-APA or 4-ABA was not successful.

(A)



(B)



* the combinations selected.

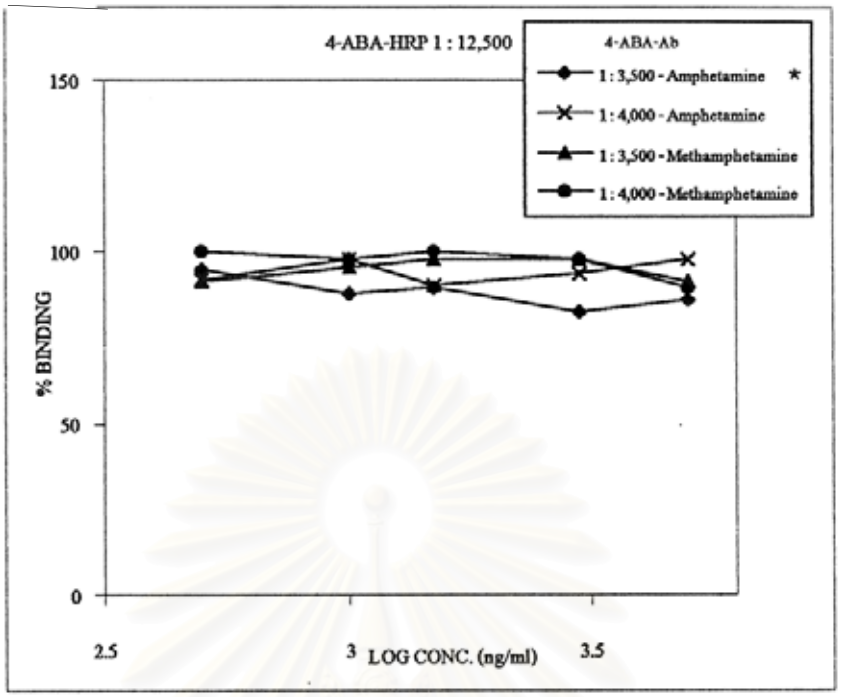
Figure 11 Dose response curve for competitive reaction of amphetamine and methamphetamine with the combinations of 3-APA-HRP and 3-APA-Ab.
(A) 3-APA-HRP 1 : 10,000 (B) 3-APA-HRP 1 : 12,500

Table 9 Detection of amphetamine and methamphetamine with the homologous combinations of 3-APA-HRP and 3-APA-Ab.

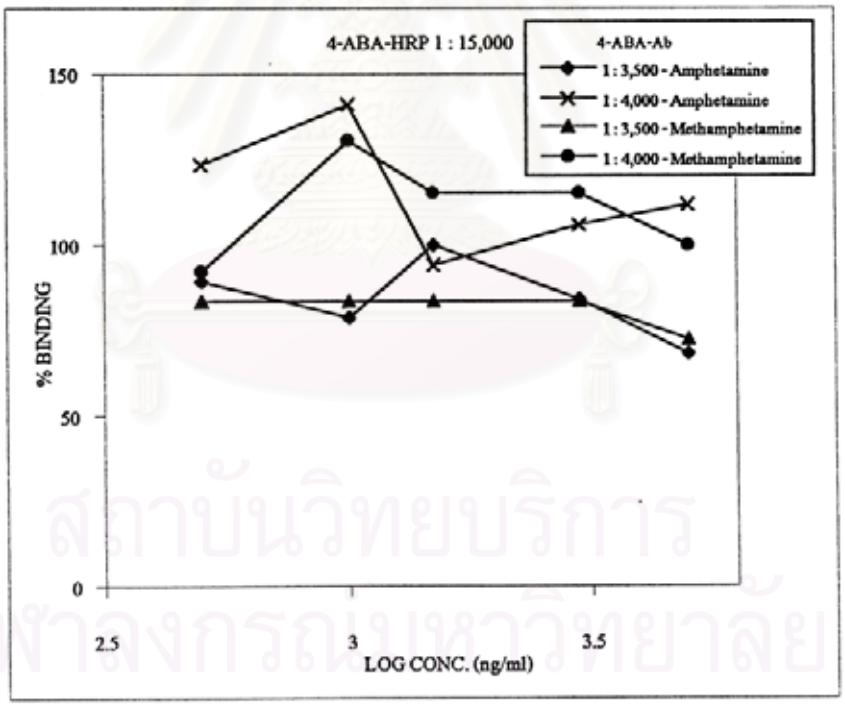
Combinations		Regression analysis			
Dilution of 3-APA-HRP	Dilution of 3-APA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 4,000	0.7801*	NR	11.07	NR
	1 : 5,500	NR	NR	NR	NR
1 : 12,500	1 : 4,000	NR	NR	NR	NR
	1 : 5,500	NR	NR	NR	NR

* the combinations selected. , NR no competitive response.

(A)



(B)



* the combinations selected.

Figure 12 Dose response curve for competitive reaction of amphetamine and methamphetamine with the combinations of 4-ABA-HRP and 4-ABA-Ab.

(A) 4-ABA-HRP 1 : 12,500 (B) 4-ABA-HRP 1 : 15,000

Table 10 Detection of amphetamine and methamphetamine with the homologous combinations of 4-ABA-HRP and 4-ABA-Ab.

Combinations		Regression analysis			
Dilution of 4-ABA-HRP	Dilution of 4-ABA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 12,500	1 : 3,500	0.8266*	NR	9.498	NR
	1 : 4,000	NR	0.7672	NR	8.592
1 : 15,000	1 : 3,500	NR	0.6958	NR	8.795
	1 : 4,000	NR	NR	NR	NR

* the combinations selected., NR no competitive response.

Homologous combinations between 3-APM-HRP and 3-APM-Ab

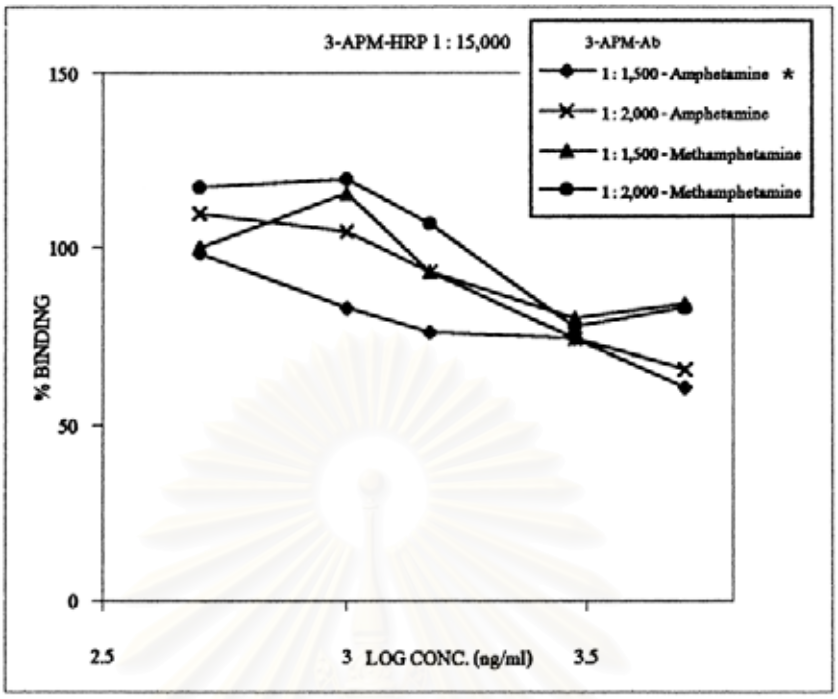
The competitive reaction of amphetamine with 3-APM-HRP in binding to 3-APM-Ab was better than methamphetamine as shown in Figure 13(A) and indicated by the r value in Table 11. The combinations of 3-APM-HRP 1 : 15,000 and 3-APM-Ab 1 : 1,500 gave the better competitive reaction for amphetamine with the r value of 0.9635. The competitive response for amphetamine was 34% binding change per log amphetamine concentration (Table 11).

Homologous combinations between 4-ABM-HRP and 4-ABM-Ab

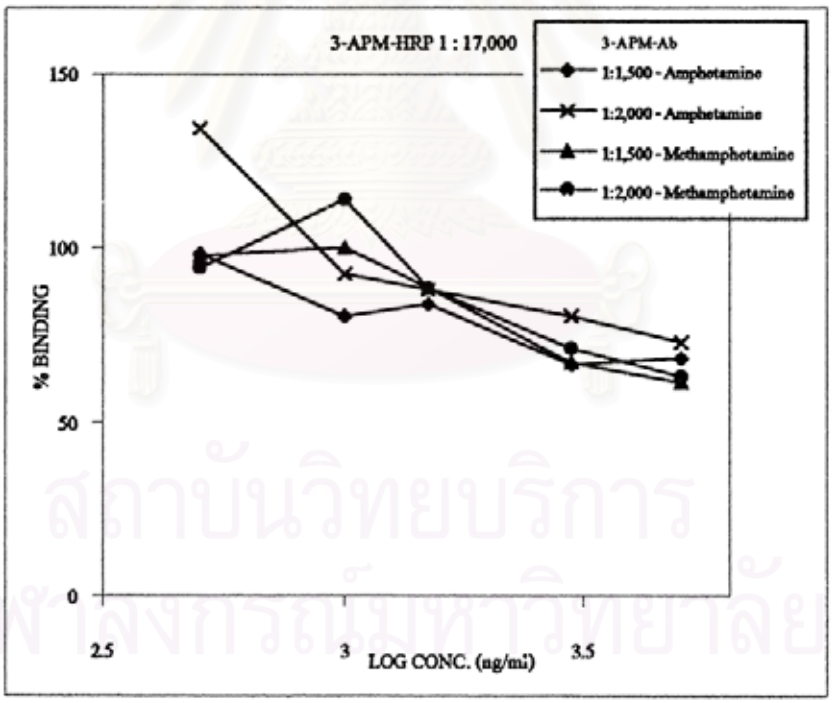
Methamphetamine could compete with the combinations of 4-ABM-HRP 1 : 10,000 and 4-ABM-Ab 1 : 3,000 [Figure 14(A)] as indicated by the r value of 0.9398 (Table 12). No competitive response of both amphetamine and methamphetamine would be notified in Figure 14(B).

From all four homologous combinations studied, the combinations that could exert the competition were summarized in Table 13. Their competitive performance were displayed in Figure 15. By comparison, it is clearly shown that amphetamine could be best detected with the combinations of 3-APM-HRP 1 : 15,000 and 3-APM-Ab 1 : 1,500 ($r = 0.9635$).

(A)



(B)



* the combinations selected.

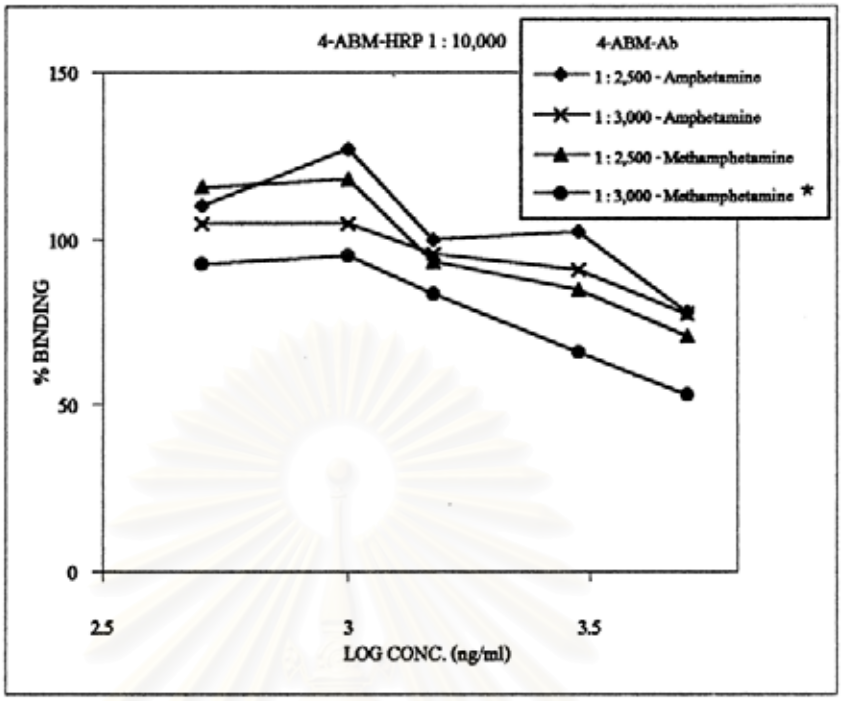
Figure 13 Dose response curve for competitive reaction of amphetamine and methamphetamine with the combinations of 3APM-HRP and 3-APM-Ab.
(A) 3-APM-HRP 1 : 15,000 (B) 3-APM-HRP 1 : 17,000

Table 11 Detection of amphetamine and methamphetamine with the homologous combinations of 3-APM-HRP and 3-APM-Ab.

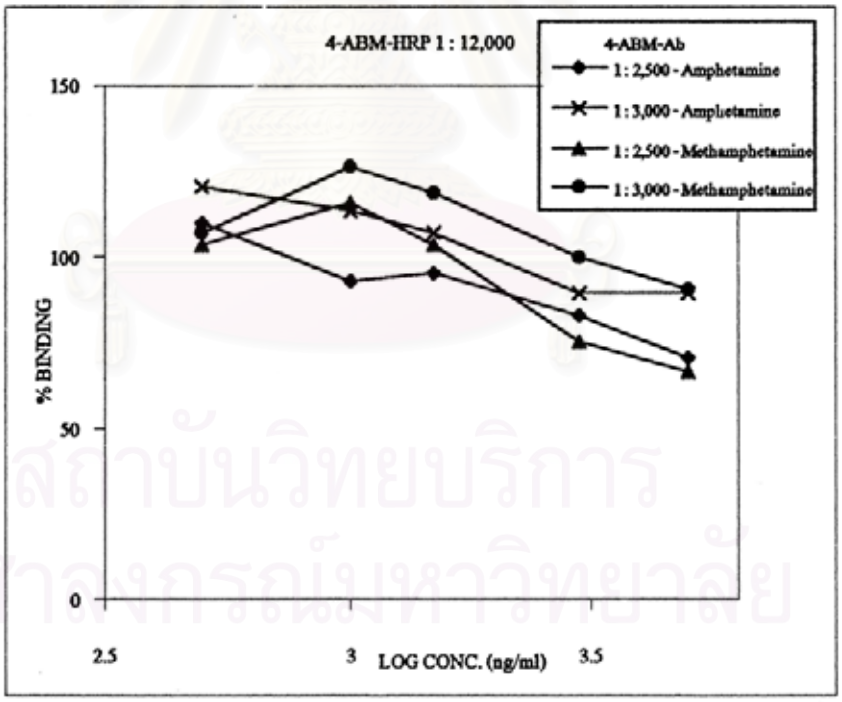
Combinations		Regression analysis			
Dilution of 3-APM-HRP	Dilution of 3-APM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 15,000	1 : 1,500	0.9635*	NR	33.97	NR
	1 : 2,000	NR	NR	NR	NR
1 : 17,000	1 : 1,500	0.9323	0.9406	30.50	42.44
	1 : 2,000	NR	NR	NR	NR

* the combinations selected. , NR no competitive response.

(A)



(B)



* the combinations selected.

Figure 14 Dose response curve for competitive reaction of amphetamine and methamphetamine with the combinations of 4-ABM-HRP and 4-ABM-Ab.
(A) 4-ABM-HRP 1 : 10,000 (B) 4-ABM-HRP 1 : 12,000

Table 12 Detection of amphetamine and methamphetamine with the homologous combinations of 4-ABM-HRP and 4-ABM-Ab.

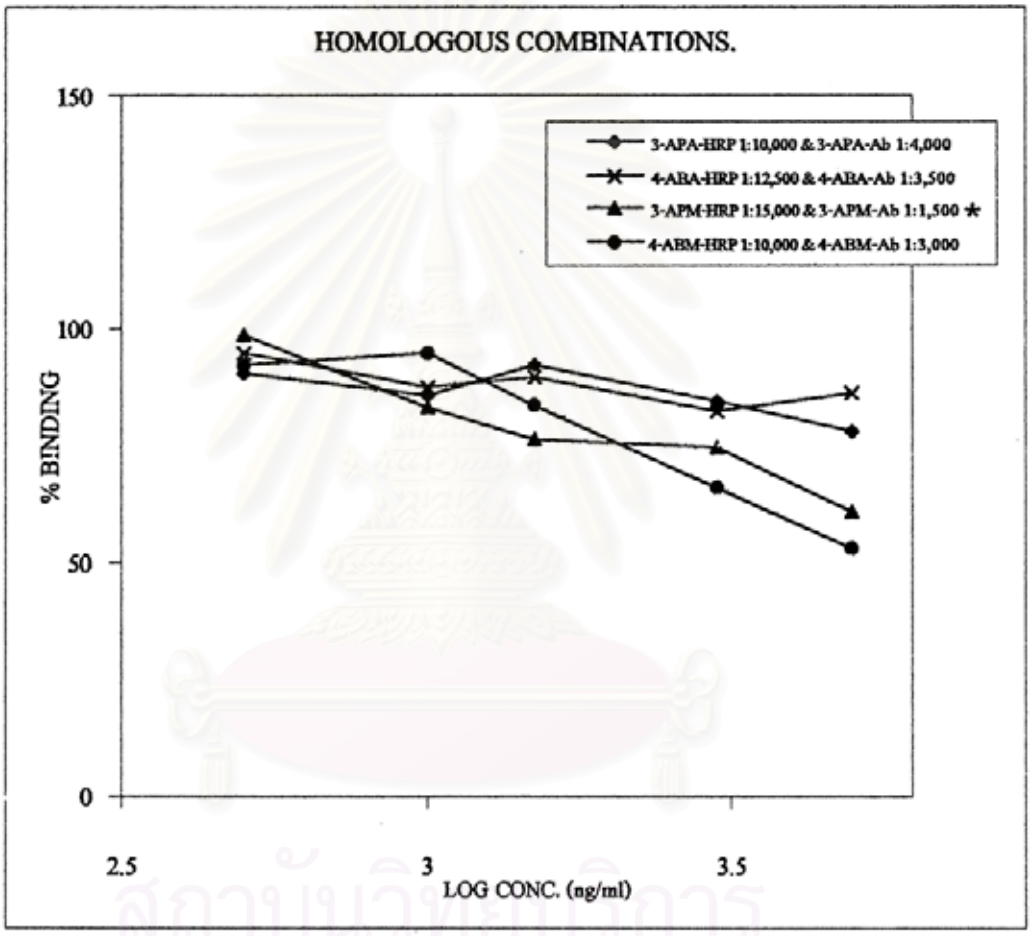
Combinations		Regression analysis			
Dilution of 4-ABM-HRP	Dilution of 4-ABM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 2,500	NR	NR	NR	NR
	1 : 3,000	NR	0.9398*	NR	42.93
1 : 12,000	1 : 2,500	NR	NR	NR	NR
	1 : 3,000	NR	NR	NR	NR

* the combinations selected. , NR no competitive response.

Table 13 The summary for homologous combinations study.

Combinations		Analyte	Regression analysis	
Enzyme-labeled (Dilution used)	Antibodies (Dilution used)		Correlation coefficient (r)	Slope
3-APA-HRP (1 : 10,000)	3-APA-Ab (1 : 4,000)	Amphetamine	0.7801	11.07
4-ABA-HRP (1 : 12,500)	4-ABA-Ab (1 : 3,500)	Amphetamine	0.8266	9.498
3-APM-HRP (1 : 15,000)	3-APM-Ab (1 : 1,500)	Amphetamine	0.9635*	33.97
4-ABM-HRP (1 : 10,000)	4-ABM-Ab (1 : 3,000)	Methamphetamine	0.9398	42.93

* the combinations selected.



* the combinations selected.

Figure 15 The competitive reaction selected for homologous study.

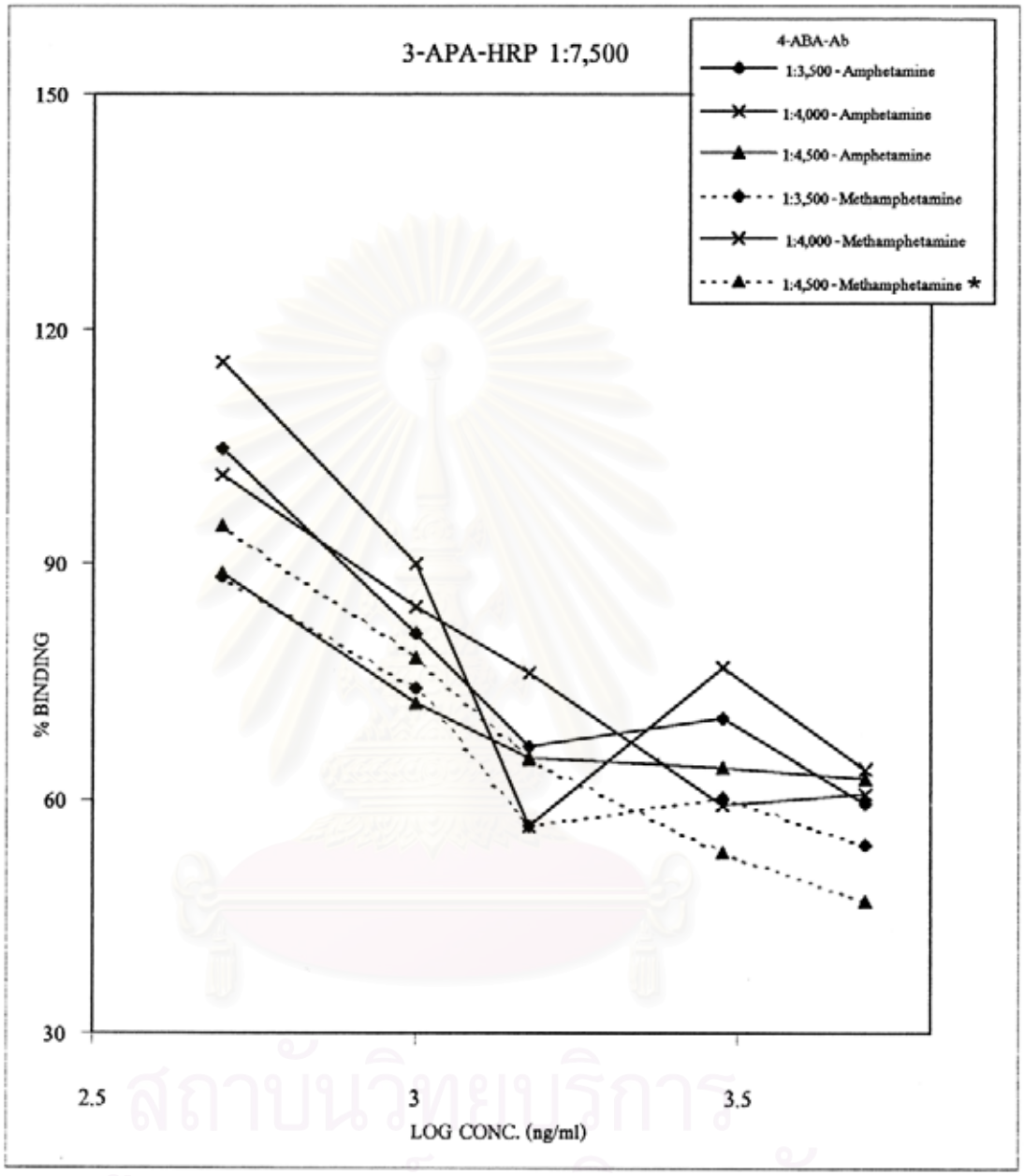
5.2 Bridge heterologous study

The combinations of 3-APA-HRP and 4-ABA-Ab

The combinations of 3-APA-HRP 1 : 7,500 and 4-ABA-Ab 1 : 4,500 , in Figure 16, gave the better competitive reaction for methamphetamine than amphetamine with the r values of 0.9881 and 0.8904, respectively. For amphetamine detection, Figure 16, the competitive reaction in the concentration range of 1,500 - 5,000 ng/ml (log concentration of 3.176 – 3.699) was not progress. The improper competitive reaction with the scattered reaction was observed, in Figure 17. Although, amphetamine and methamphetamine could be determined using 3-APA-HRP 1 : 8,500 , the competitive response showed the less r values comparing to utilizing 3-APA-HRP 1 : 7,500 . Therefore, the suitable combinations of 3-APA-HRP 1 : 7,500 and 4-ABA-Ab 1 : 4,500 for methamphetamine determination was selected with the sensitivity of the competitive binding of 48%.

The combinations of 4-ABA-HRP and 3-APA-Ab

As shown in Figure 18 for 4-ABA-HRP 1 : 10,000 , the better competitive response was observed for amphetamine at the dilution of 3-APA-Ab (1 : 6,000) with the r value of 0.9498 (Table 15). For methamphetamine detection, the saturation effect was found at methamphetamine concentration higher than 1,500 ng/ml (Figure 18). With the use of 4-ABA-HRP 1 : 12,500 , competitive response couldn't be observed from methamphetamine at any dilution of 3-APA-Ab. But at the dilution of 3-APA-Ab 1 : 5,500 and 1 : 6,000, amphetamine could be determined with the r value of 0.8827 and 0.9026, respectively. Thus, the combination of 3-APA-HRP 1 : 10,000 and 4-ABA-Ab 1 : 6,000 was then chosen for amphetamine determination with the sensitivity of competitive binding of 29%.



* the combinations selected.

Figure 16 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 3-APA-HRP 1 : 7,500 and 4-ABA-Ab.

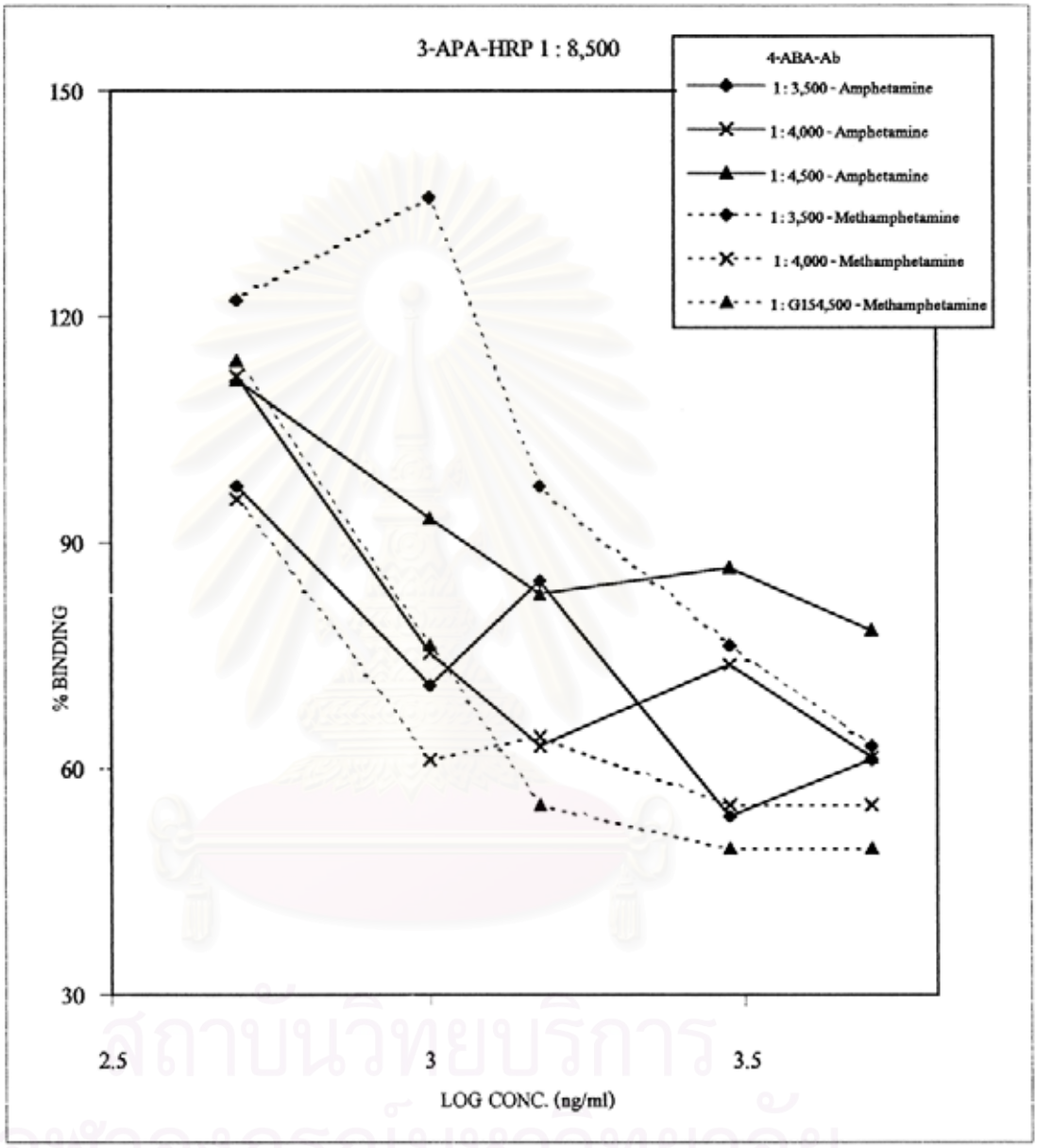
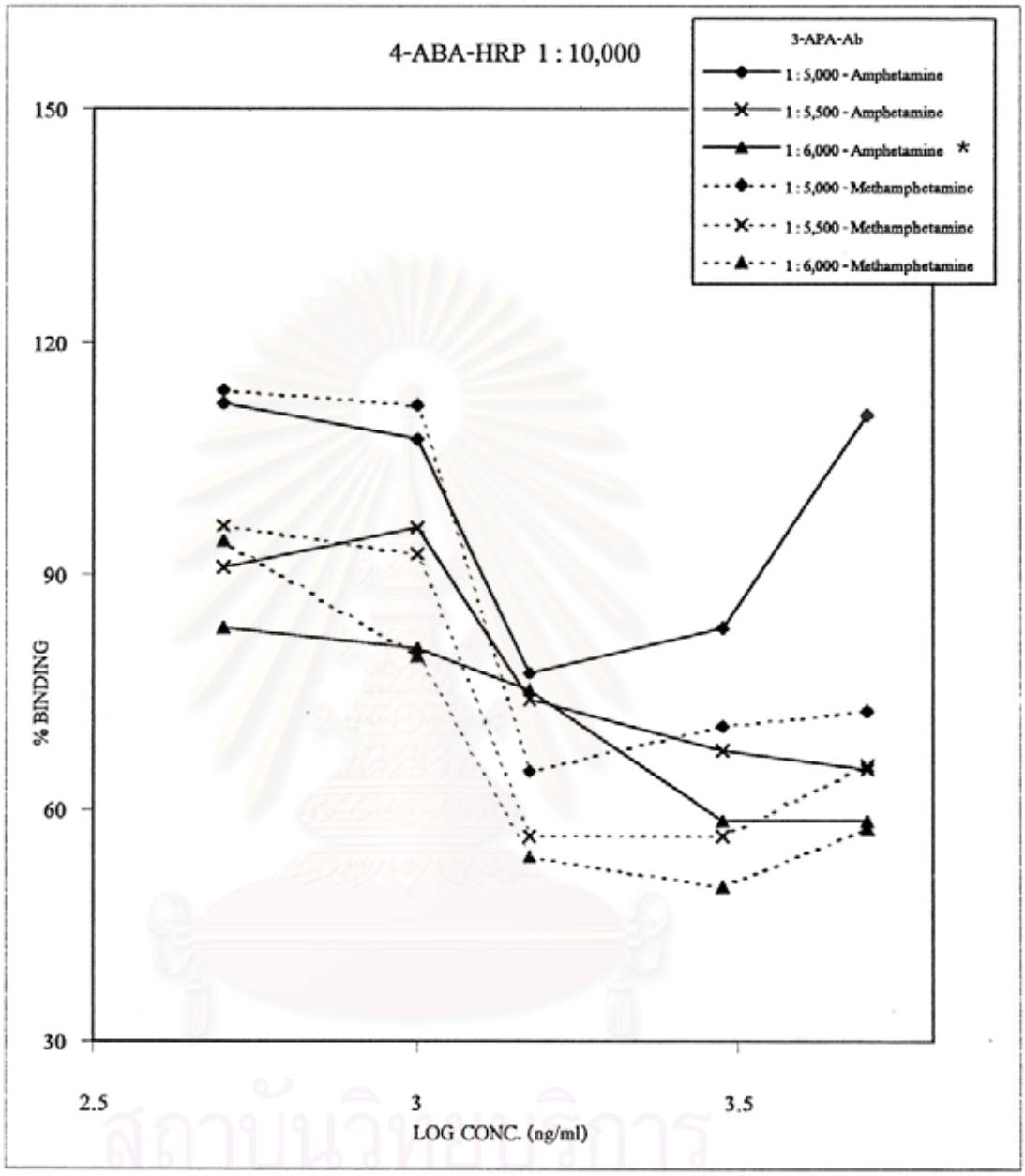


Figure 17 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 3-APA-HRP 1 : 8,500 and 4-ABA-Ab.

Table 14 Detection of amphetamine and methamphetamine with the bridge heterologous combinations of 3-APA-HRP and 4-ABA-Ab.

Combinations		Regression analysis			
Dilution of 3-APA-HRP	Dilution of 4-ABA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 7,500	1 : 3,500	NR	0.8932	NR	32.66
	1 : 4,000	NR	NR	NR	NR
	1 : 4,500	0.8904	0.9881*	24.74	48.62
1 : 8,500	1 : 3,500	0.8435	NR	38.02	NR
	1 : 4,000	NR	0.8405	NR	36.40
	1 : 4,500	NR	NR	NR	NR

* the combinations selected ., NR no competitive response.



* the combinations selected.

Figure 18 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 4-ABA-HRP 1 : 10,000 and 3-APA-Ab.

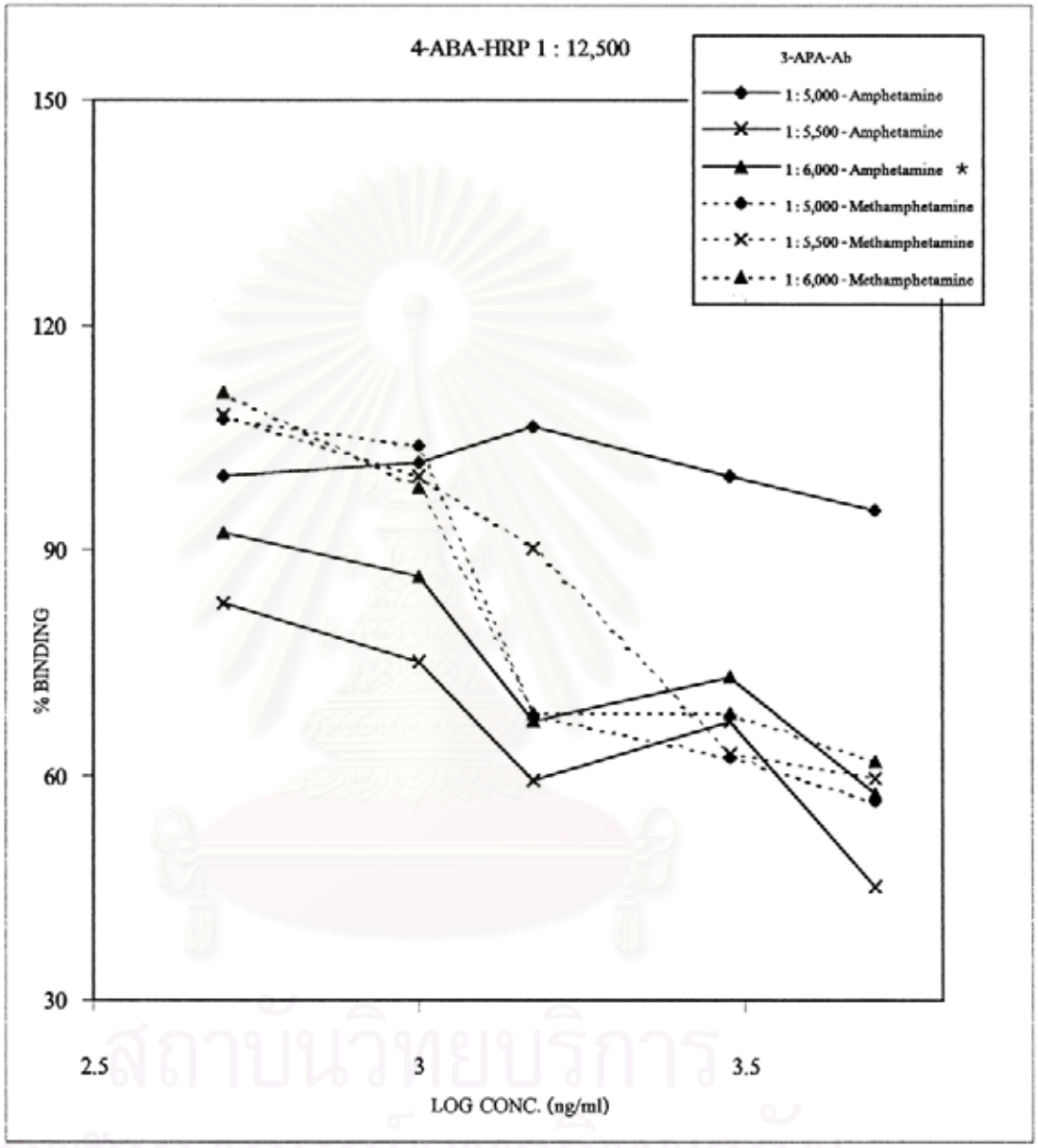


Figure 19 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 4-ABA-HRP 1 : 12,500 and 3-APA-Ab.

Table 15 Detection of amphetamine and methamphetamine with the bridge heterologous combinations of 4-ABA-HRP and 3-APA-Ab.

Combinations		Regression analysis			
Dilution of 4-ABA-HRP	Dilution of 3-APA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 5,000	NR	NR	NR	NR
	1 : 5,500	0.8821	NR	31.56	NR
	1 : 6,000	0.9498*	0.8452	28.94	41.23
1 : 12,500	1 : 5,000	NR	NR	NR	NR
	1 : 5,500	0.8827	NR	32.51	NR
	1 : 6,000	0.9026	NR	32.36	NR

* the combinations selected., NR no competitive response.

The combinations of 3-APM-HRP and 4-ABM-Ab

As shown in Figure 20 and 21, neither amphetamine nor methamphetamine could compete for 3-APM-HRP. Therefore, this combination was not suitable for amphetamine and methamphetamine detection.

The combinations of 4-ABM-HRP and 3-APM-Ab

Methamphetamine could compete with 4-ABM-HRP in the dilution of 1 : 9,000 at 3-APM-Ab of 1 : 2,000 (Figure 22) as indicated in Table 17 with the r value of 0.8094 . No competitive response for amphetamine was found. For the competitive reaction in Figure 23 at the dilution of 4-ABM-HRP of 1 : 11,000 and 3-APM-Ab of 1 : 2,000 , could be determined with the r value of 0.9883 . Therefore, the combinations of 4-ABM-HRP and 3-APM-Ab could be better used for determining amphetamine than for methamphetamine as shown in Table 17.

The competitive reaction suited for amphetamine and methamphetamine were summarized in Table 18 and portrayed in Figure 24. It is the combinations of 4-ABM-HRP 1 : 11,000 and 3-APM-Ab 1 : 2,000 that was the selected couple for amphetamine detection with the r value equal to 0.9883. The competitive binding was 13% per concentration.

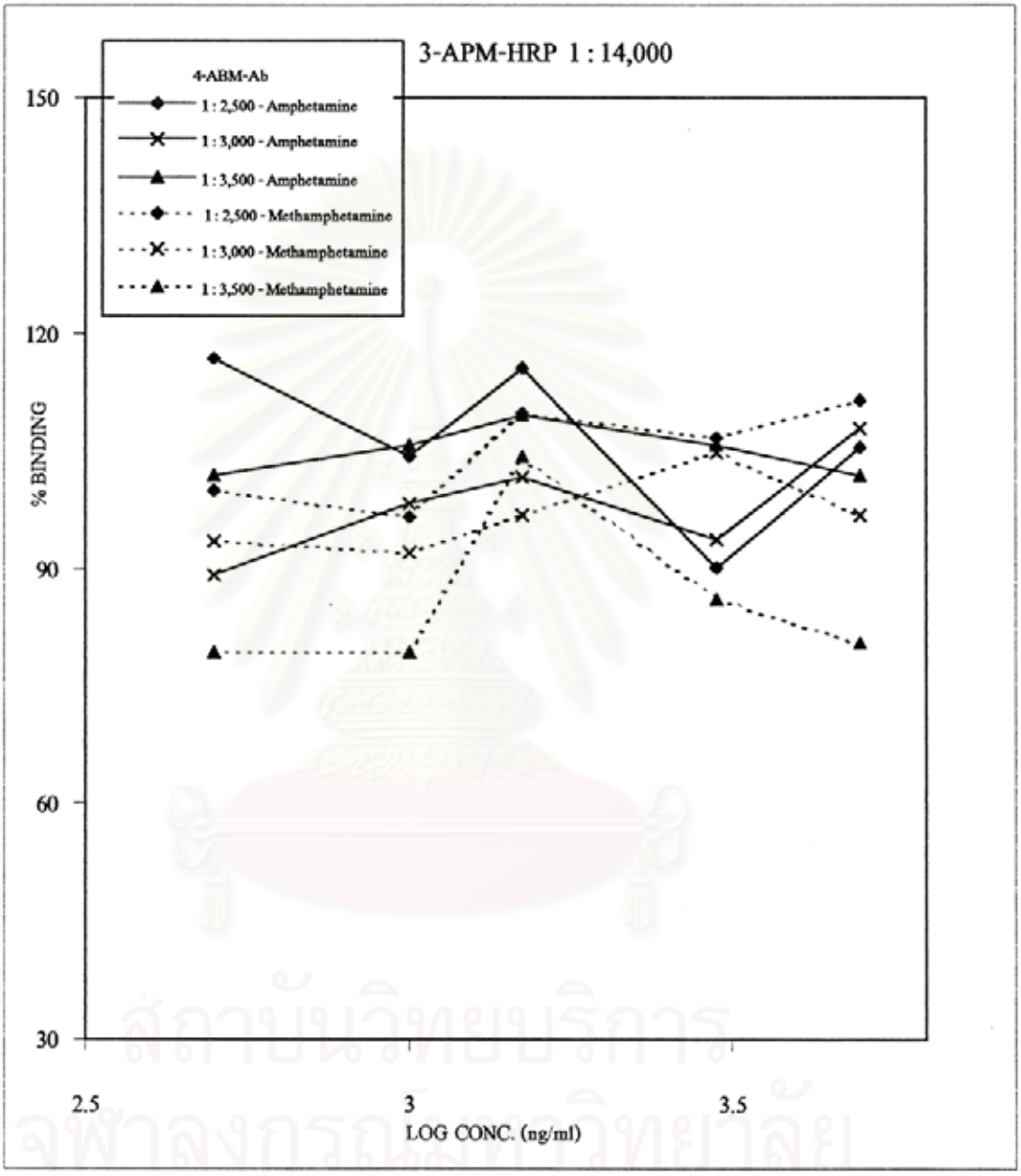


Figure 20 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 3-APM-HRP 1 : 14,000 and 4-ABM-Ab.

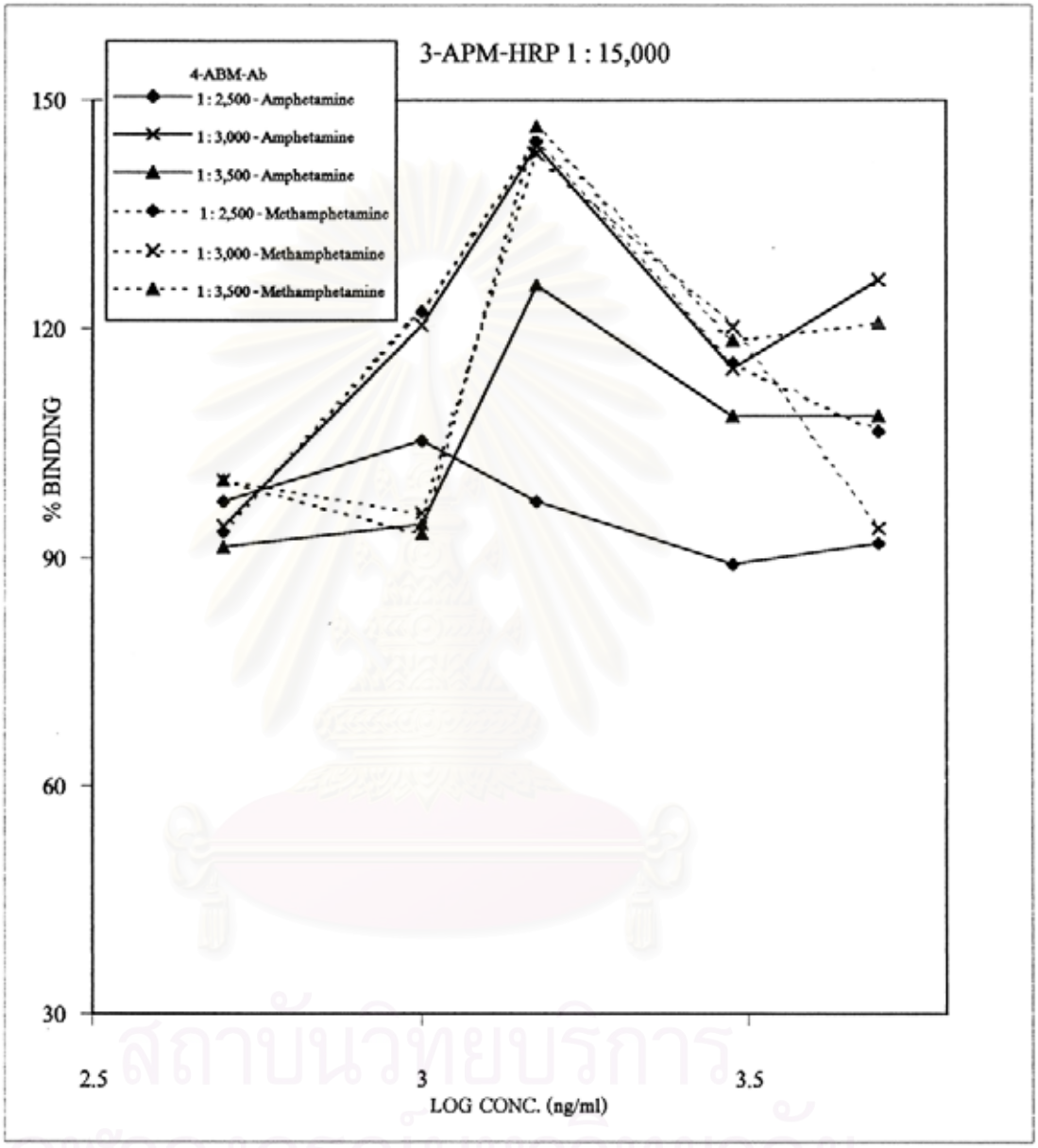


Figure 21 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 3-APM-HRP 1 : 15,000 and 4-ABM-Ab.

Table 16 Detection of amphetamine and methamphetamine with the bridge heterologous combinations of 3-APM-HRP and 4-ABM-Ab.

Combinations		Regression analysis			
Dilution of 3-APM-HRP	Dilution of 4-ABM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 14,000	1 : 2,500	NR	NR	NR	NR
	1 : 3,000	NR	NR	NR	NR
	1 : 3,500	NR	NR	NR	NR
1 : 15,000	1 : 2,500	NR	NR	NR	NR
	1 : 3,000	NR	NR	NR	NR
	1 : 3,500	NR	NR	NR	NR

NR no competitive response.

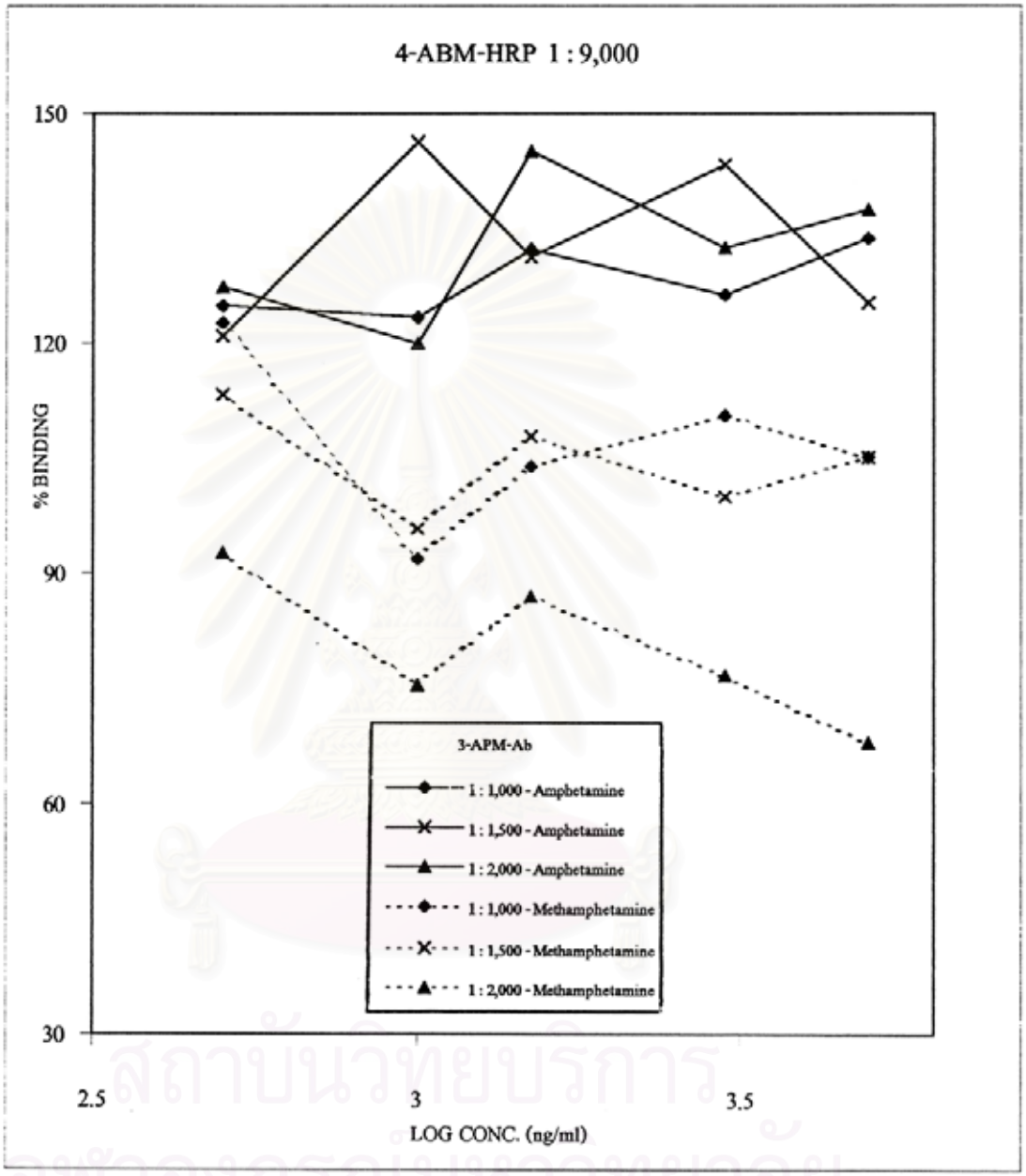
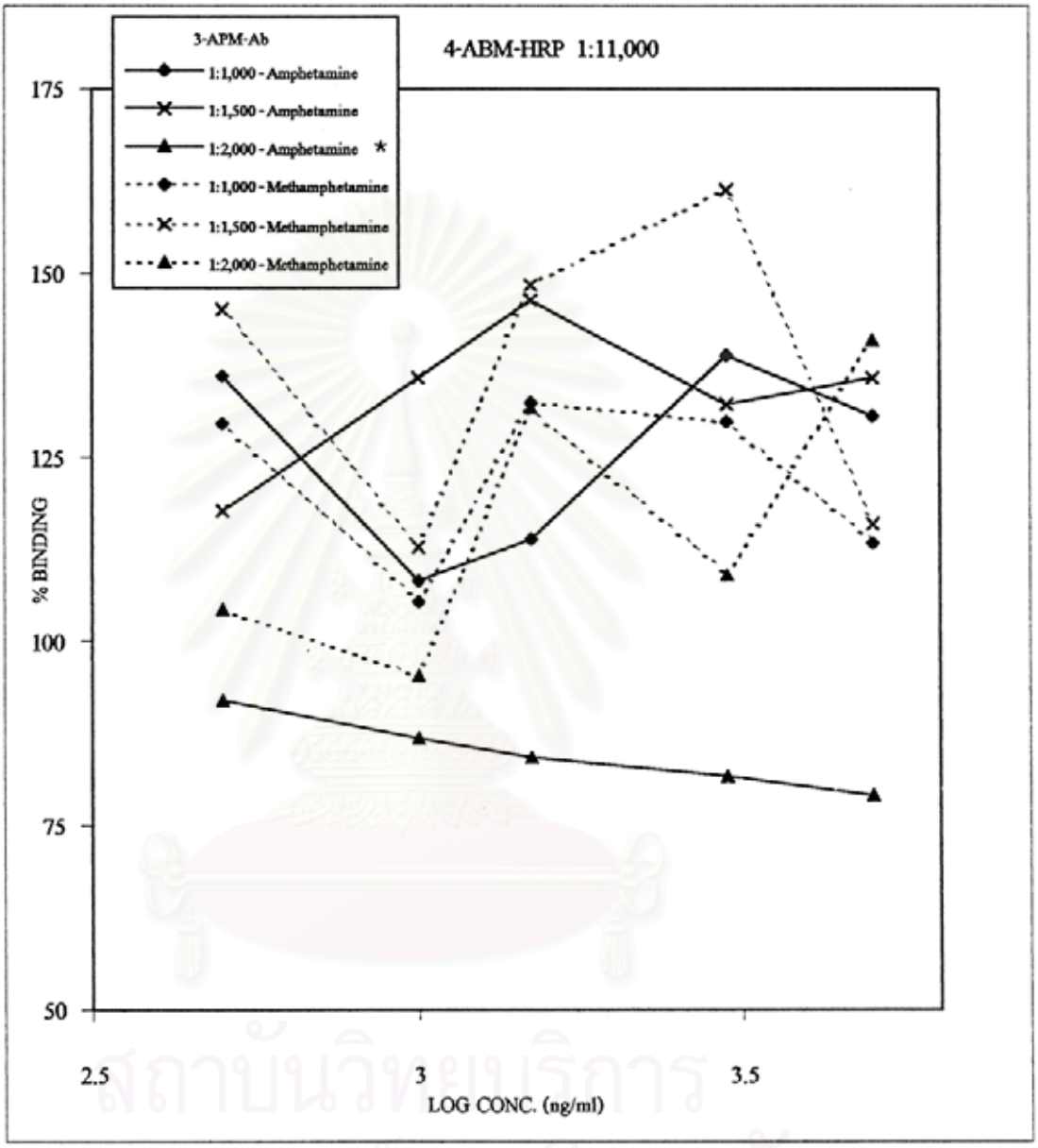


Figure 22 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 4-ABM-HRP 1 : 9,000 and 3-APM-Ab.



* the combinations selected.

Figure 23 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 4-ABM-HRP 1 : 11,000 and 3-APM-Ab.

Table 17 Detection of amphetamine and methamphetamine with the bridge heterologous combinations of 4-ABM-HRP and 3-APM-Ab.

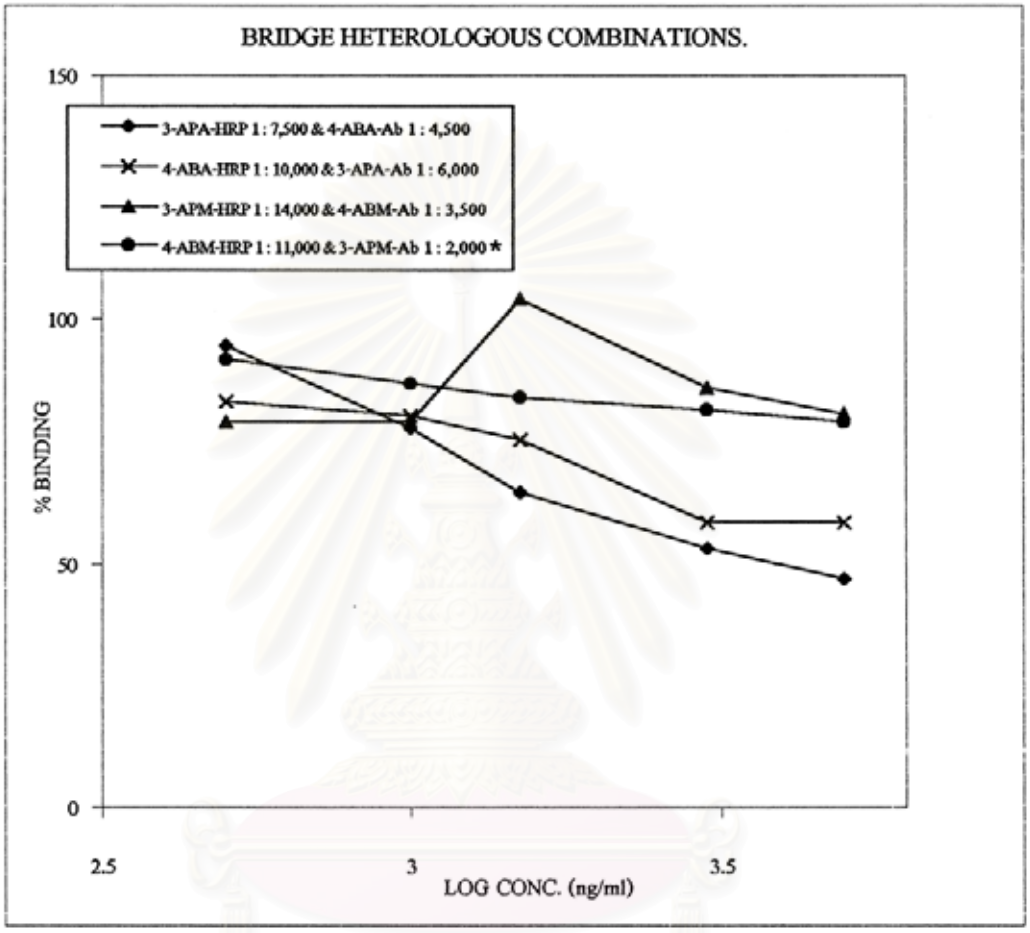
Combinations		Regression analysis			
Dilution of 4-ABM-HRP	Dilution of 3-APM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 9,000	1 : 1,000	NR	NR	NR	NR
	1 : 1,500	NR	NR	NR	NR
	1 : 2,000	NR	0.8094	NR	20.24
1 : 11,000	1 : 1,000	NR	NR	NR	NR
	1 : 1,500	NR	NR	NR	NR
	1 : 2,000	0.9883*	NR	12.68	NR

* the combinations selected. , NR no competitive response.

Table 18 The summary for bridge heterologous combinations.

Combinations		Analyte	Regression analysis	
Enzyme-labeled (Dilution used)	Antibodies (Dilution used)		Correlation coefficient (r)	Slope
3-APA-HRP (1 : 7,500)	4-ABA-Ab (1 : 4,500)	Methamphetamine	0.9881	48.62
4-ABA-HRP (1 : 10,000)	3-APA-Ab (1 : 6,000)	Amphetamine	0.9498	28.94
3-APM-HRP (1 : 14,000)	4-ABM-Ab (1 : 3,500)	Methamphetamine	NR	NR
4-ABM-HRP (1 : 11,000)	3-APM-Ab (1 : 2,000)	Amphetamine	0.9883*	12.68

* the combinations selected. , NR no competitive response.



* the combinations selected.

Figure 24 The competitive reaction selected for bridge heterologous study.

5.3 Hapten heterologous study.

The combinations of 3-APA-HRP and 3-APM-Ab

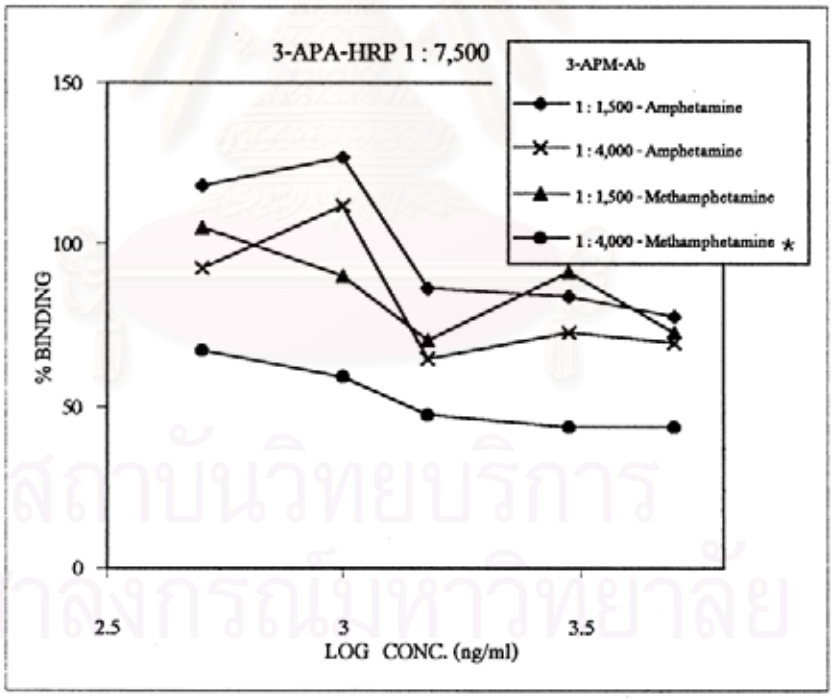
As shown in Figure 25, only the combination of 3-APA-HRP 1 : 7,500 and 3-APM-Ab 1 : 4,000 that could detect methamphetamine at the r value of 0.9351 . (Table 14) with the competitive binding of 25% per concentration

The combinations of 4-ABA-HRP and 4-ABM-Ab

Only methamphetamine was capable for competing with 4-ABA-HRP 1 : 12,500 and 4-ABM-Ab 1 : 3,000 [Figure 26(B)] was indicated from the r value of 0.9321 (Table 20). No competitive response for amphetamine and methamphetamine was observed at other combinations. Therefore, the combinations of 4-ABA-HRP and 4-ABM-Ab was not suitable for the competitive reaction for amphetamine while methamphetamine could be possibly determined with the combinations of 4-ABA-HRP 1 : 12,500 and 4-ABM-Ab 1 : 3,000.

Table 19 Detection of amphetamine and methamphetamine with the hapten heterologous combinations of 3-APA-HRP and 3-APM-Ab.

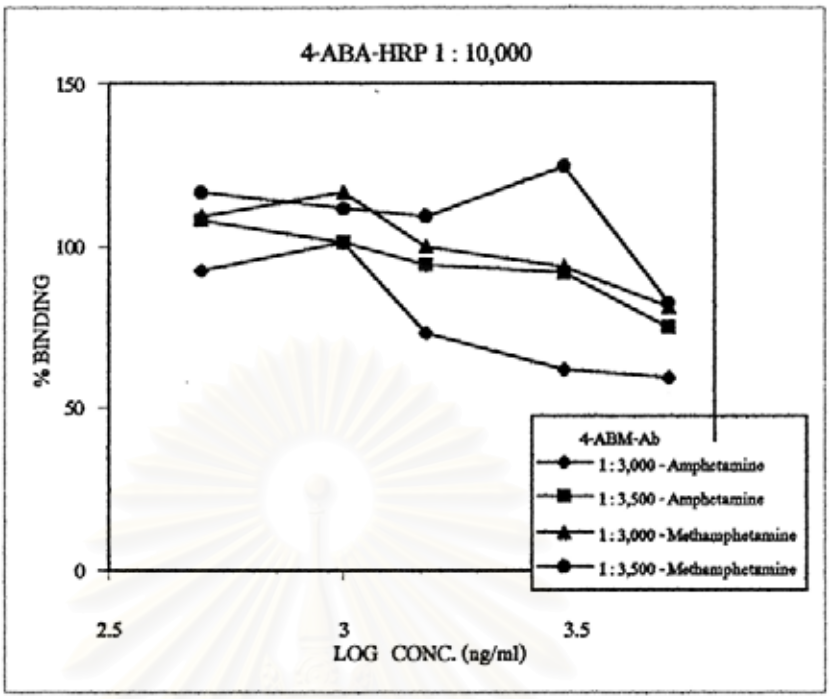
Combinations		Regression analysis			
Dilution of 3-APA-HRP	Dilution of 3-APM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 7,500	1 : 1,500	NR	NR	NR	NR
	1 : 4,000	NR	0.9351*	NR	25.39



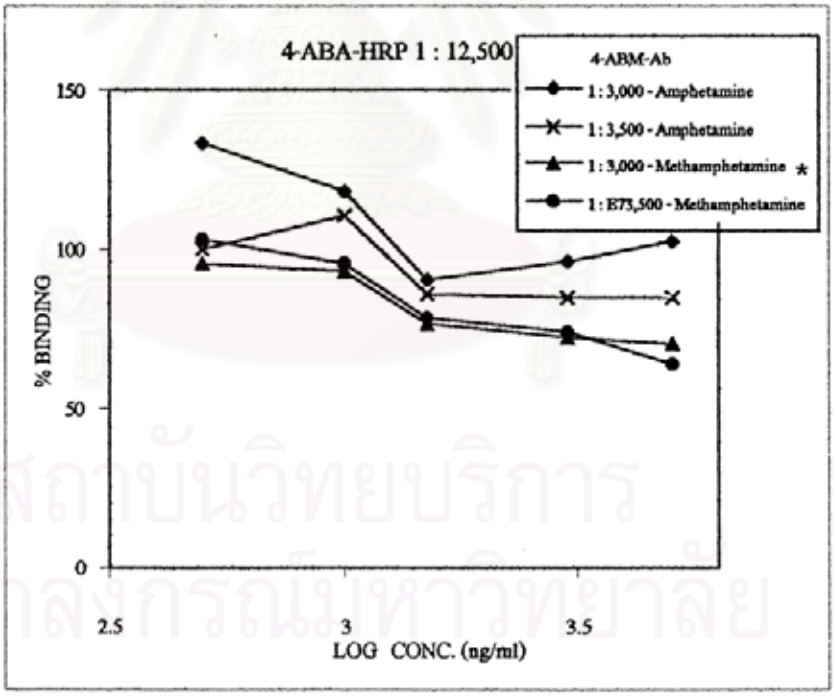
* the combinations selected ., NR no competitive response.

Figure 25 Dose response curve for competitive reaction of amphetamine and methamphteamine combined with 3-APA-HRP and 3-APM-Ab.

(A)



(B)



* the combinations selected.

Figure 26 Dose response curve for competitive reaction of amphetamine and methamphteamine combined with 4-ABA-HRP and 4-ABM-Ab.
(A) 4-ABA-HRP 1 : 10,000 (B) 4-ABA-HRP 1 : 12,500

Table 20 Detection of amphetamine and methamphetamine with the hapten heterologous combinations of 4-ABA-HRP and 4-ABM-Ab.

Combinations		Regression analysis			
Dilution of 4-ABA-HRP	Dilution of 4-ABM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 3,000	NR	NR	NR	NR
	1 : 3,500	NR	NR	NR	NR
1 : 12,500	1 : 3,000	NR	0.9321*	NR	28.58
	1 : 3,500	NR	NR	NR	NR

* the combinations selected . , NR no competitive response.

The combinations of 3-APM-HRP and 3-APA-Ab

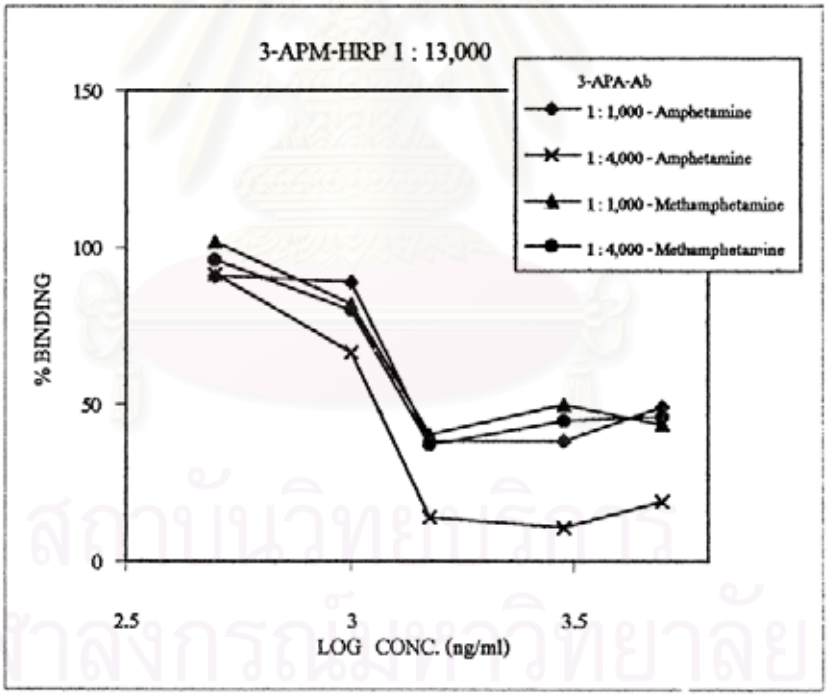
At the dilution of 3-APM-HRP 1 : 13,000 , either amphetamine or methamphetamine could be determined. But the better competitive reaction for amphetamine at the dilution of 3-APA-Ab 1 : 4,000 was confirmed by the r value of 0.8538 (Table 21). Binding saturation was clearly shown in Figure 27 at the concentration more than 1,500 ng/ml. Therefore, it was suggested that the concentration of amphetamine for competitive reaction should not more than 1,500 ng/ml.

The combinations of 4-ABM-HRP and 4-ABA-Ab

Both amphetamine and methamphetamine could compete with 4-ABM-HRP 1 : 10,000 in binding to 4-ABA-Ab 1 : 3,000 or 1 : 3,500 as shown in Figure 28. Nevertheless, the better reaction was proposed for methamphetamine with the r value of 0.9025 (Table 22). The saturation of binding occured at the concentration higher than 1,500 ng/ml (Figure 28). Thus, the concentration range of 0 - 1,500 ng/ml would be proper for methamphetamine.

Table 21 Detection of amphetamine and methamphetamine with the hapten heterologous combinations of 3-APM-HRP and 3-APA-Ab.

Combinations		Regression analysis			
Dilution of 3-APM-HRP	Dilution of 3-APA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 13,000	1 : 1,000	0.7687	NR	52.39	NR
	1 : 4,000	0.8538*	0.8112	79.33	53.31

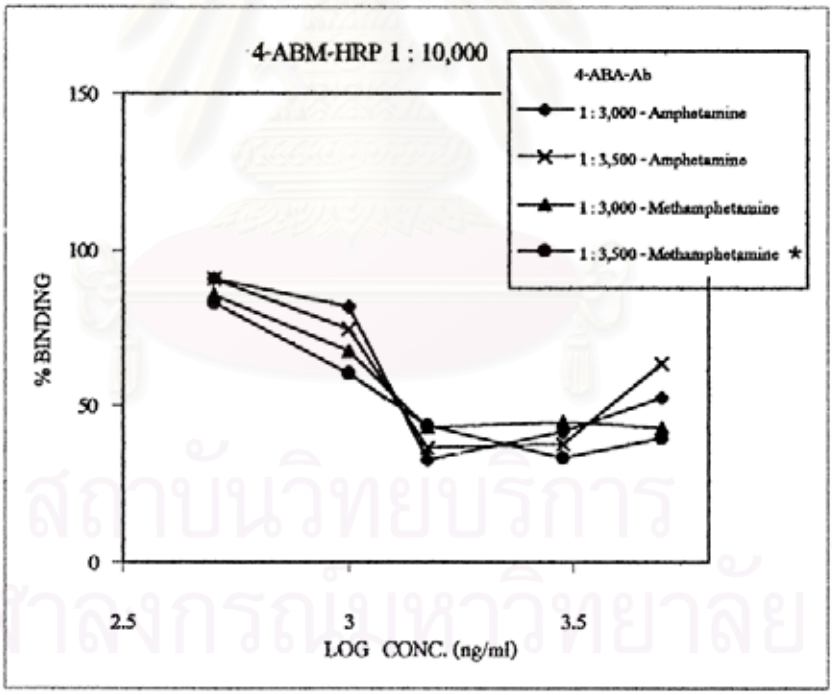


* the combinations selected. , NR no competitive response.

Figure 27 Dose response curve for competitive reaction of amphetamine and methamphteamine combined with 3-APM-HRP and 3-APA-Ab.

Table 22 Detection of amphetamine and methamphetamine with the hapten heterologous combinations of 4-ABM-HRP and 4-ABA-Ab.

Combinations		Regression analysis			
Dilution of 4-ABM-HRP	Dilution of 4-ABA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 3,000	0.7014	0.8782	45.18	42.92
	1 : 3,500	0.5987	0.9025*	35.82	46.27



* the combinations selected.

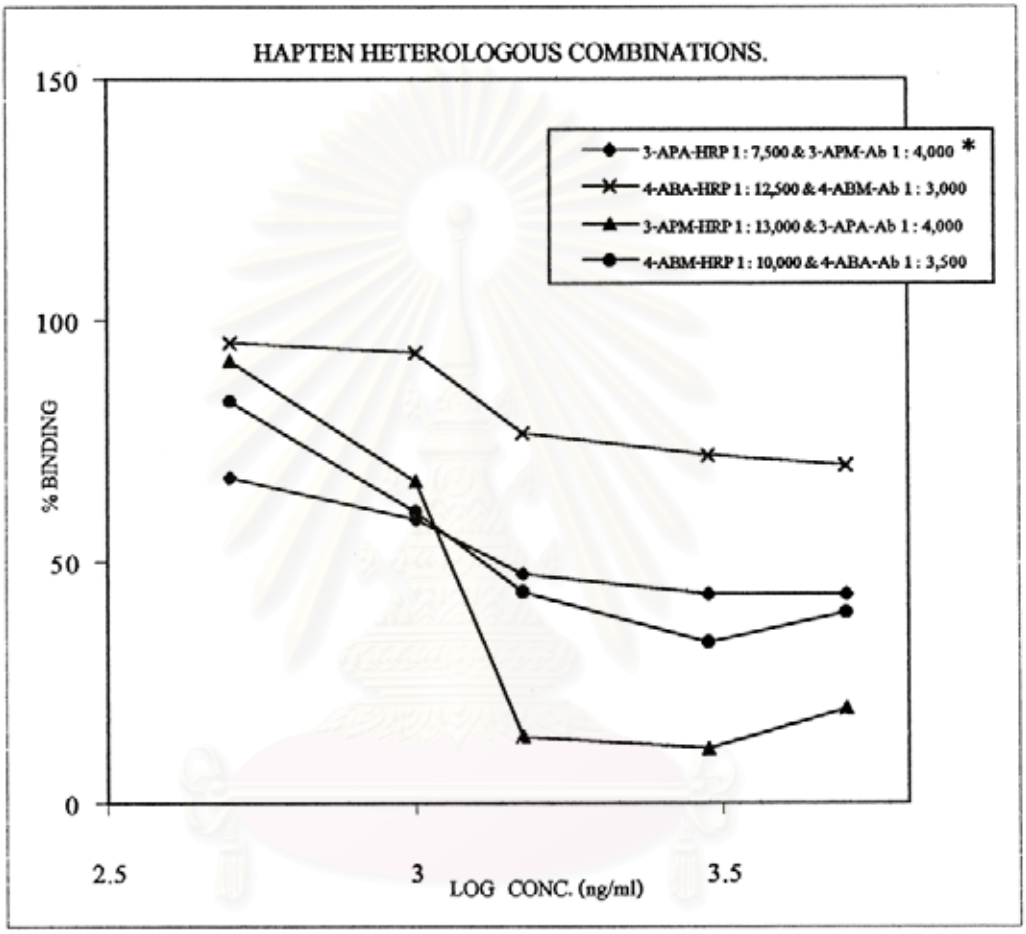
Figure 28 Dose response curve for competitive reaction of amphetamine and methamphteamine combined with 4-ABM-HRP and 4-ABA-Ab.

The competitive reactions for hapten heterologous combination study was summarized in Table 23 and depicted in Figure 29. It could then be concluded that hapten heterologous combination of 3-APA-HRP 1 : 7,500 and 3-APM-Ab 1 : 4,000 was suitable for methamphetamine rather than amphetamine with the competitive binding of 25% per concentration.

Table 23 The summary for hapten heterologous combinations study.

Combinations		Analyte	Regression analysis	
Enzyme-labeled (Dilution used)	Antibodies (Dilution used)		Correlation coefficient (r)	Slope
3-APA-HRP (1 : 7,500)	3-APM-Ab (1 : 4,000)	Methamphetamine	0.9351*	25.39
4-ABA-HRP (1 : 12,500)	4-ABM-Ab (1 : 3,000)	Methamphetamine	0.9321	28.58
3-APM-HRP (1 : 13,000)	3-APA-Ab (1 : 4,000)	Amphetamine	0.8538	79.33
4-ABM-HRP (1 : 10,000)	4-ABA-Ab (1 : 3,500)	Methamphetamine	0.9025	46.27

* the combinations selected., NR no competitive response.



* the combinations selected.

Figure 29 The competitive reaction selected for hapten heterologous study.

5.4 Bridge and hapten heterologous study

The combination of 3-APA-HRP and 4-ABM-Ab

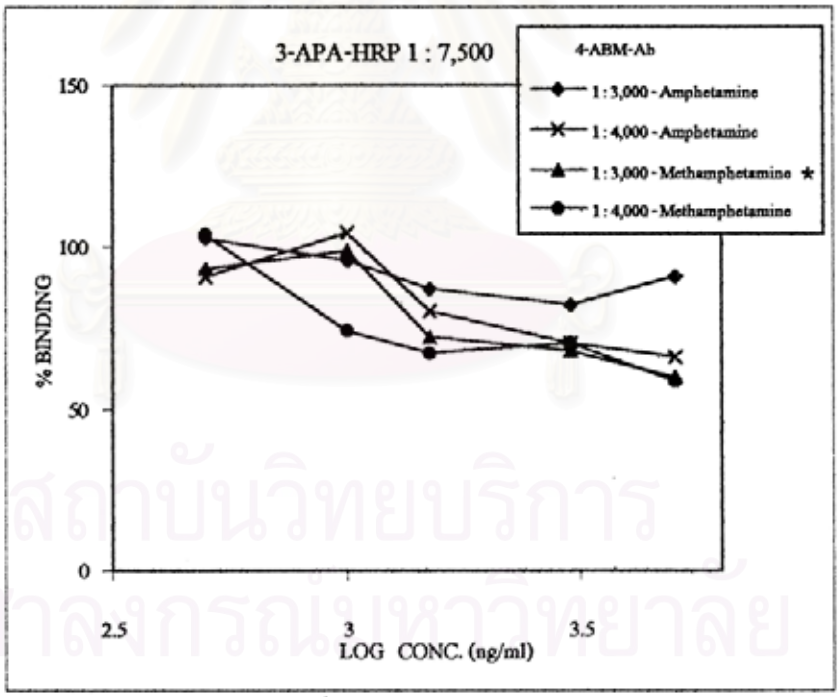
It is shown in Figure 30 that combinations of 3-APA-HRP 1 : 7,500 and 4-ABM-Ab 1 : 3,000 could be used for methamphetamine detection with the r value as shown in Table 24 to be 0.8947 No competitive response for amphetamine reaction was obtained from the reaction of the combinations between 3-APA-HRP 1 : 7,500 and 4-ABM-Ab 1 : 3,000 as well as the combinations of 3-APA-HRP 1 : 7,500 and 4-ABM-Ab 1 : 4,000 in analysis of amphetamine and methamphetamine. Therefore, with this combination only methamphetamine could be determined.

The combinations of 4-ABA-HRP and 3-APM-Ab

As depicted in Figure 31(A), the combinations of 4-ABA-HRP 1 : 10,000 and 3-APM-Ab 1 : 1,500 or 3-APM-Ab 1 : 3,500 could not propose the competitive reaction for amphetamine and methamphetamine as well as the combinations of 4-ABA-HRP 1 : 12,500 and 3-APM-Ab 1 : 3,500 for methamphetamine detection in Figure 31(B). Amphetamine could competed with 4-ABA-HRP 1 : 12,500 in binding to 3-APM-Ab 1 : 3,500 with the r value of 0.9380 Therefore, the combinations of 4-ABA-HRP and 3-APM-Ab was suitable only for amphetamine detection as shown in Table 25.

Table 24 Detection of amphetamine and methamphetamine with the bridge and hapten heterologous combinations of 3-APA-HRP and 4-ABM-Ab.

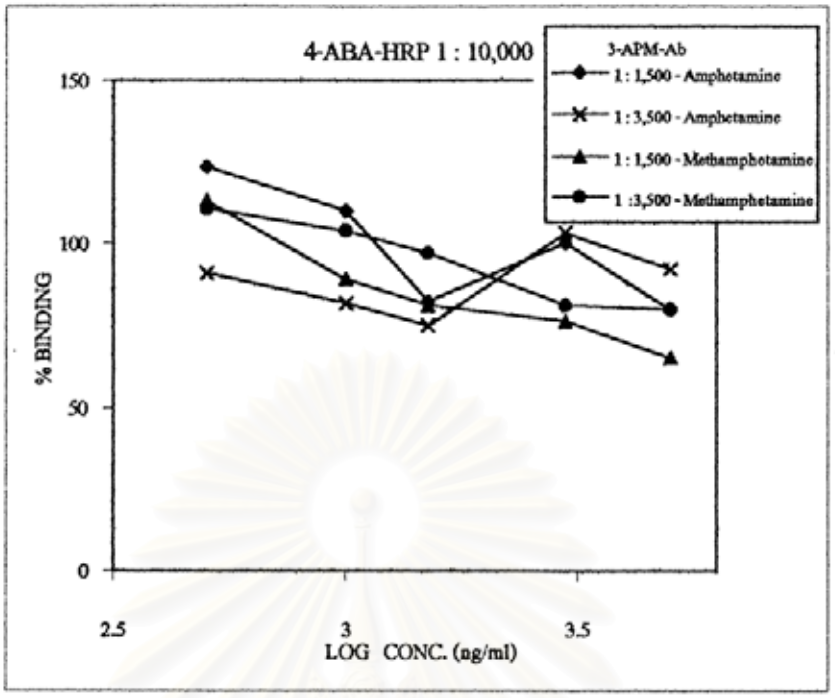
Combinations		Regression analysis			
Dilution of 3-APA-HRP	Dilution of 4-ABM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 7,500	1 : 3,000	NR	0.8947*	NR	38.77
	1 : 4,000	NR	NR	NR	NR



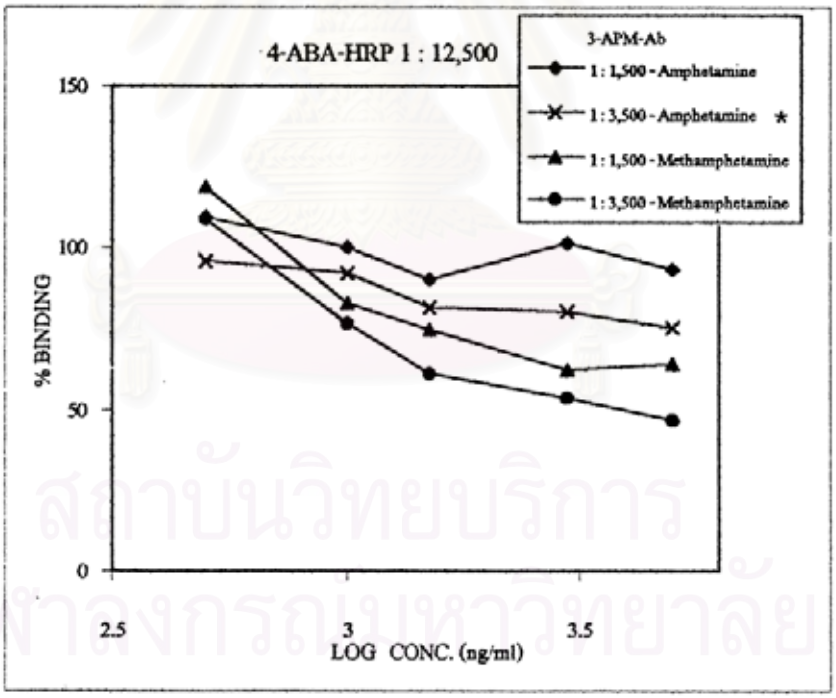
* the combinations selected., NR no competitive response.

Figure 30 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 3-APA-HRP and 4-ABM-Ab.

(A)



(B)



* the combinations selected.

Figure 31 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 4-ABA-HRP and 3-APM-Ab.
(A) 4-ABA-HRP 1 : 10,000 (B) 4-ABA-HRP 1 : 12,500

Table 25 Detection of amphetamine and methamphetamine with the bridge and hapten heterologous combinations of 4-ABA-HRP and 3-APM-Ab.

Combinations		Regression analysis			
Dilution of 4-ABA-HRP	Dilution of 3-APM-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 1,500	NR	NR	NR	NR
	1 : 3,500	NR	NR	NR	NR
1 : 12,500	1 : 1,500	NR	NR	NR	NR
	1 : 3,500	0.9380*	NR	21.11	NR

* the combinations selected., NR no competitive response.

The combinations of 3-APM-HRP and 4-ABA-Ab

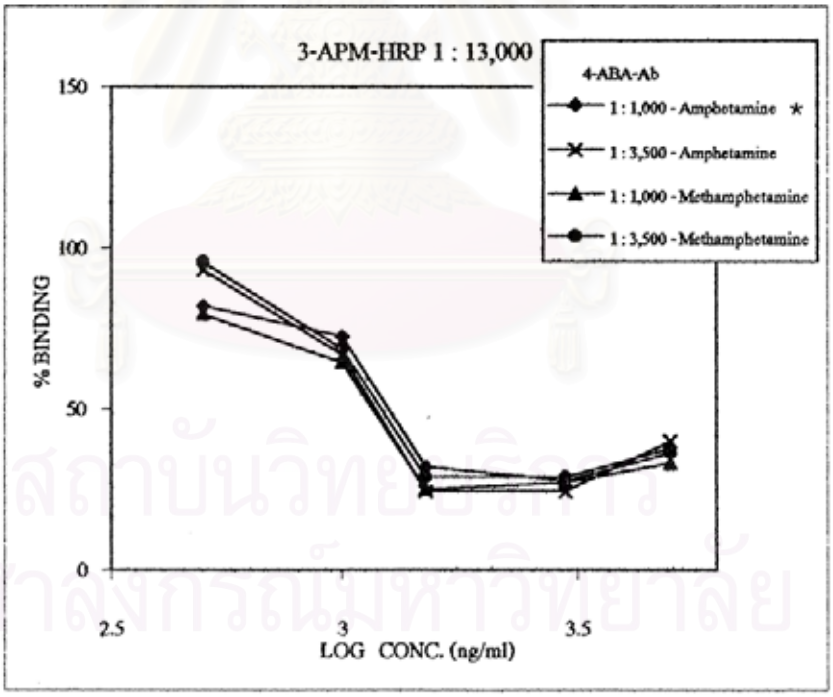
From Figure 32, amphetamine and methamphetamine showed the similar competitive response. The combinations of 3-APM-HRP 1 : 13,000 and 4-ABA-Ab 1 : 1,000 was appropriate for amphetamine detection with the r value of 0.8336 The combinations of 3-APM-HRP 1 : 13,000 and 4-ABA-Ab 1 : 3,500 gave the identical competitive reaction for amphetamine and methamphetamine as indicated in Table 26 that the r values were 0.8265 and 0.8256, respectively. The competitive reaction for amphetamine and methamphetamine, in Figure 32, showed the binding saturation of antibody binding site at concentration more than 3,000 ng/ml.

The combinations of 4-ABM-HRP and 3-APA-Ab

From Figure 33, the combinations of 4-ABM-HRP 1 : 10,000 and 3-APA-Ab 1 : 4,000 was the possible couple for amphetamine and methamphetamine detection in which the r value as indicated in Table 27 to be 0.9472 and 0.9017, respectively. The result showed that the competition seem not to progress at analyte concentration higher than 3,000 ng/ml. The couple of 4-ABM-HRP 1 : 10,000 and 3-APA-Ab 1 : 4,000 gave the better competitive reaction for amphetamine than methamphetamine but the sensitivity for amphetamine was less than for methamphetamine such that the slope values shown to be 37.96 and 51.57, respectively.

Table 26 Detection of amphetamine and methamphetamine with the bridge and hapten heterologous combinations of 3-APM-HRP and 4-ABA-Ab.

Combinations		Regression analysis			
Dilution of 3-APM-HRP	Dilution of 4-ABA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 13,000	1 : 1,000	0.8336*	0.8152	53.68	50.93
	1 : 3,500	0.8265	0.8256	59.16	61.86

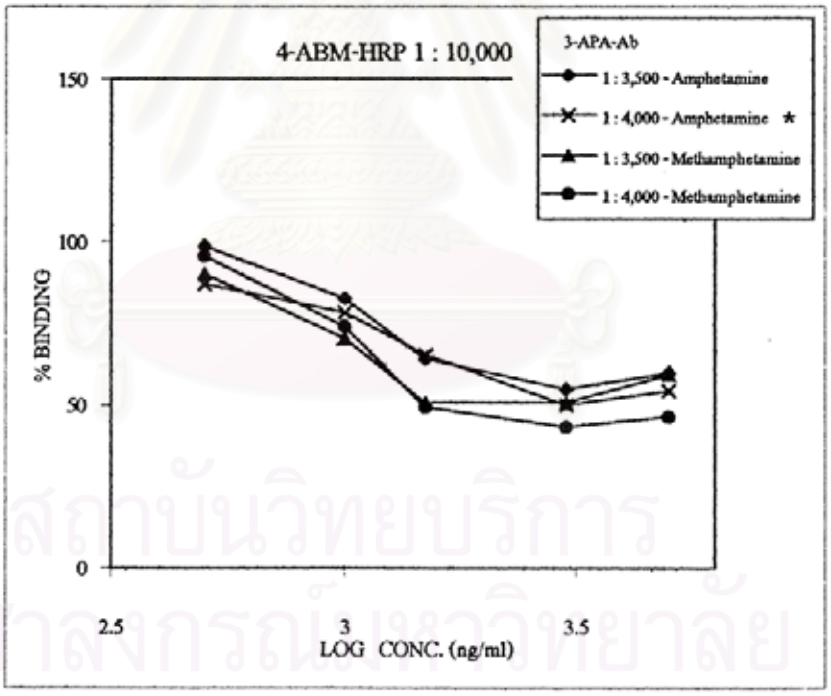


* the combinations selected.

Figure 32 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 3-APM-HRP and 4-ABA-Ab.

Table 27 Detection of amphetamine and methamphetamine with the bridge and hapten heterologous combinations of 4-ABM-HRP and 3-APA-Ab.

Combinations		Regression analysis			
Dilution of 4-ABM-HRP	Dilution of 3-APA-Ab	Correlation coefficient (r)		Slope	
		Amphetamine	Methamphetamine	Amphetamine	Methamphetamine
1 : 10,000	1 : 3,500	0.9099	0.7777	42.33	32.87
	1 : 4,000	0.9472*	0.9017	37.96	51.57



* the combinations selected.

Figure 33 Dose response curve for competitive reaction of amphetamine and methamphetamine combined with 4-ABM-HRP and 3-APA-Ab.

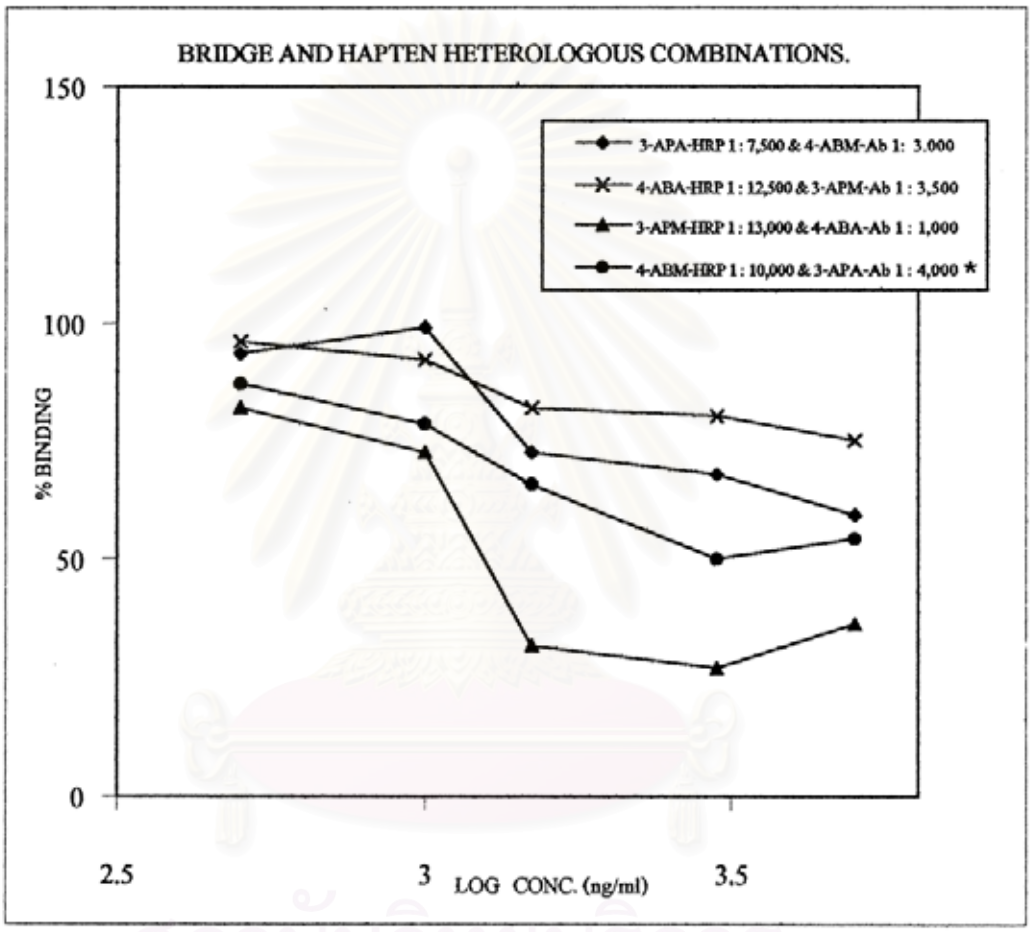
From all four types of bridge and hapten heterologous combinations, the possible competitive reaction for amphetamine and methamphetamine detection were summarized in Table 28 and portrayed Figure 34. The final selection of competitive reaction was the combinations of 4-ABM-HRP 1 : 10,000 and 3-APA-Ab 1 : 4,000 for amphetamine with the r value equals to 0.9472. At the 5,000 ng/ml, the final selected competitive reaction seem to be inactive for amphetamine detection.

Finally, the competitive reactions with the highest value of r in analysis of amphetamine or methamphetamine were tabulized in Table 29 and displayed in Figure 35. These combinations were carried through for subsequent cross-reacted determination. However, bridge heterologous between 4-ABM-HRP and 3-APM-Ab showed the worst competitive binding (slope = 12.68) and they would omitted for cross-reaction determination.

Table 28 The summary for bridge and hapten heterologous combinations study.

Combinations		Analyte	Regression analysis	
Enzyme-labeled (Dilution used)	Antibodies (Dilution used)		Correlation coefficient (r)	Slope
3-APA-HRP (1 : 7,500)	4-ABM-Ab (1 : 3,000)	Methamphetamine	0.8947	38.77
4-ABA-HRP (1 : 12,500)	3-APM-Ab (1 : 3,500)	Amphetamine	0.9380	21.11
3-APM-HRP (1 : 13,000)	4-ABA-Ab (1 : 1,000)	Amphetamine	0.8336	53.68
4-ABM-HRP (1 : 10,000)	3-APA-Ab (1 : 4,000)	Amphetamine	0.9472*	37.96

* the combinations selected.



* the combinations selected.

Figure 34 The competitive reaction selected for bridge and hapten heterologous study.

Table 29 Heterologous studies for amphetamine and methamphetamine.

Type of combinations	Combinations		Analyte	Regression analysis	
	Enzyme-labeled	Antibodies		Correlation coefficient (r)	Slope
Homologous	3-APM-HRP	3-APM-Ab	Amphetamine	0.9635	33.97
Bridge heterologous	4-ABM-HRP	3-APM-HRP	Amphetamine	0.9883	12.68
Hapten heterologous	3-APA-HRP	3-APM-Ab	Methamphetamine	0.9351	25.39
Bridge and hapten heterologous	4-ABM-HRP	3-APA-Ab	Amphetamine	0.9472	37.93

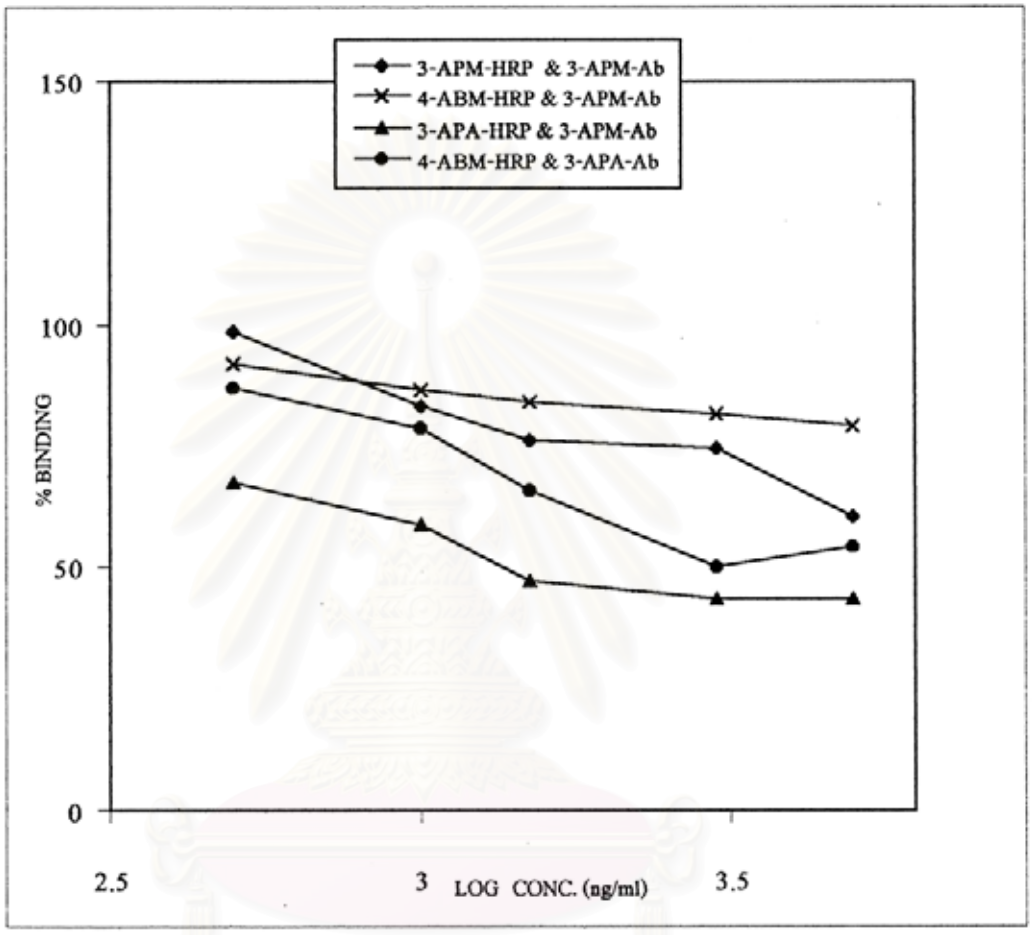


Figure 35 Selected heterologous studies for amphetamine and methamphetamine

6. Cross-reaction determination.

Following the competitive reaction in Table 30, it was shown that the homologous combination of 3-APM-HRP and 3-APM-Ab combination was usable for both amphetamine and methamphetamine. The detection was 20 percent more active for amphetamine than methamphetamine. Ephedrine, pseudoephedrine and phenylpropanolamine could be cross-reacted only at the concentrations higher than amphetamine 3.7, 7.0 and 7.0 times, respectively.

3-APA-HRP combined with 3-APM-Ab for methamphetamine determination but the method was 6 times more reactive than amphetamine. Ephedrine would be determined eventhough the cross-reaction was only 70 percent of methamphetamine. Pseudoephedrine and phenylpropanolamine would not be cross-reacted since the cross-reaction was only 5 and 10 percent, respectively.

4-ABM-HRP combined with 3--APA-Ab in amphetamine detection, could also be used for methamphetamine, ephedrine, pseudoephedrine and phenylpropranolamine. Methamphetamine was equally to amphetamine (RR = 0.91) in competitive reaction while ephedrine was 30 percent less active. Pseudoephedrine and phenylpropanolamine were 2.5 times more active than amphetamine.

Therefore from all these three combinations selected in this study. Only the combinations of 3-APM-HRP and 3-APM-Ab that would be appropriate for both amphetamine and methamphetamine detection and the combinations of 3-APA-HRP and 3-APM-Ab that could possibly detected amphetamine, methamphetamine and ephedrine.

Analyte	Homologous combinations		Hapten heterologous combinations		Bridge and hapten heterologous combinations	
	3-APM-HRP & 3-APM-Ab [a]		3-APA-HRP & 3-APM-Ab [b]		4-ABM-HRP & 3-APA-Ab [a]	
	Concentration [c] (ng/ml)	Relative reactivity [d]	Concentration (ng/ml)	Relative reactivity	Concentration (ng/ml)	Relative reactivity
Amphetamine	1,000	1.0	150	6.67	1,000	1.0
Methamphetamine	1,200	0.83	1,000	1.0	1,100	0.91
Ephedrine	3,700	0.27	1,300	0.77	1,300	0.77
Pseudoephedrine	7,000	0.14	20,000	0.05	400	2.5
Phenylpropanolamine	7,000	0.14	10,000	0.10	400	2.5

a : The assay drug was amphetamine.

b : The assay drug was methamphetamine.

c : The concentration of cross-reactants that binding equivalent to the assay drug 1,000 ng/ml.

d : The ratio obtained by dividing the assay drug equivalent of 1,000 ng/ml by the ng/ml concentration of the cross-reactants.

CHAPTER IV

CONCLUSION

Two derivatives of amphetamine, N-(3-aminopropyl)amphetamine (3-APA), N-(4-aminobutyl)amphetamine(4-ABA), and two derivatives of methamphetamine , N-(3-aminopropyl)methamphetamine (3-APM) and N-(4-aminobutyl)methamphetamine (4-ABM), were chemically synthesized in this study.

The peroxidase enzyme-labeled of all derivatives, 3-APA-HRP, 4-ABA-HRP, 3-APM-HRP and 4-ABM-HRP, were prepared in which the activity of the peroxidase enzyme was still remained. Four immunogens, 3-APA-BSA, 4-ABA-BSA, 3-APM-BSA and 4-ABM-BSA, were also prepared for induced antibodies in rabbits. The presence of antibody was indicated by the titer values of each antiserum.

Immuno-detection of amphetamine and methamphetamine was successfully proposed in three different combination of HRP-labeled hapten and antibody using heterologous principle. They were the combination of 3-APM-HRP and 3-APM-Ab, 3-APA-HRP and 3-APM-Ab and 4-ABM-HRP and 3-APA-Ab.

The selective determination of amphetamine and methamphetamine could be obtained from the combination of 3-APM-HRP and 3-APM-Ab. With this combination, ephedrine, pseudoephedrine or phenylpropanolamine couldn't interfere since the relative reactivity were only 0.27, 0.14, and 0.14, respectively.

Amphetamine, methamphteamine and ephedrine were detected when the combination of 3-APA-HRP and 3-APM-Ab was utilized. The relative reactivity of

pseudoephedrine and phenylpropanolamine comparing to methamphetamine was only 0.05 and 0.10, respectively

The non-selective immuno-detection of amphetamine and methamphetamine was resulted from the combinations of 4-ABM-HRP and 3-APA-Ab. All amine compounds studied, ephedrine, pseudoephedrine and phehylpropanolamine could also be detected.

From these varieties of enzyme-labeled and the use of polyclonal antibodies in this study, the immuno-detection that selective to amphetamine and methamphetamine was suitably created. This implied the possibility of applying polyclonal antibody for immunoasssay of these compounds.

Suggestion for further work

The result of this study have to be further developed as an instant test-kit before used in drug-abuse test in Thailand.

REFERENCES

Aoki, A., and Kuroiwa, Y. 1985. A screening method for urinary methamphetamine latex agglutination inhibition reaction test. Forensic.Sci.Int. 27: 49-56.

Barrett, J.T., ed. Textbook of Immunology, pp. 48-52. USA: The C.V. Mosby, 1974.

Caraway, K.L., and Koshland, Jr., D.E. 1972. Carbodiimide modification of proteins. Methods.Enzymol. 25: 616-623.

Catty, D., and Raykundalia, C. Production and quality control of polyclonal antibodies. In D. Catty (ed.), Antibodies. vol. I: A practical approach, pp.19-80. Oxford: IRL press, 1988.

Cheng, L.T., Kim, S.Y., Chung, A., and Castro, A. 1973. Amphetamine: New radioimmunoassay. FEBS LETTERS 36 No.3: 339-342.

Choi, M.J. 1994. A visual membrane immunoassay for the detection of methamphetamine using an enzyme-labeled tracer derived from methaphetamine and amphetamine. Biol.Pharm.Bull. 17 No. 7: 875-880.

Ciabattoni, G. Production of antisera by conventional techniques. In C. Patrono; and B.A. Pedskar (eds.), Radioimmunoassay in basic and clinical pharmacology, pp.23-69. Berlin: Springer Verlag, 1987.

Colbert, D.L., Gallacher, G., and Mainwaring-Burton, R.W. 1985. Single-reagent polarization fluoroimmunoassay for amphetamine in urine. Clin.Chem. 31 No 7: 1193-1195.

D'Nicuola, J., et al. 1992. Evaluation of six commercial amphetamine and methamphetamine immunoassays for cross-reactivity to phenylpropanolamine and ephedrine in urine. J.Anal.Toxicol. 16: 211-213.

Erlanger, B.F., Borex, F., Beiser, S.M., and Lieberman, S, 1957. Steroid-protein conjugates:I Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. J.Biol.Chem. 228: 713-727.

Hosoda, H., Yoshida, H., Sakai, Y., Miyairi, S., and Nambara, T. 1980. Sensitivity and specificity in enzyme immunoassay of testosterone. Chem.Pharm.Bull. 28 No.10: 3035-3040.

Hosoda, H., Kawamura N., and Nambara, T. 1981. Effect of bridge heterologous combination on sensitivity in enzyme immunoassay for cortisol. Chem.Pharm.Bull. 29 No. 7: 1969-1974.

Hosoda, H., Kobayashi, N., and Nambara, T. 1983. Effect of bridge heterologous combination on sensitivity in enzyme immunoassay for 11-deoxycortisol. Chem.Pharm.Bull. 31 No. 3: 953-958.

Hosoda, H., Kobayashi, N., Kawamoto, H., and Nambara, T. 1983. The specificity of enzyme immunoassays for Plasma 11-Deoxycortisol. Chem.Pharm.Bull. 31 No. 10: 3595-3600.

Hosoda, H., Kobayashi, N., Ishii, N., and Nambara, T. 1985. The bridge length effect on sensitivity in steroid enzyme immunoassays. Chem.Pharm.Bull. 33 No. 2: 902-904.

Hosoda, H., Kobayashi, N., Ishii, N., and Nambara, T. 1986. Bridging phenomena in steroid immunoassays. The effect of bridge length effect on sensitivity in steroid enzyme immunoassays. Chem.Pharm.Bull. 34 No. 5: 2105-2111.

Khosravi, M.J., and Papanastasiou-Diamandi, A. 1993. Hapten-heterologous conjugates evaluated for application to free thyroxine immunoassays. Clin.Chem. 39(2): 256-262.

Kemeny, D.M. 1992. Titration of antibodies. J.Immunol.Methods. 150: 57-76.

Kohler, G., and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

Landsteiner, K. ed. The specificity of serological reactions. Massachusetts: Harvard university Press, 1945.

Mongkolsirichaikul, D., Tarnchompoo, B., and Ratanabanangkoon, K. 1993. Development of latex agglutination inhibition reaction test for amphetamine in urine. J.Immunol.Methods. 157: 189-195.

Nakane, P.K., and Kawaoi, A. 1974. Peroxidase-labeled antibody a new method of conjugation. J.Histochem.Cytochem. 22 No.12:1084-1091.

Nishikawa, T., Saito, M., and Kubo, H. 1984. Effect of heterologous combination on competitive nephelometric immunoassay II. Bridge-heterologous combination for ethosuximide immunoassay. Chem.Pharm.Bull. 32 No.12: 4958-4962 .

Porstmann, T., and Kiessig, S.T. 1992. Enzyme immunoassay techniques: An overview J.Immunol.Methods. 150: 5-21.

Piran, U., Riordan, W.J., and Silbert, D.R. 1990. Effect of hapten heterology on thyroid hormone immunoassays. J.Immunol.Methods. 133: 207-214.

Pitchayawasin, S. Effect of heterologous combinations between enzyme labeled theophylline and immunogen on the sensitivity and specificity of enzyme immunoassay for theophylline. Master' s Thesis, Department of Pharmaceutical Chemistry, Graduate School, Chulalongkorn University, 1997.

Ruangyuttikarn, K., and Moody, D.E. 1988. Comparison of three commercial amphetamine immunoassays for detection of methamphetamine , methylenedioxyethylamphetamine. J.Anal.Toxicol. 12: 229-233.

Standerfer, J.C., and Saunders, G.C. 1978. Enzyme immunoassay for gentamicin. Clin.Chem. 24:1903-1907.

Suttijitpasal, P., and Ratanabanangkorn, K. 1992. Immunoassays of amphetamines : immunogen structure & antibody specificity. Asian Pacific J. of Allergy and Immunology 10: 159-164.

Tijssen, P. Practice and theory of enzyme immunoassay. New York: Elsevier-North Holland, 1985.

Van Weemen, B.K., and Schuurs, A.H.W.M. 1972. Immunoassay using hapten-enzyme conjugates. FEBS LETTERS 24 No. 1: 77-81.

Van Weemen, B.K., and Schuurs, A.H.W.M. 1975. The influence of heterologous combinations of antiserum and enzyme-labeled estrogen on the characteristic of estrogen enzyme-immunoassays. Immunochemistry. 12: 667-670.

Wilson, M.B., and Nakane, P.K. Recent developments in the periodate method of conjugation horseradish peroxidase (HRPO) to antibodies. In W. Knapp; R. Holubar; and G. Wick (eds.), Immunofluorescence and related staining techniques, pp. 215-224. New York: Elsevier-North Holland Biochemical Press, 1978.

Yalow, R.S., and Berson, S.A. Immunoassay of plasma insulin. In D. Grick (ed.), Methods of biochemical analysis, Vol.12, pp. 69-96. New York: Interscience, 1962.

APPENDIX

APPENDIX A

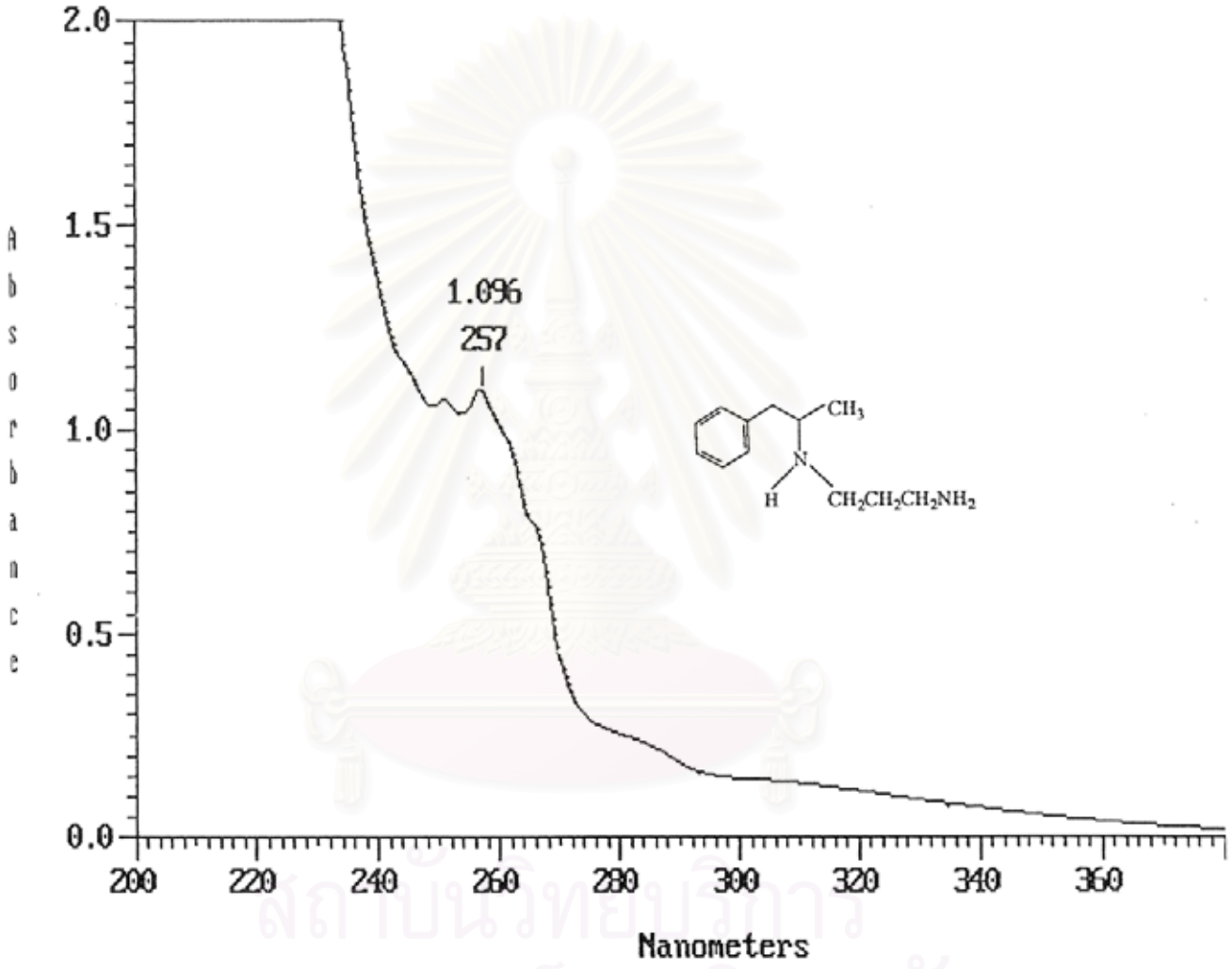


Figure A1 The UV spectrum of aqueous solution of N-(3-aminopropyl)amphetamine at the concentration of 0.65 mg/ml.

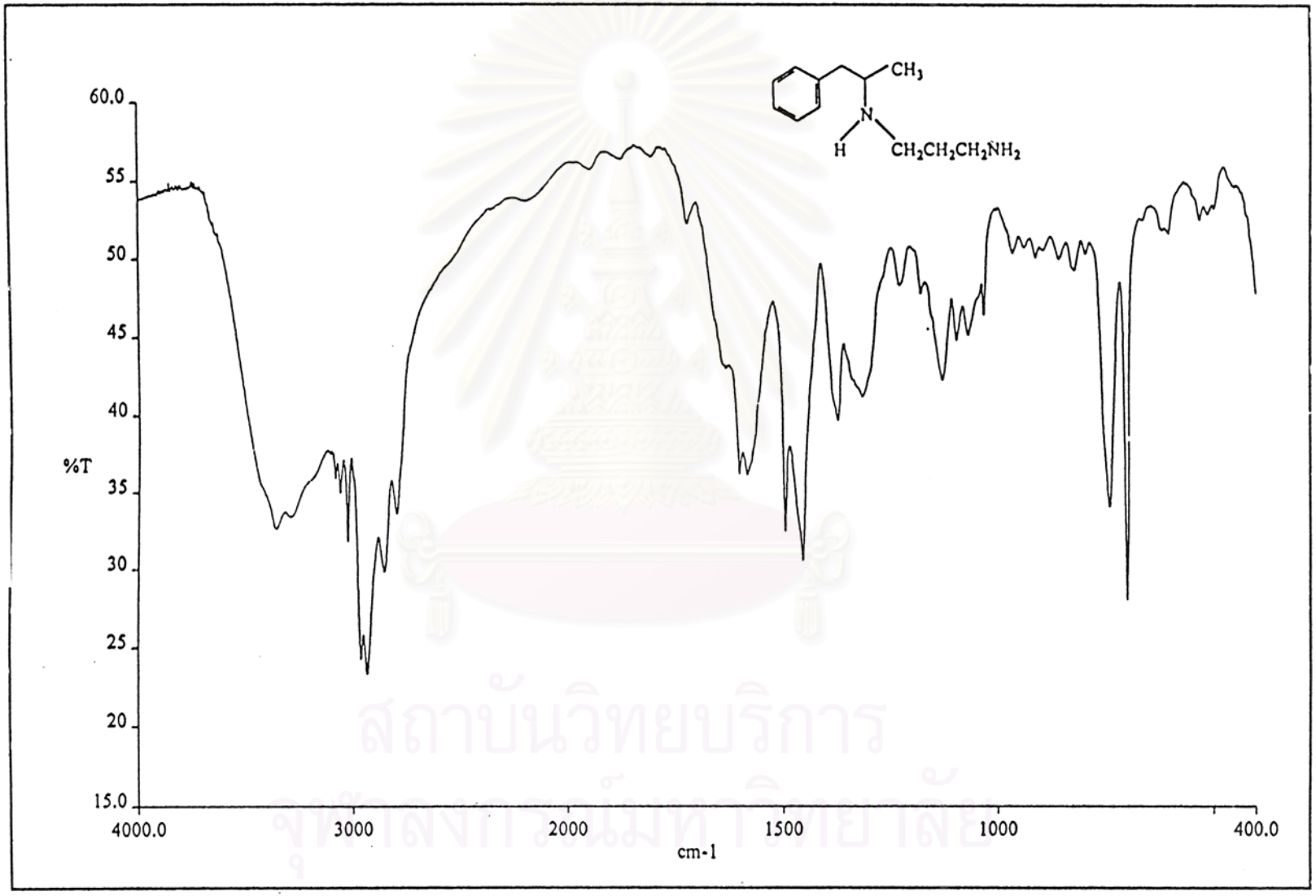
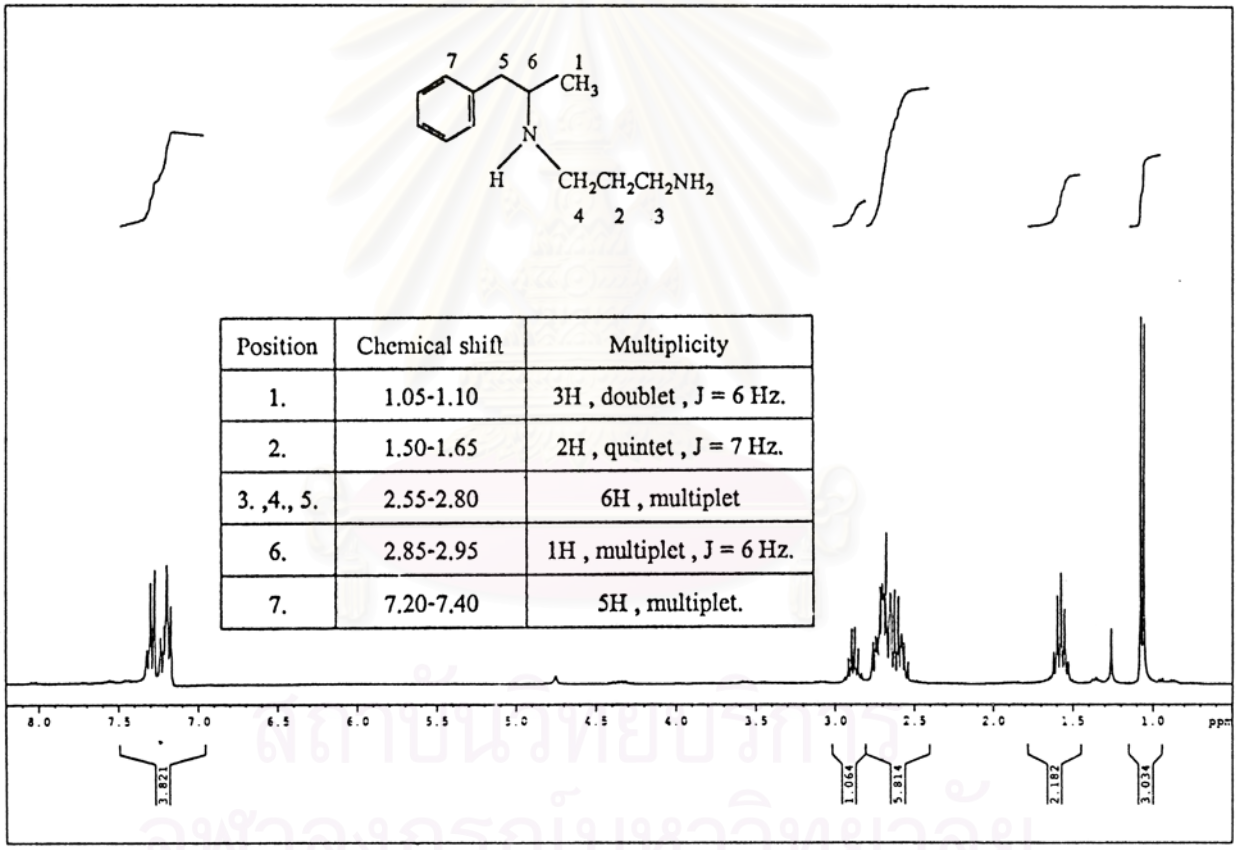


Figure A2 The IR spectrum of N-(3-aminopropyl)amphetamine (Neat).

Table A1 Assignment of IR spectrum of N-(3-aminopropyl)amphetamine.

Wavenumbers (cm^{-1})	Functional groups
3360 , 3284	N-H stretching
3100 - 3025	C-H stretching , aromatic
3000 – 2800	C-H stretching , aliphatic
1650 - 1580	N-H bending
1494 - 1300	C-H bending
1150 - 1000	C-N stretching
741 , 699	Aromatic C-H out-of-plane bending (monosubstituted benzene)



Position	Chemical shift	Multiplicity
1.	1.05-1.10	3H , doublet , J = 6 Hz.
2.	1.50-1.65	2H , quintet , J = 7 Hz.
3. ,4., 5.	2.55-2.80	6H , multiplet
6.	2.85-2.95	1H , multiplet , J = 6 Hz.
7.	7.20-7.40	5H , multiplet.

Figure A3 The ^{1}H-NMR spectrum of N-(3-aminopropyl)amphetamine in $CDCl_3$.

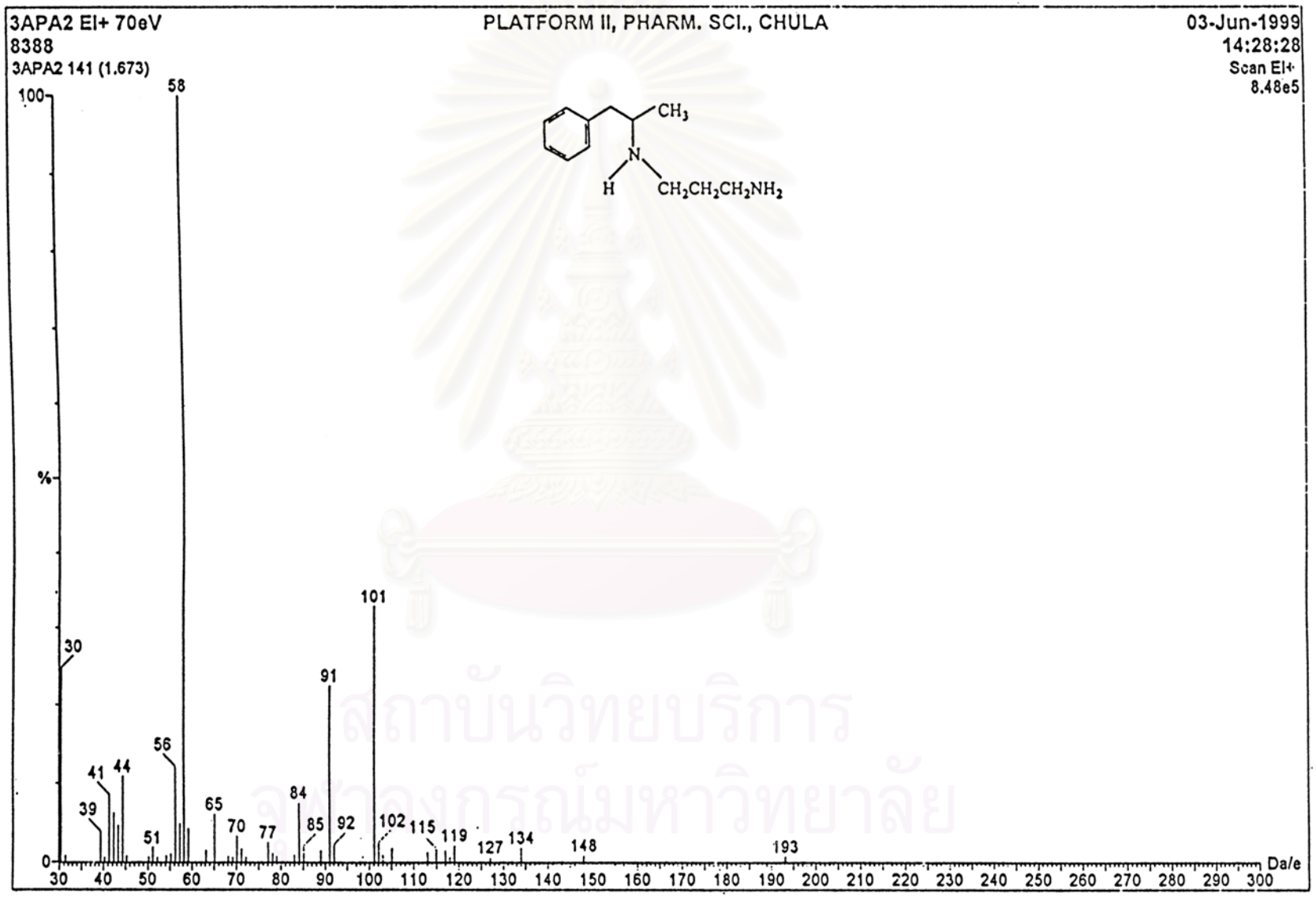


Figure A4 The EIMS spectrum of N-(3-aminopropyl)amphetamine.

108

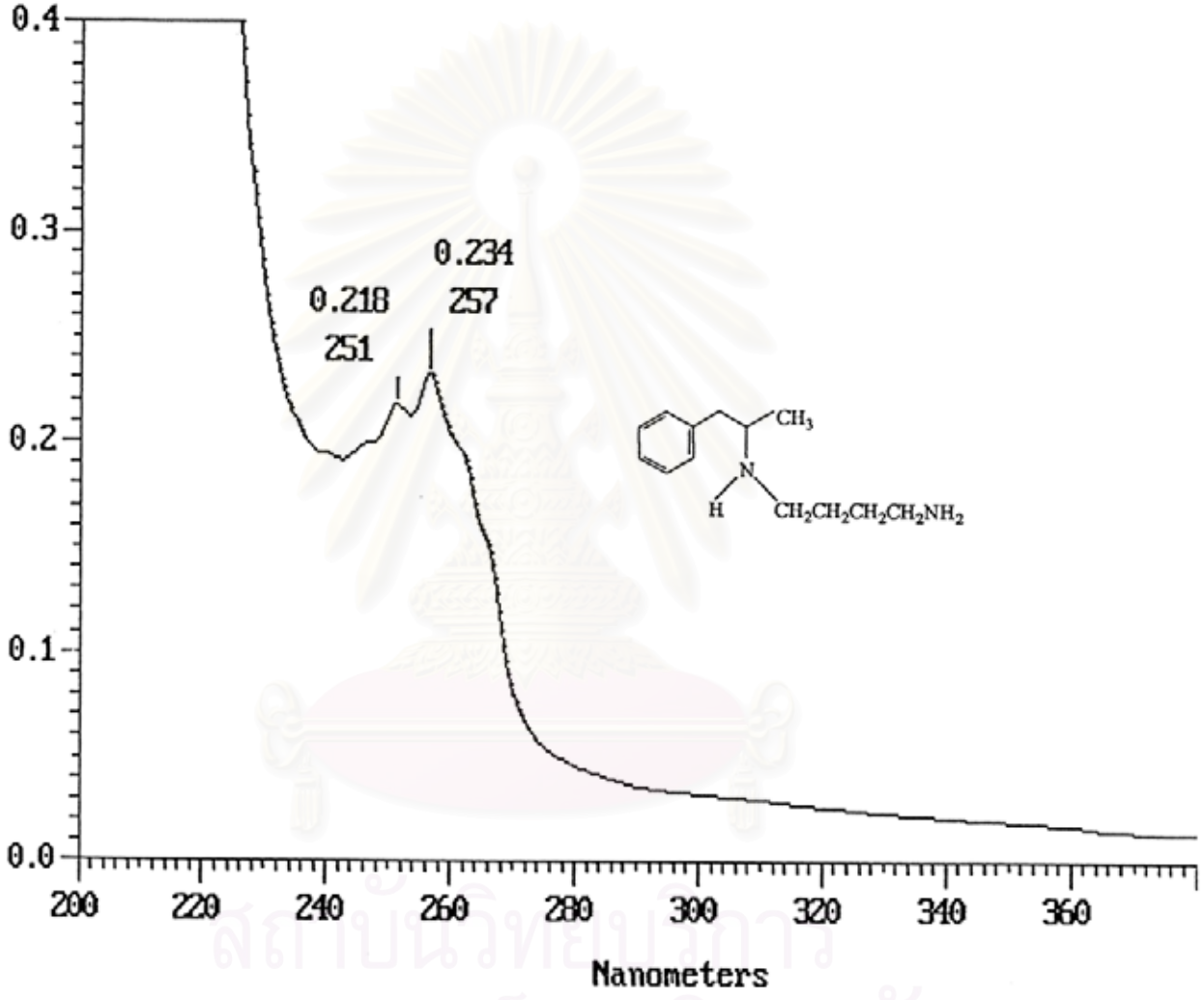


Figure A5 The UV spectrum of aqueous solution of N-(4-aminobutyl)amphetamine at the concentration of 0.20 mg/ml.

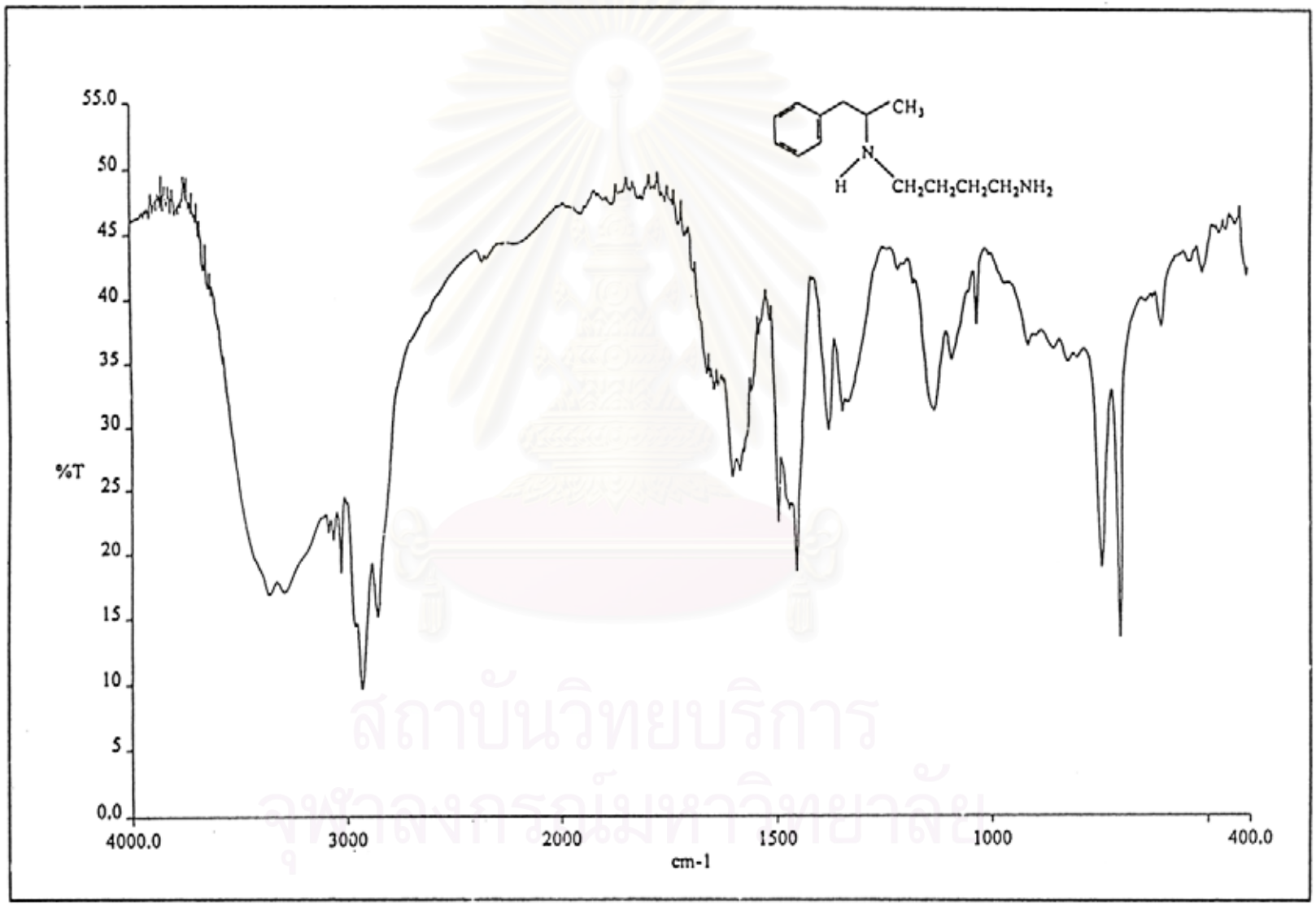
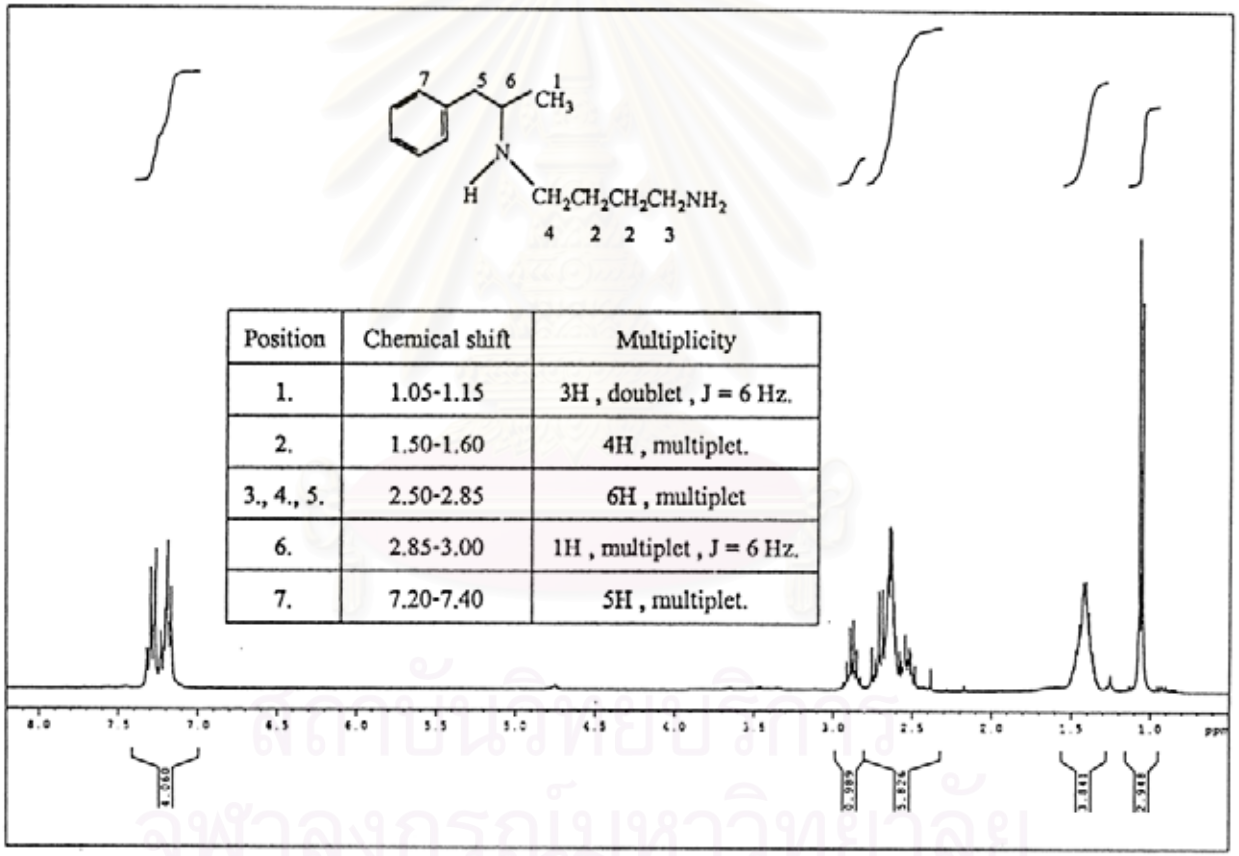


Figure A6 The IR spectrum of N-(4-aminobutyl)amphetamine (Neat).

110

Table A2 Assignment of IR spectrum of N-(4-aminobutyl)amphetamine

Wavenumbers (cm^{-1})	Functional groups
3360 , 3284	N-H stretching
3100 - 3026	C-H stretching , aromatic
3000 - 2850	C-H stretching , aliphatic
1650 - 1580	N-H bending
1494 - 1300	C-H bending
1150 – 1000	C-N stretching
744 , 701	Aromatic C-H out-of-plane bending (monosubstituted benzene)



Position	Chemical shift	Multiplicity
1.	1.05-1.15	3H , doublet , J = 6 Hz.
2.	1.50-1.60	4H , multiplet.
3., 4., 5.	2.50-2.85	6H , multiplet
6.	2.85-3.00	1H , multiplet , J = 6 Hz.
7.	7.20-7.40	5H , multiplet.

Figure A7 The ^{1}H-NMR spectrum of N-(4-aminobutyl)amphetamine in $CDCl_3$.

112

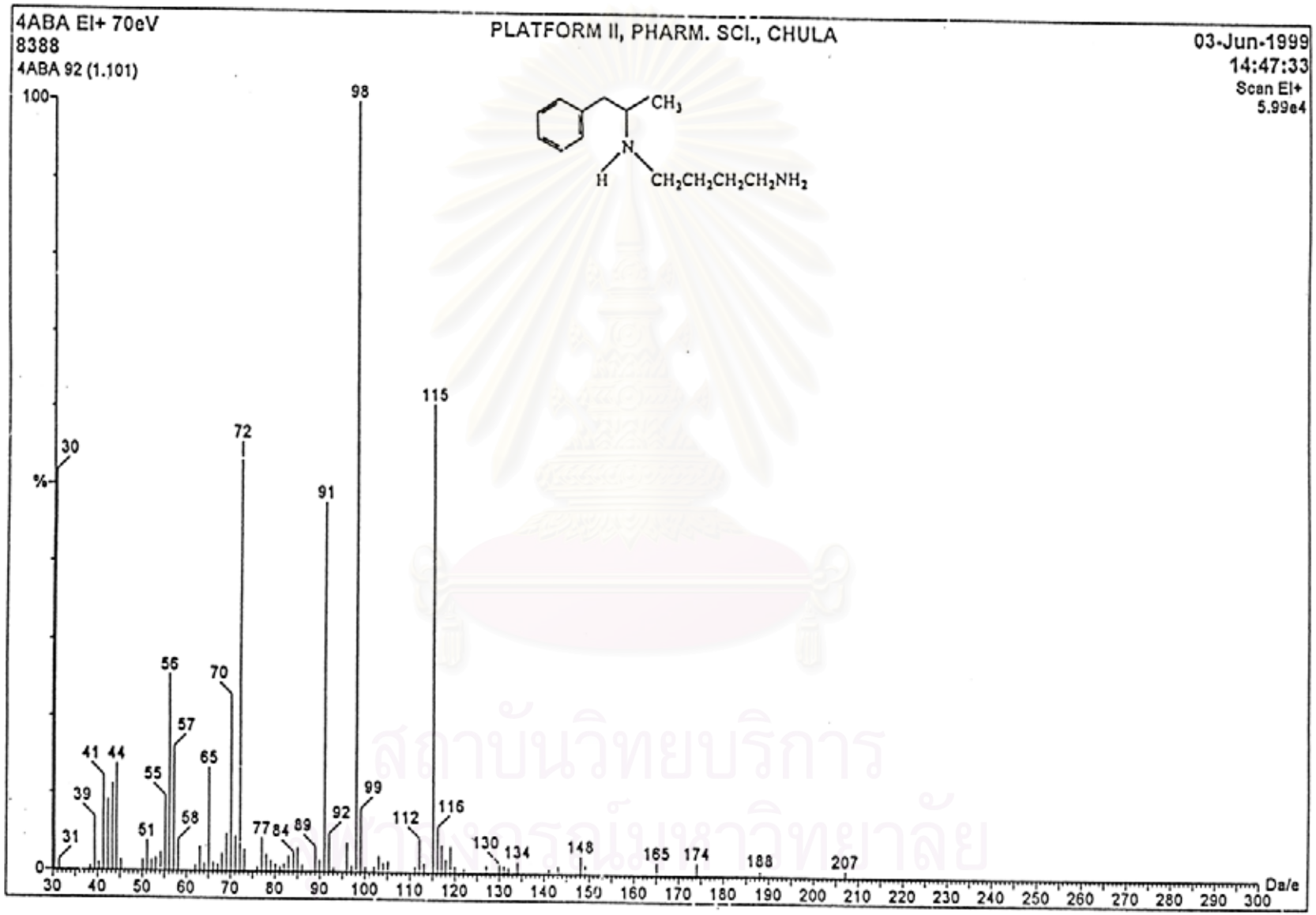


Figure A8 The EIMS spectrum of N-(4-aminobutyl)amphetamine.

113

APPENDIX B

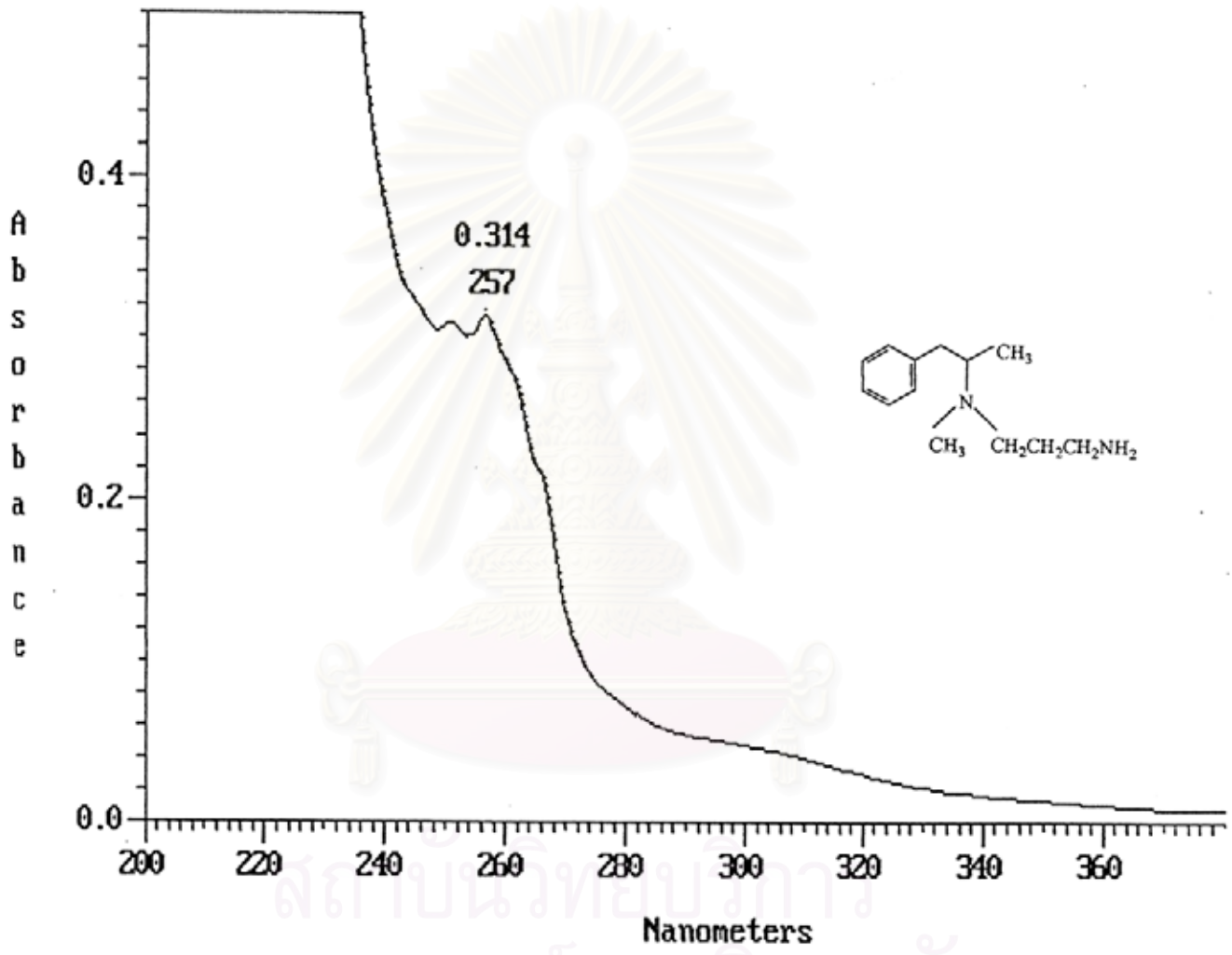


Figure B1 The UV spectrum of aqueous solution of N-(3-aminopropyl) methamphetamine at the concentration of 0.20 mg/ml.

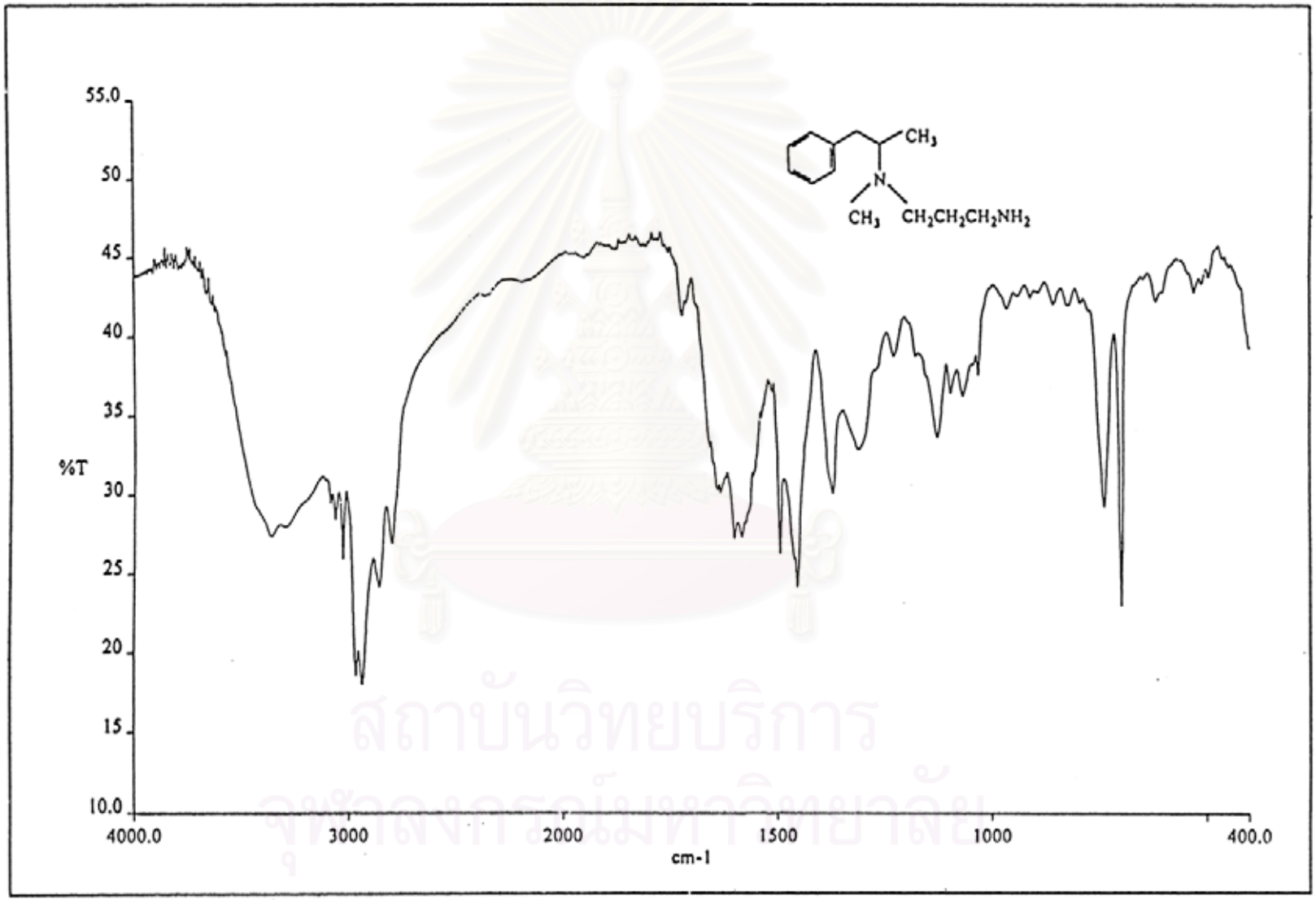
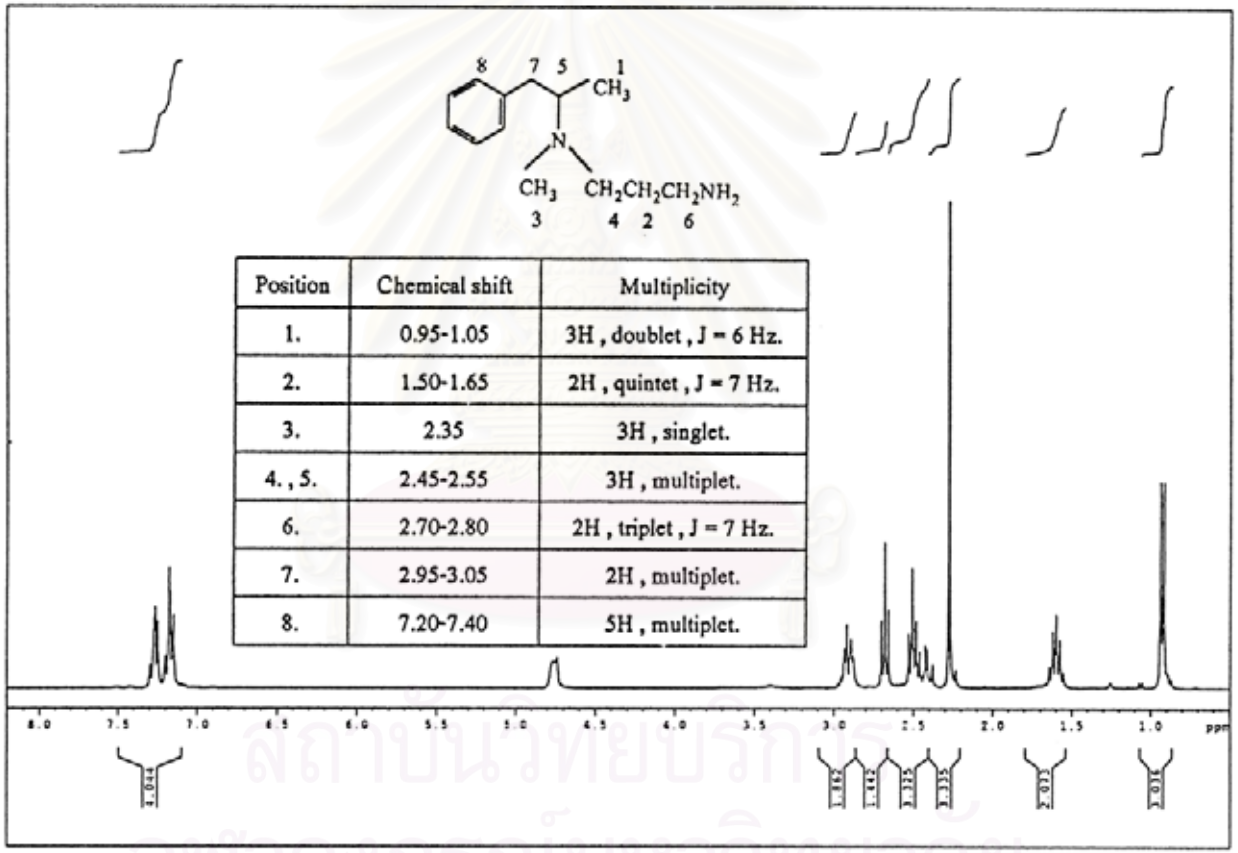


Figure B2 The IR spectrum of N-(3-aminopropyl)methamphetamine (Neat).

115

Table B1 Assignment of IR spectrum of N-(3-aminopropyl)methamphetamine.

Wavenumbers (cm^{-1})	Functional groups
3359 , 3284	N-H stretching
3100 - 3025	C-H stretching , aromatic
3000 - 2800	C-H stretching , aliphatic
1650 - 1580	N-H bending
1494 - 1300	C-H bending
1150 - 1000	C-N stretching
739 , 700	Aromatic C-H out-of-plane bending (monosubstituted benzene)



Position	Chemical shift	Multiplicity
1.	0.95-1.05	3H , doublet , J = 6 Hz.
2.	1.50-1.65	2H , quintet , J = 7 Hz.
3.	2.35	3H , singlet.
4. , 5.	2.45-2.55	3H , multiplet.
6.	2.70-2.80	2H , triplet , J = 7 Hz.
7.	2.95-3.05	2H , multiplet.
8.	7.20-7.40	5H , multiplet.

Figure B3 The ^{1}H-NMR spectrum of N-(3-aminopropyl)methamphetamine in $CDCl_3$.

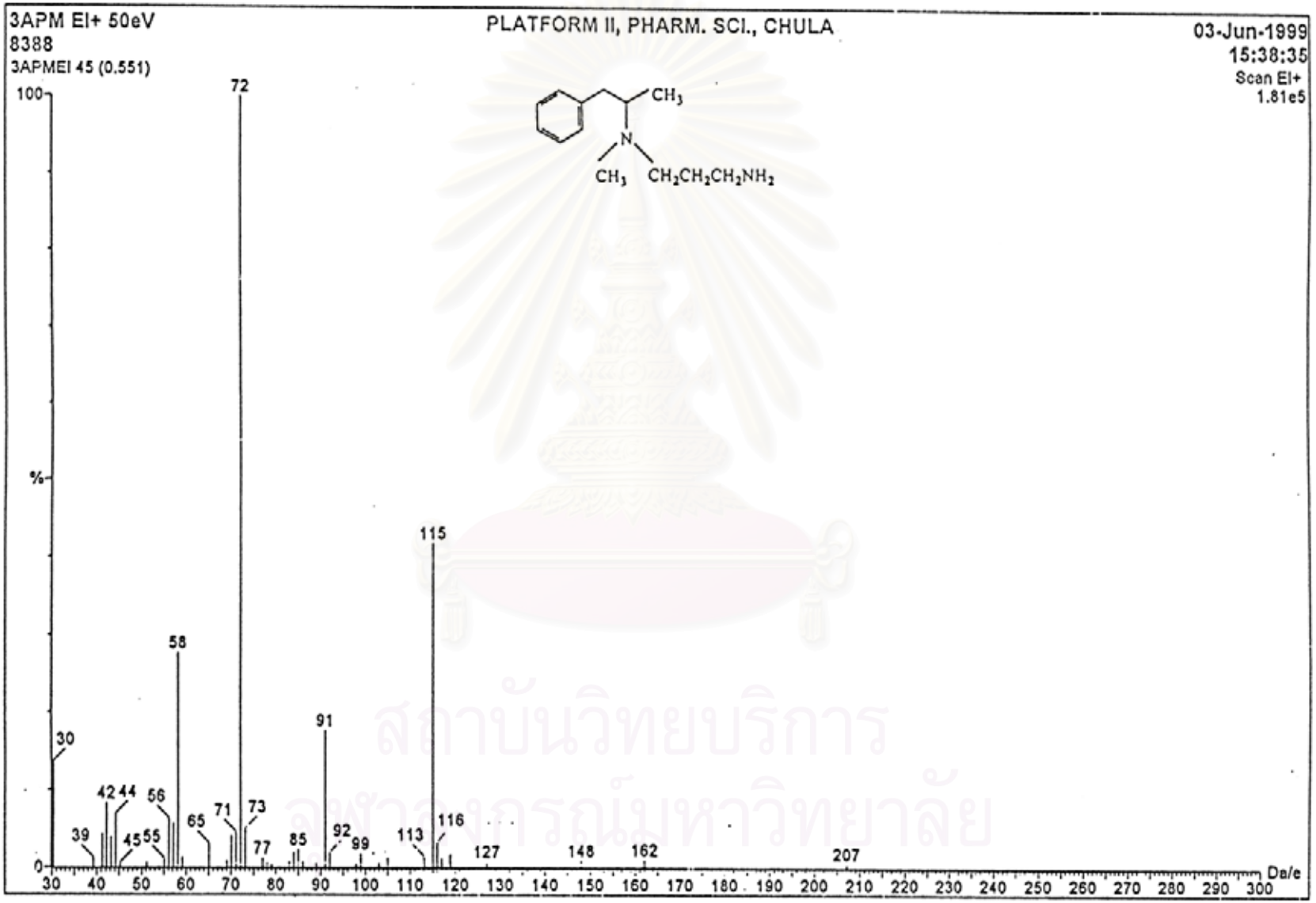


Figure B4 The EIMS spectrum of N-(3-aminopropyl)methamphetamine.

118

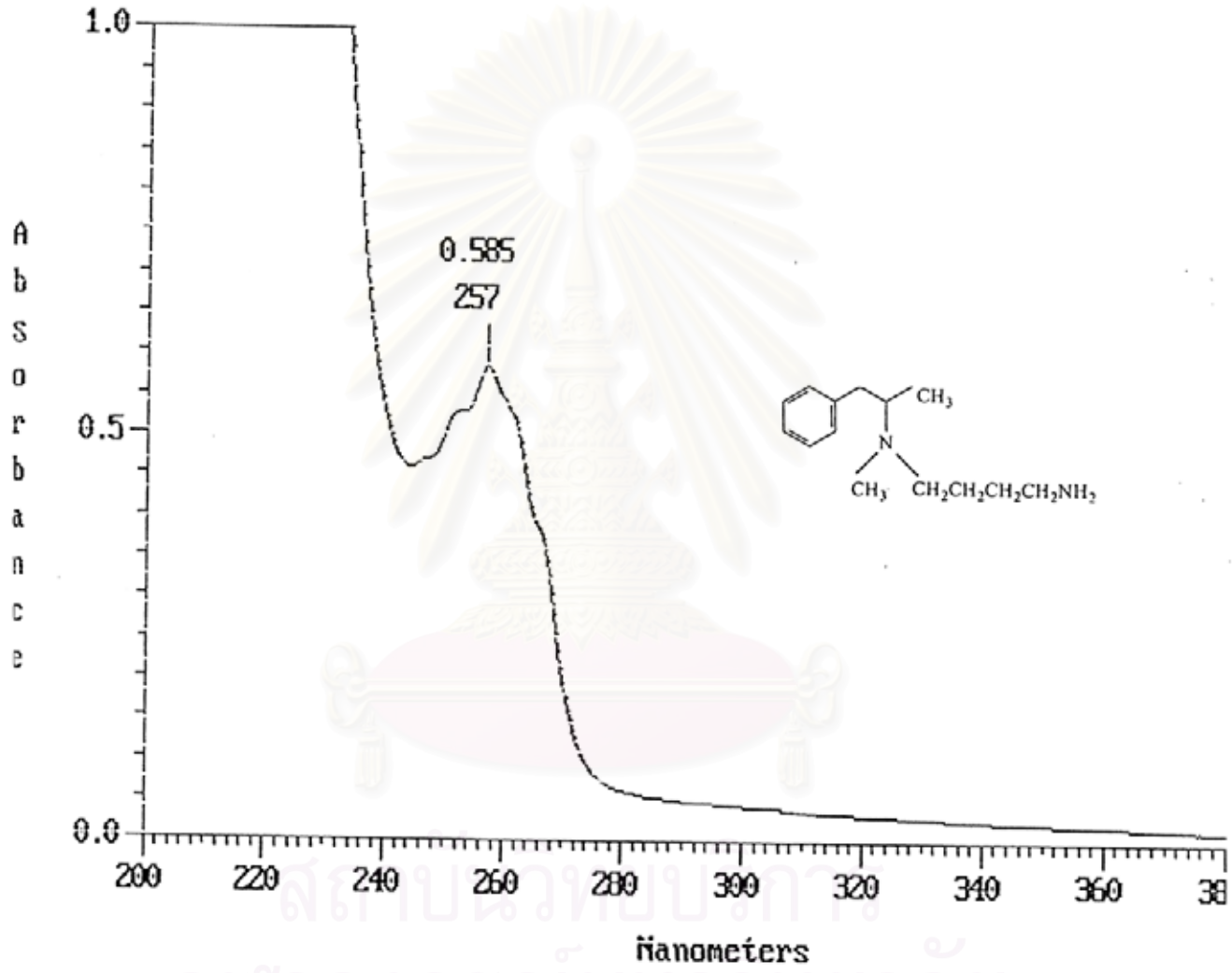


Figure B5 The UV spectrum of aqueous solution of N-(4-aminobutyl) methamphetamine at the concentration of 0.40 mg/ml.

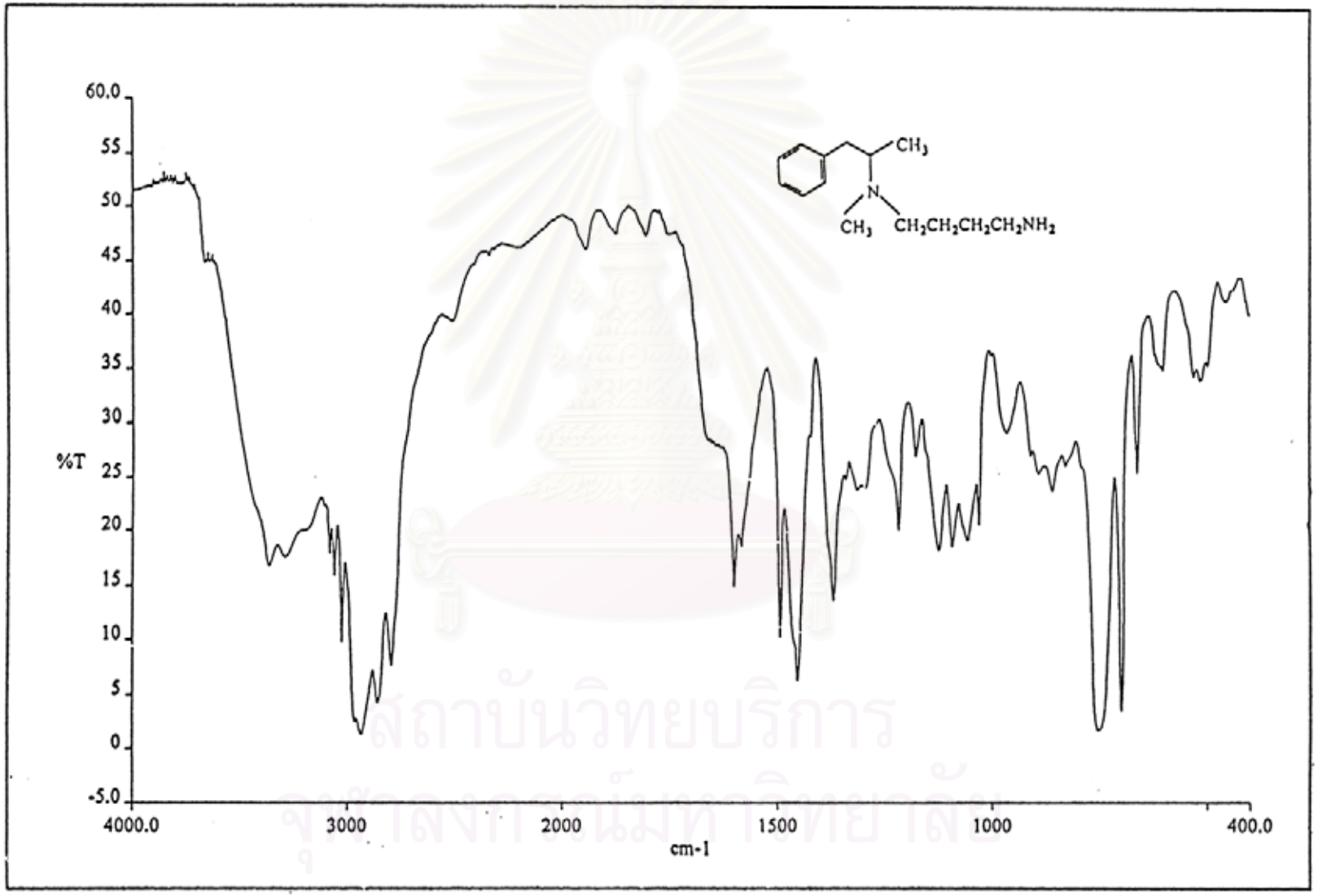
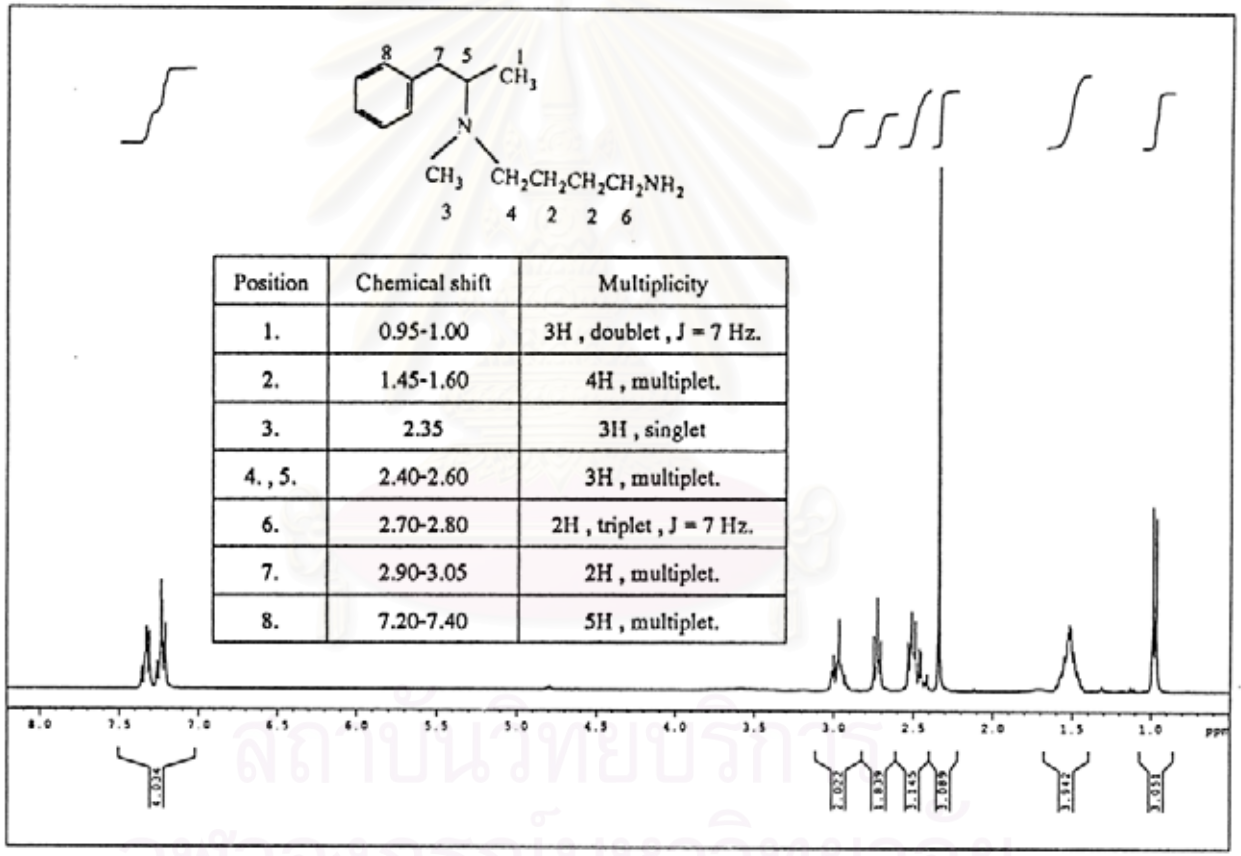


Figure B6 The IR spectrum of N-(4-aminobutyl)methamphetamine (Neat).

120

Table B2 Assignment of IR spectrum of N-(4-aminobutyl)methamphetamine.

Wavenumbers (cm^{-1})	Functional groups
3367 , 3292	N-H stretching
3100 - 3025	C-H stretching , aromatic
3000 - 2800	C-H stretching , aliphatic
1650 - 1580	N-H bending
1494 - 1300	C-H bending
1150 - 1000	C-N stretching
753 , 700	Aromatic C-H out-of-plane bending (monosubstituted benzene)



Position	Chemical shift	Multiplicity
1.	0.95-1.00	3H , doublet , J = 7 Hz.
2.	1.45-1.60	4H , multiplet.
3.	2.35	3H , singlet
4. , 5.	2.40-2.60	3H , multiplet.
6.	2.70-2.80	2H , triplet , J = 7 Hz.
7.	2.90-3.05	2H , multiplet.
8.	7.20-7.40	5H , multiplet.

Figure B7 The ^{1}H-NMR spectrum of N-(4-aminobutyl)methamphetamine in $CDCl_3$.

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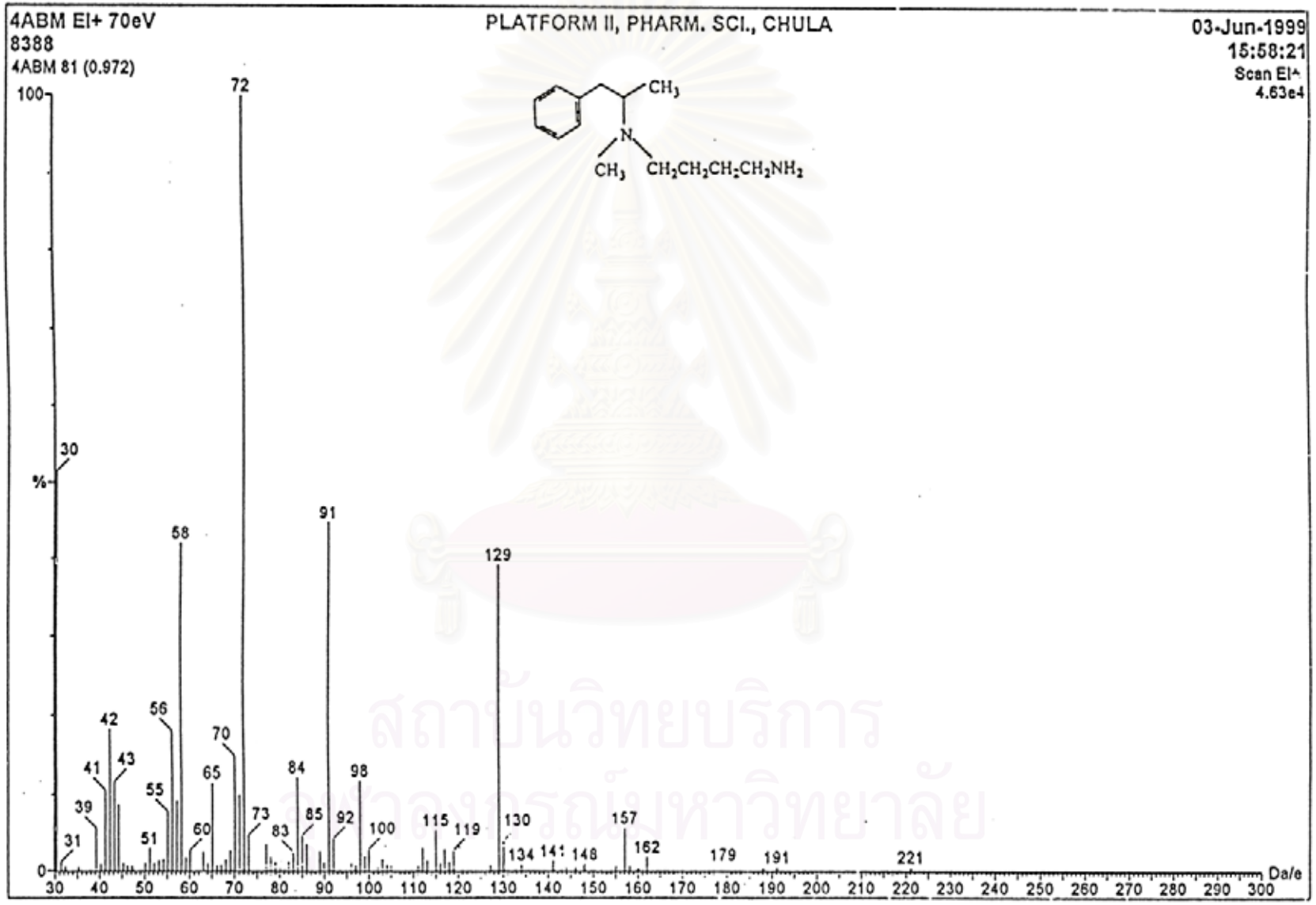

Figure B8 The EIMS spectrum of N-(4-aminobutyl)methamphetamine.

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VITA

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