CHAPTER III

RESULT AND DISCUSSION

Part A. Immunoanalytical method for amphetamine, methamphetamine and ephedrine detection using polyclonal antibodies

A-1 Promptness of antisera and peroxidase-labeled derivatives

The activity of peroxidase enzyme in every enzyme-labeled amphetamine, methamphetamine and ephedrine derivatives was proven to be remained as shown in Table 5. The activity of peroxidase in all conjugated labeled, except 4-ABM as hapten, was decreased in comparative to the former testing as shown in Table 5. To compensate the decrease in the enzyme conjugated activity, the concentration used was increased to gain the sufficient activity of the labeling peroxidase in further ELISA study. It was not unusual that the rate of activity decreasing was different for each conjugated-peroxidase enzyme. Therefore, these enzyme-label compounds would be ready to be used.

Table 5 The activity of HRP-labeled hapten conjugates determination

HRP-labeled hapten	Enzyme label activity after conjugation (%)	Enzyme label activity in this study	
		Remained activity (%)	Decreasing activity per year (%)
HRP	100.0	100.0	-
3-APA	20.0	10.0	2.0
4-ABA	25.0	10.0	3.0
3-APM	34.0	20.0	2.8
4-ABM	20.0	20.0	0
3-APE	4.0	3.0	0.2
4-ABE	8.0	5.0	0.6

The titer of antibody induced from 3-APA and 4-ABA against amphetamine was determined to be 1:500 and 1:1,000, respectively. For antibody against methamphetamine, the titer values were 1:30,000 and 1:1,000 for 3-APM and 4-ABM, respectively. Antibody titer for ephedrine detection was 1:250 and 1:350 when 3-APE and 4-ABE, respectively, were immunogen hapten.

A-2 Heterology study

A-2.1 Selection of proper heterologous combinations

As shown in Table 6, many heterology combinations with the high value of r and slope were selected for amphetamine, methamphetamine and ephedrine detection. The number of bridge-hapten combinations for detection of amphetamine, methamphetamine and ephedrine was greater than bridge and hapten heterology. It was therefore implied that combined heterology was more selective than simple heterology as presented in Table 7-17 and Figure 6 -16.

3-APE-HRP and 4-ABE-HRP was slightly bound to 3-APA-Ab, 4-ABA-Ab, 3-APM-Ab, and 4-ABM-Ab with very low response. The extra hydroxyl group of ephedrine might be steric to bind the amphetamine and methamphetamine antibody. Therefore, no competitive reaction was observed in these combinations, H2, H4, H6, H8, BH2, BH4, BH6, and BH8.

Table 6 The selective heterologous combinations

Type of combination	Combination code
Bridge	B2, B6
Hapten	H5, H9 , H10 , H12
Bridge-hapten	BH1, BH9, BH10, BH11, BH12

Bridge heterologous of 4-ABA-Ab and 3-APA-HRP (B2)

The relationship between the percentage of binding and the log analyte concentration was depicted in Figure 6. Amphetamine, methamphetamine and ephedrine could compete with 3-APA-HRP in binding to 4-ABA-Ab. The linear response of amphetamine, methamphetamine and ephedrine within the concentration range of 500 – 3,000 µg/L were observed as indicated in Table 7. The B2 was the proper combinations for amphetamine detection with the highest r value of 0.9644 whereas the detection of methamphetamine and ephedrine with the scatter response was observed in the competitive reaction with the r value of 0.7481 and 0.8479, respectively. Although, the slope values for methamphetamine and ephedrine detection were higher than the slope value for amphetamine detection (Table 7), the concentration at the 50% binding for amphetamine detection was lower than 500 µg/L, whereas for methamphetamine and ephedrine was about 500 µg/L. Therefore, this combination would be the proper for amphetamine detection.

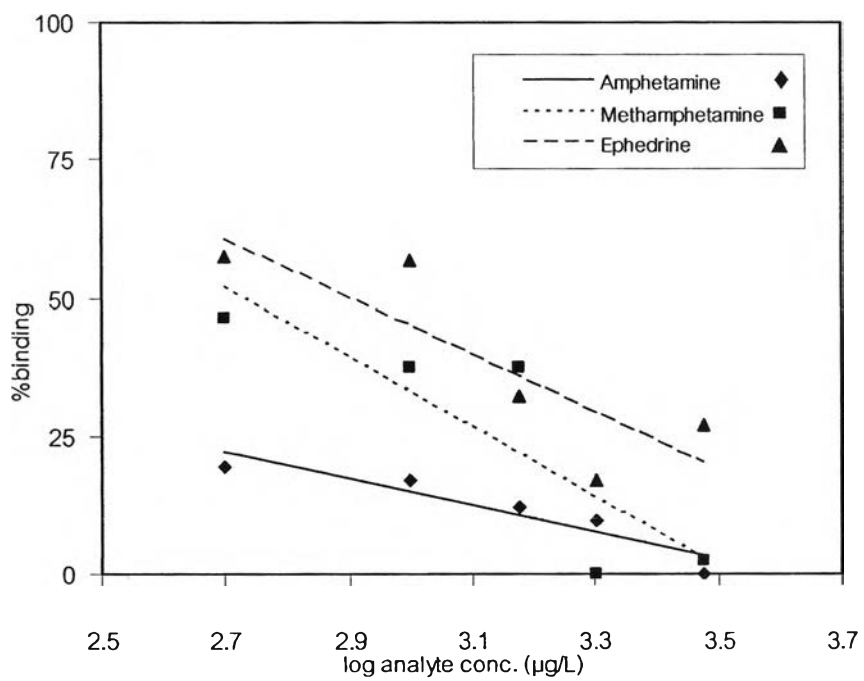


Figure 6 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge heterologous combination (B2)

Table 7 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge heterologous combination (B2)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 86.143 - 23.773x	0.9644 *
Methamphetamine	Y = 222.87 - 63.32x	0.7481
Ephedrine	Y = 201.83 - 52.299x	0.8479

* the proper competitive reaction

Bridge-hapten heterologous of 3-APA-Ab and 4-ABM-HRP (BH1)

The logic plots of amphetamine, methamphetamine and ephedrine detection were shown in Figure 7. No competitive reaction for amphetamine and methamphetamine were observed. Only ephedrine could compete with 4-ABM-HRP in binding to 3-APA-Ab. The linear response of ephedrine within the concentration range of 500 -3,000 µg/L was evidence with the r values of 0.8568 (Table 8). Therefore, ephedrine could be detected by this combination.

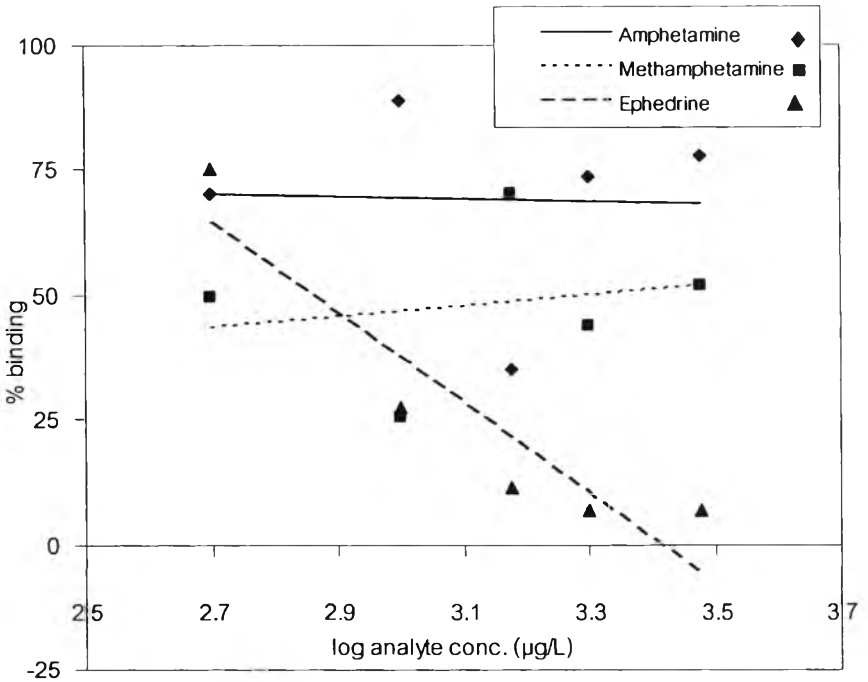


Figure 7 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH1)

Table 8 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH1)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 76.341 - 2.2746x	0.0011
Methamphetamine	Y = 13.48 - 11.04x	0.0416
Ephedrine	Y = 307.26 - 90.015x	0.8568 *

* the proper competitive reaction

Hapten heterologous of 3-APM-Ab and 3-APA-HRP (H5)

As presented in Figure 8, only methamphetamine and ephedrine could compete with 3-APA-HRP in binding to 3-APM-Ab. Within the concentration range of 500 – 3,000 µg/L, the linear response of methamphetamine and ephedrine was observed with the r value of 0.8942 and 0.8602, respectively (Table 9).

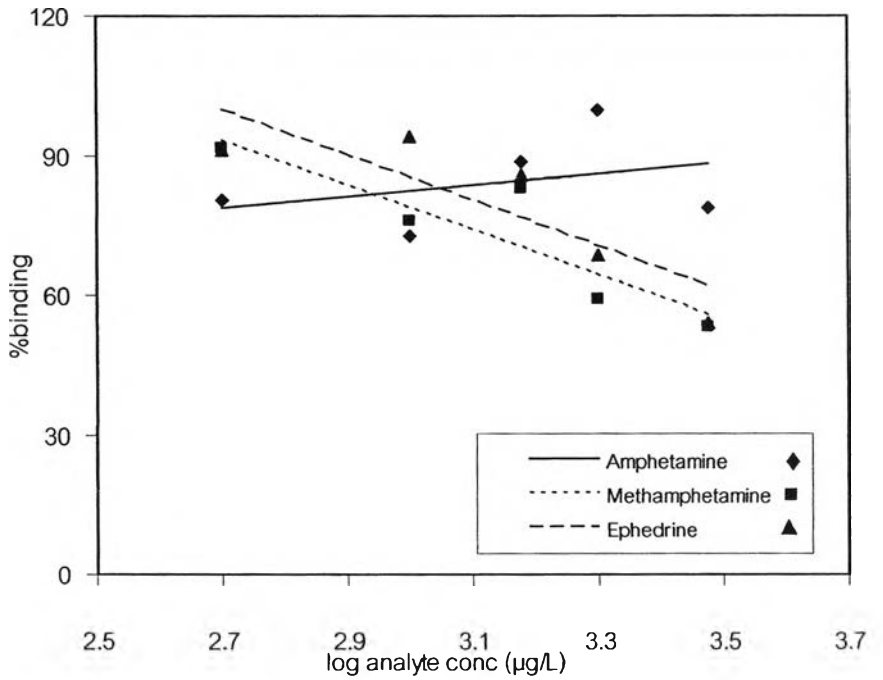


Figure 8 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H5)

Table 9 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H5)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 47.399 + 11.749x	0.1101
Methamphetamine	Y = 223.17 - 48.07x	0.8942 *
Ephedrine	Y = 232.32 - 49.02x	0.8602 *

* the proper competitive reaction

Bridge heterologous of 4-ABE-Ab and 3-APE-HRP (B6)

The logic plots of amphetamine, methamphetamine and ephedrine indicated the competition of 3-APM-HRP in binding to 4-ABE-Ab (Figure 9). From the linear regression in the concentration range of 0 – 100 µg/L as indicated in Table 10, the r values were observed to be 0.9876, 0.9518 and 0.9868, respectively. In contrast to amphetamine and methamphetamine, ephedrine showed the highest sensitivity with the slope value of 47.86 therefore this combination was proper for ephedrine detection.

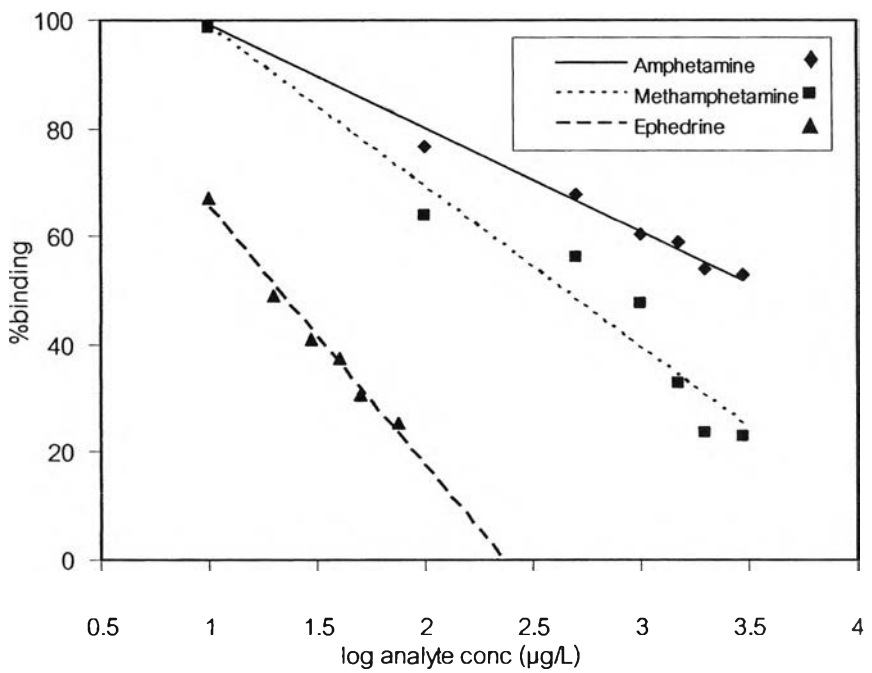


Figure 9 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge heterologous combination (B6)

Table 10 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge heterologous combination (B6)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 118.62 - 19.196x	0.9876
Methamphetamine	Y = 128.6 - 29.725x	0.9518
Ephedrine	Y = 113.21 - 47.861x	0.9868 *

* the proper competitive reaction

Hapten heterologous of 3-APE-Ab and 3-APA-HRP (H9)

Within the concentration range of 500 – 3,000 µg/L, methamphetamine showed the highest sensitivity with the highest slope and r values (Table 11). Therefore, methamphetamine could be detected with hapten heterologous combination H9.

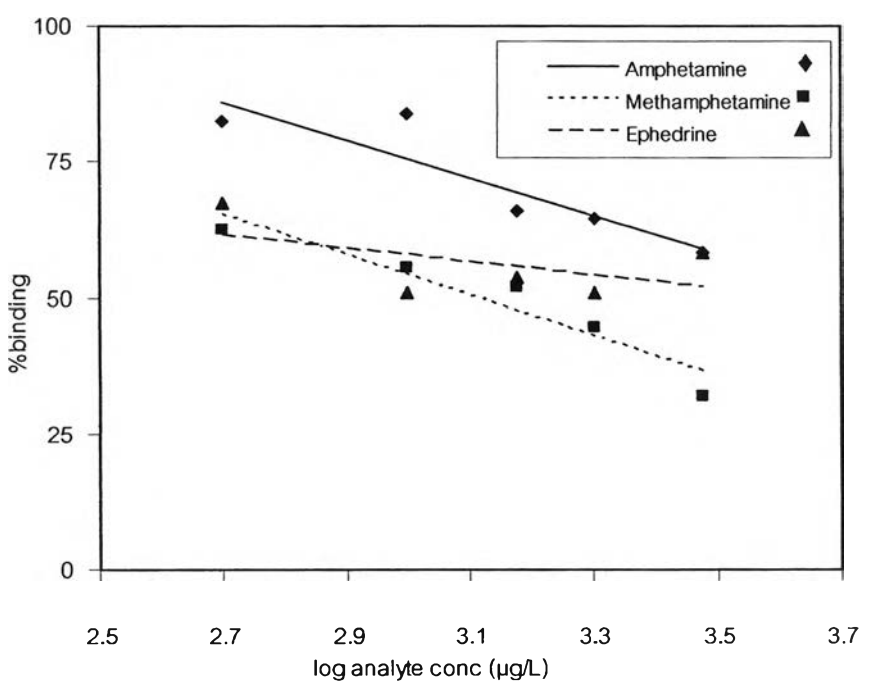


Figure 10 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H9)

Table 11 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H9)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 180.16 - 34.875x	0.8183
Methamphetamine	Y = 165.34 - 37.07x	0.9536*
Ephedrine	Y = 94.857 - 12.367x	0.2860

* the proper competitive reaction

Hapten heterologous of 3-APE-Ab and 3-APM-HRP (H10)

As shown in Figure 11, only methamphetamine and ephedrine could compete with 3-APM-HRP in binding to 3-APE-Ab. The linear response of methamphetamine and ephedrine within the concentration range of 500 – 3,000 µg/L was evidence with the r value of 0.9686 and 0.8288 (Table 12). Therefore, this combination was proper for detection of methamphetamine and ephedrine.

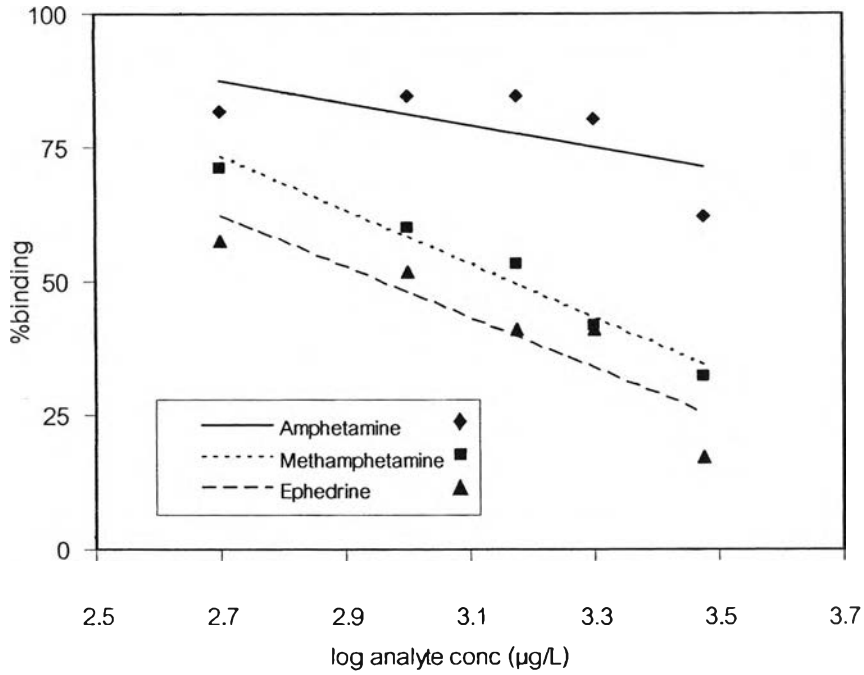


Figure 11 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H10)

Table 12 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H10)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 143.09 - 20.6x	0.4205
Methamphetamine	Y = 208.62 - 50.139x	0.9686*
Ephedrine	Y = 188.89 - 46.992x	0.8288*

* the proper competitive reaction

Bridge-hapten heterologous of 3-APE-Ab and 4-ABA-HRP (BH9)

Amphetamine, methamphetamine and ephedrine detection could compete with 4-ABA-HRP in binding to 3-APE-Ab in the concentration range of 500 – 3,000 µg/L with the r value of 0.9669, 0.9720 and 0.9361, respectively, (Table 13). In contrast to methamphetamine and ephedrine, amphetamine showed more than 50% binding in the concentration range of assay. Therefore, this combination was proper for detection of methamphetamine and ephedrine.

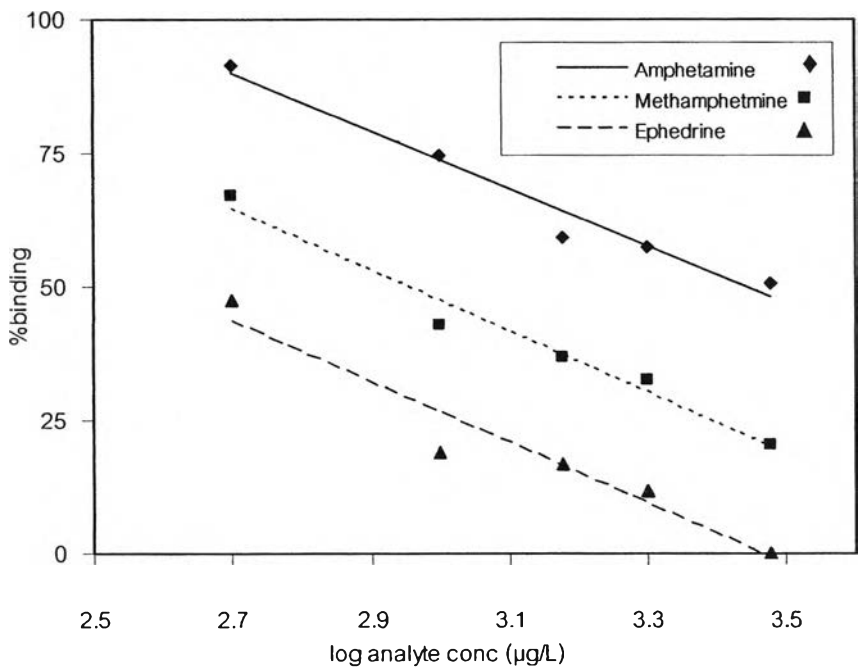


Figure 12 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH9)

Table 13 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH9)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 235.73 - 53.974x	0.9669
Methamphetamine	Y = 219.83 - 57.45x	0.9720 *
Ephedrine	Y = 197.81 - 57.105x	0.9361 *

* the proper competitive reaction

Bridge-hapten heterologous of 3-APE-Ab and 4-ABM-HRP (BH10)

As depicted in Figure 13, amphetamine, methamphetamine and ephedrine could compete with 4-ABM-HRP in binding to 3-APE-Ab with the r value of 0.6977, 0.8323 and 0.9680, respectively. As indicated in Table 14, ephedrine was presented the highest slope value of 41.70. Therefore, this combination was proper for ephedrine detection.

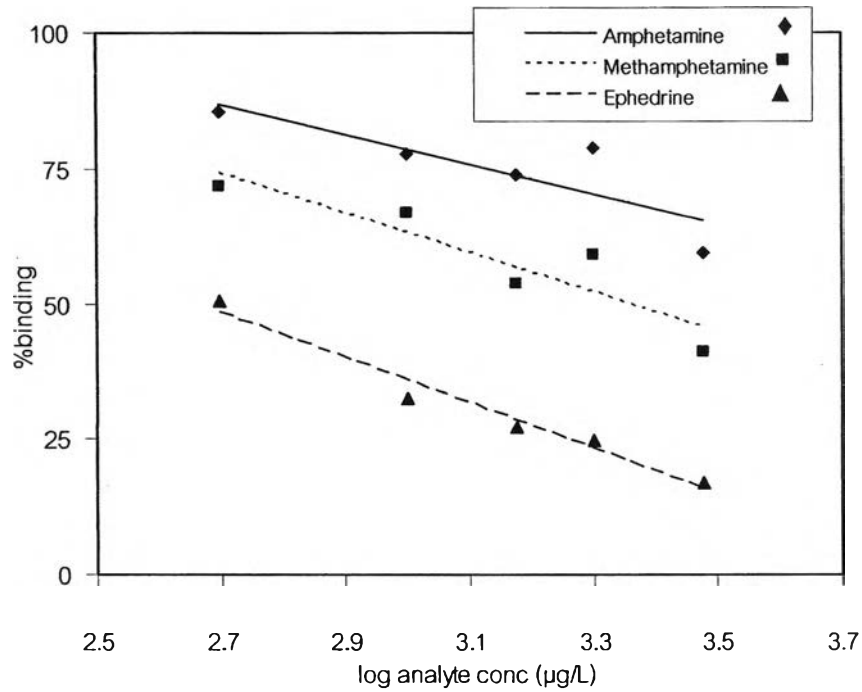


Figure 13 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH10)

Table 14 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH10)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 161.0 - 27.478x	0.6977
Methamphetamine	Y = 173.3 - 36.683x	0.8328
Ephedrine	Y = 160.96 - 41.704x	0.9680 *

* the proper competitive reaction

Bridge-hapten heterologous of 4-ABE-Ab and 3-APA-HRP (BH11)

No competitive reaction for amphetamine was observed. Only methamphetamine and ephedrine could compete with the 3-APA-HRP in binding to 4-ABE-Ab (Figure 14) within the concentration range of 500 – 3,000 µg/L with the r values of 0.7696 and 0.8917, respectively. However, this combination was selected for ephedrine detection because of the higher sensitivity and r values than methamphetamine.

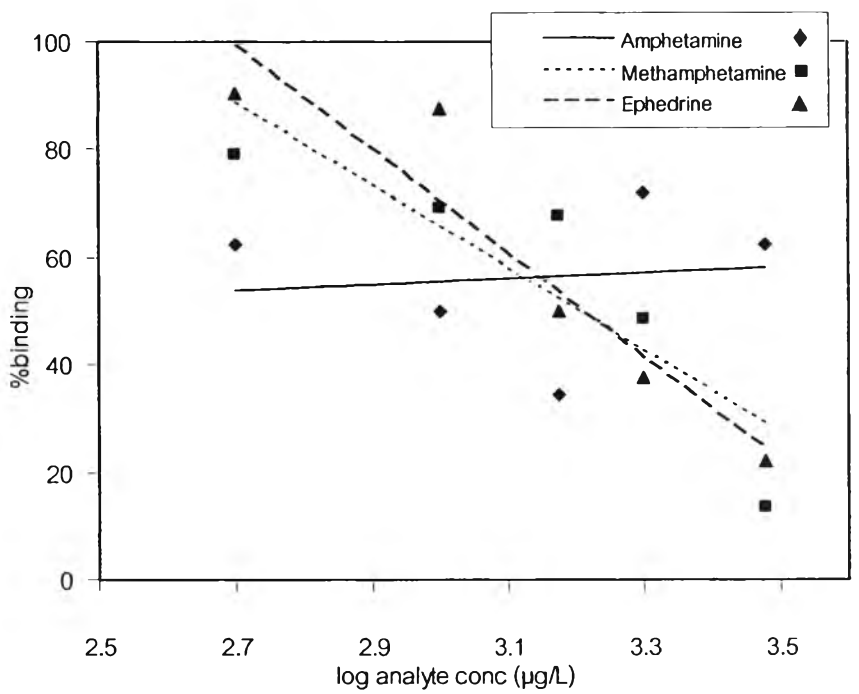


Figure 14 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH11)

Table 15 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH11)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 38.961 + 5.5259x	0.0129
Methamphetamine	Y = 295.28 - 76.567x	0.7696
Ephedrine	Y = 360.33 - 96.732x	0.8917 *

* the proper competitive reaction

Bridge-hapten heterologous of 4-ABE-Ab and 3-APM-HRP (BH12)

Only methamphetamine and ephedrine could compete with the 3-APM-HRP in binding to 4-ABE-Ab (Figure 15) within the concentration range of 500 – 3,000 µg/L with the r values of 0.9990 and 0.9399, respectively. Therefore, this combination was selected for methamphetamine and ephedrine detection.

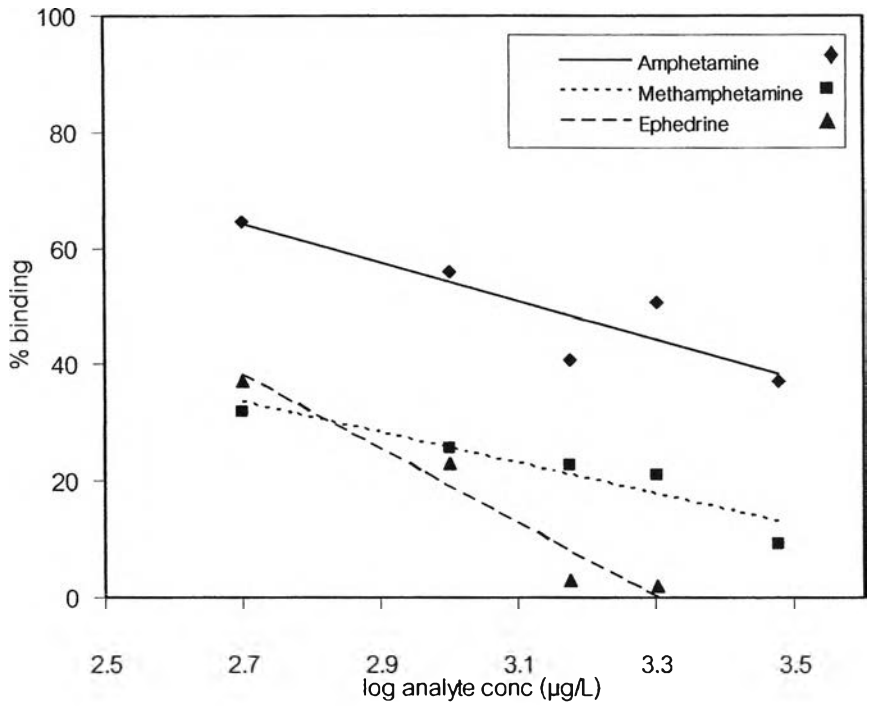


Figure 15 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH12)

Table 16 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (BH12)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 152.2 - 31.373x	0.5722
Methamphetamine	Y = 104.26 - 26.237x	0.9990 *
Ephedrine	Y = 208.46 - 63.151x	0.9399 *

* the proper competitive reaction

Hapten heterologous of 4-ABE-Ab and 4-ABM-HRP (H12)

Amphetamine, methamphetamine and ephedrine could compete with the 3-APA-HRP in binding to 4-ABE-Ab (Figure 16) within the concentration range of 500 – 3,000 µg/L with the r values of 0.9828, 0.8024 and 0.9100, respectively. Although, the sensitivity for methamphetamine and ephedrine detection was lower than amphetamine detection as the presented of the slope value in Table 17 but the concentration of amphetamine for decreasing the %binding to 50 was 3000 µg/L. Whereas, methamphetamine and ephedrine was less than 500 µg/L. Therefore, this combination should be more appropriate for detection of methamphetamine and ephedrine detection than amphetamine.

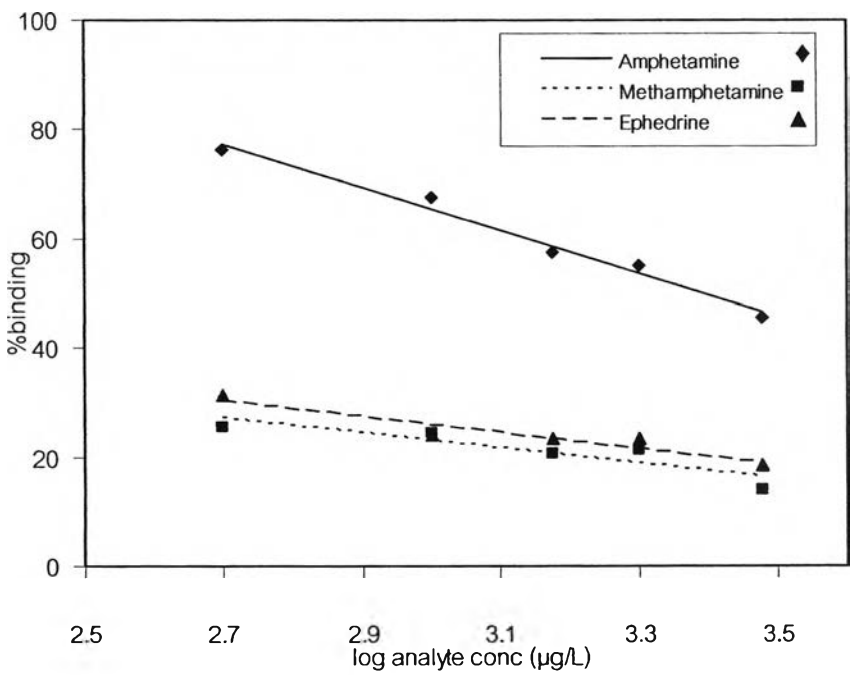


Figure 16 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H12)

Table 17 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by hapten heterologous combination (H12)

Analyte	Equation	Correlation coefficient (r)
Amphetamine	Y = 183.36 - 39.289x	0.9828
Methamphetamine	Y = 64.468 - 13.847x	0.8024 *
Ephedrine	Y = 70.108 - 14.728x	0.9100 *

* the proper competitive reaction

Selection of proper competitive reactions

From the logic plot of each possible combinations studied, the best competitive reaction with the highest value of r from each heterology studied was selected and summarized in Table 18. The B2 combination was the only proper combination for amphetamine detection with the slope of 23.773. For methamphetamine detection, no proper combination based on methamphetamine derivatives antibody could be observed. The H9 combination was selected because of the best sensitivity with the high slope value of 37.072. There were three combinations that were proper for ephedrine detection. Only the B6 and BH10 combinations with the higher r values were selected for ephedrine detection. The combinations for methamphetamine and ephedrine detection were the BH12 combinations as well as the candidate H12 combinations. Both combinations presented the possibility to detect methamphetamine and ephedrine at the concentration lower than that 500 µg/L.

As shown in Table 18, the combination of antibodies from amphetamine derivatives, 4-ABA, could detect only amphetamine whereas methamphetamine could not detected from the combination of antibodies from amphetamine derivatives. Similar observation was also reported in the other studies (Cheng et al., 1973; Faraj, Israili and Kight, 1976; Colbert et al., 1985; Aoki et al, 1990; Mongkolsirichaikul et al., 1993). It

would be possible that methamphetamine antibodies were directly bound to the secondary amine of methamphetamine. In addition amphetamine antibodies could well react with amphetamine and fail to interact with methamphetamine.

Choi et al. (1994) found that the combinations of antiserum from 4-ABM and enzyme labeled of 4-ABA and 4-ABM could detect methamphetamine. Moreover, the bridge combination of antiserum from 4-ABM and enzyme-labeled of 4-ABM exerted the better sensitivity than the other combination (Choi et al., 1994). In contrast to the present study, no proper combination of antiserum from 4-ABM was observed. This showed that the similar derivatives were used in the assay, the proper pairing of the antibody and enzyme-labeled for immuno-detection could be variated from one experiment to another.

The data given in Table 18 showed that increased the different of hapten for preparing the immunogen and the enzyme labeled, increased heterology, lead to effect on the selectivity. Therefore, only BH12 and H12, could detect both of methamphetamine and ephedrine.

No proper combination could detect all three analytes. This possible due to size of haptens using in the combination design (Danilova, 1994). For haptens with the relative large molecular weight using in immunogen preparation, the binding site of their antibodies were large enough for both small and large molecule analyte. In the contrary, the small molecule could presented the low affinity in binding to the antibodies. Therefore, the small molecule of amphetamine could show the low affinity in binding to methamphetamine and ephedrine antibodies as well as ephedrine and methamphetamine could not fit in the amphetamine antibody binding site.

These six appropriate combinations for amphetamine and/or methamphetamine and/or ephedrine detection would be carried through the cut-off value determination.

Table 18 The summarized of the proper competitive combinations

No.	Analyte	Combinations code	Equation	r
1	Amphetamine	B2	Y = -23.773x + 86.143	0.9644
2	Methamphetamine	H9	Y = -37.07x + 165.34	0.9536
3	Ephedrine	B6	Y = -47.861x + 113.21	0.9868
4	Ephedrine	BH10	Y = -41.704x + 160.96	0.9680
5	Methamphetamine ephedrine	BH12	Y = -26.237x + 104.26 Y = -63.151x + 208.46	0.9990 0.9399
6	Methamphetamine ephedrine	H12	Y = -13.847x + 64.468 Y = -14.728x + 70.108	0.8024 0.9100

A-2.2 Cut-off determination and confirmation

The proper combinations as shown in Table 18 were determined for cut-off value of their analytes and these cut-off values were confirmed for their accuracy. The different heterology presented the different ability for increasing the sensitivity as shown in Table 19.

As shown in Table 19, the bridge-hapten combinations, BH10 and BH12, were not absolutely presented the lower cut-off value than bridge (B2 and B6) or hapten (H9 and H12) combinations. However, these cut-off values were all sensitive enough for further studies. H9 and BH10 showed the cut-off value at 55.0 µg/L for methamphetamine and 30.0 µg/L for ephedrine.

Table 19 Cut-off value determination of the proper combinations

No.	Combinations code	Analyte	r	Cut-off (μg/L)
1	B2	Amphetamine	0.9644	15.0
2	H9	Methamphetamine	0.9536	55.0
3	B6	Ephedrine	0.9868	7.0
4	BH10	Ephedrine	0.9680	30.0
5	BH12	Methamphetamine ephedrine	0.9990 0.9399	12.0 5.0
6	H12	Methamphetamine ephedrine	0.8024 0.9100	32.0 4.0

A-3. Cross-reactivity

As indicated in Table 21, the combination B2 using antiserum from 4-ABA and the enzyme labeled of 3-APA was used for amphetamine detection. Ephedrine seems to be more action for detection of ephedrine than amphetamine with the percent cross-reaction of 200.0. The combination B6 could specifically detected ephedrine without any cross-reaction from amphetamine, methamphetamine and the other cross-reactants, respectively (Table 21). The combination BH12 for detection of methamphetamine and ephedrine. Only the phenethylamine drugs, amphetamine and phenylpropanolamine, could cross-reacted with the percentage of cross-reactivity at 5.0.

In B2 combination, 4-ABA-Ab showed the cross-reactivity to all of the phenethylamine and alkyl amine compound except ranitidine and cimetidine. It was

showed that the 4-ABA-Ab might bind with the amine moiety in the drug molecule. Methamphetamine was excreted about less than 5.0 mg/L as parent form therefore it was not interfere in detection of amphetamine. Ephedrine could detect with B2 because 70% of ephedrine was excreted in urine as unchanged form. Pseudoephedrine, more than 90% of the dose is excreted as unchanged form in urine, and vitamin c, the excretion as unchanged in urine excretion at 70%, could detected as false positive result with B2. In contrast to chlorpromazine that could not detected as positive result because of the less than 1% urine excretion as unchanged form (Table 20).

Whereas 4-ABE-Ab in the combination B6 and BH12 would be more selective to phenethylamine than alkyl amine, only the phenethylamine compounds could cross-react with both combinations.

Phenylpropanolamine could interfered BH12 for detection of methamphetamine and ephedrine because it is excreted as unchanged form in urine about 90%. It was reported that amphetamine concentration in urine after ingested 15.0 to 30.0 mg of amphetamine, was about 1.0 mg/L (Cleeland et al, 1976), it is therefore concluded that within the range of methamphetamine and ephedrine detection, amphetamine would not interfere.

It showed that 4-ABE-Ab was highly stereo-specific for ephedrine structure. In addition to the using of 3-APE-HRP as enzyme label, amphetamine, methamphetamine and other cross-reactants could not compete to bind the ephedrine antibodies that corresponding to the previous studies (Midha et al., 1983; Sirikatithum, 1999).

Table 20 The excretion of cross-reactant in urine

Name	Oral dose per day	Urine excretion in 24 hours
Amphetamine	5-20 mg	Unchanged 35-44% (620 - 3160 µg/L)
Methamphetamine	10 mg	30 – 54 % as methamphetamine (403 to 4919 µg/L) and 7% as amphetamine
Ephedrine	90 – 180 mg	55 – 75 %
Pseudoephedrine	180 - 240 mg	1.5 – 7.5 mg/L (90%)
Phenylpropanolamine	75 - 200 mg	90 %
Ranitidine	150 - 600 mg	70 - 80%
Cimetidine	800 - 1600 mg	50 - 80%
Diphenhydramine	75 – 200 mg	1.9%
Chlorpromazine	75 - 300 mg	< 1.0%
Chlorpheniramine	12 - 24 mg	0.06 – 3.0 mg/L (3-10%)
Caffeine	50 - 400 mg	1%
Theophylline	0.18 – 1.0 gm	13%
Paracetamol	Up to maximum 4 gm	1 – 4%
Ampicillin	1.0 – 8.0 gm	30%
Aspirin	1200 – 4000 mg	10 - 85 %
Ibuprofen	600 – 2400 mg	9%
Vitamin C	200 - 3000 mg	70%

Table 21 The cross-reaction of B2, B6 and BH12 combinations

Substances	Cross reactivity					
	B2		B6		B12	
	Conc. (mg/L)	%	Conc. (mg/L)	%	Conc. (mg/L)	%
Amphetamine	1.0*	100.0	>50.0	<2.0	20.0	5.0
Methamphetamine	7.5	13.3	>20.0	<5.0	1.0*	100.0
Ephedrine	0.5	200.0	1.0*	100.0	1.0*	100.0
Phenylpropanolamine	1000.0	0.1	1000.0	0.1	20.0	5.0
Pseudoephedrine	10.0	10.0	1000.0	0.1	2000.0	0.05
Ranitidine	>1000.0	<0.1	>1000.0	<0.1	>2000.0	<0.05
Cimetidine	1000.0	0.1	>1000.0	<0.1	>2000.0	<0.05
Chlorpromazine	10.0	10.0	>1000.0	<0.1	>2000.0	<0.05
Chlorpheniramine	100.0	1.0	>1000.0	<0.1	>2000.0	<0.05
Ibuprofen	>1000.0	<0.1	>1000.0	<0.1	>2000.0	<0.05
Diphenhydramine	10.0	10.0	>1000.0	<0.1	>2000.0	<0.05
Caffeine	>1000.0	<0.1	>1000.0	<0.1	>2000.0	<0.05
Theophylline	>1000.0	<0.1	>1000.0	<0.1	>2000.0	<0.05
Paracetamol	1000.0	0.1	>1000.0	<0.1	>2000.0	<0.05
Aspirin	>1000.0	<0.1	>1000.0	<0.1	>2000.0	<0.05
Ampicillin	1000.0	0.1	>1000.0	<0.1	>2000.0	<0.05
Vitamin C	<10.0	>10.0	>1000.0	<0.1	>2000.0	<0.05

* the detected analyte of the combinations

Ranitidine was not detected under all of these immunoassay while B2 combination, chlorpromazine was detected at concentration of 10.0 mg/L because of the similarity of three ethylene bridge including in 4-ABA. Ranitidine and chlorpromazine have been reported for their cross-reactivity in various immuno-detection (Grinstead, 1989; Polkis et al., 1991; Olsen et al., 1992). Ranitidine and chlorpromazine, could give

false positive result with monoclonal EMIT d.a.u. assay, but such result was not found with polyclonal EMIT d.a.u. assay or TDx amphetamine/methamphetamine II assay (Polkis et al., 1991). Although it was unknown on the principle of immuno-detection of these commercial tests but it was confirmed that the stereo-specific of 4-ABE antibodies could affect binding to antibody.

Although 4-ABA-Ab in combination B2 presented the selective detection of amphetamine, it also presented the cross-reactivity to methamphetamine and ephedrine. Therefore, the selection of amphetamine and/or methamphetamine and/or ephedrine should be selected from the polyclone to monoclone in monoclonal antibody production. 4-ABE-Ab in combination B6 and BH12 was presented the specific selection to methamphetamine and ephedrine detection in ELISA, therefore the selective for detection of methamphetamine and/or ephedrine should be selected in immuno-detection based on monoclonal antibody. Thus, 4-ABA and 4-ABE were used as the hapten for preparing the immunogens in monoclonal antibody production.

Whereas, the combination BH12 was proper for the detection of methamphetamine and ephedrine and it was selected for the candidate to monoclonal antibody combinations in further creation of membrane immunoassay in the next part.

Part B. Monoclonal antibody for amphetamine, methamphetamine and ephedrine detection

B-1 Monoclonal antibody production

Phase 1 Induction of polyclonal antibody in mouse.

In immunogen preparation, the terminal amino groups of 4-ABA and 4-ABE were linked to C-terminal of aspartic and glutamic residues of KLH by using carbodiimide linkage. The immunogens, 4-ABA-KLH and 4-ABE-KLH, were brownish fluffy powder and slightly soluble in PBS pH 7.2 The mole ratio of hapten conjugate to KLH in each immunogen could not determine due to the poorly soluble of these immunogens in PBS pH 7.4 resulting the turbid solution. The UV spectra for the filtration of 4-ABA-KLH and 4-ABE-KLH solution were in the similar pattern as shown in Figure 17. These compounds were used to be immunogens.

Characteristic of 4-ABA-BSA and 4-ABE-BSA

Two of plate coating haptens, 4-ABA-BSA and 4-ABE-BSA, were white fluffy powder. Their aqueous solution exhibited the maximum absorption of derivative at 257 and BSA at 280 nm (Figure 18). The ultraviolet spectrum of these immunogens in water have been reported (Matapatara, W., 2005a; 2005b). Therefore, these compounds were suitable to be plate coating hapten with the mole ratio of hapten to BSA in the range of 17 : 1 to 29 : 1

The polyclonal antibodies against amphetamine and ephedrine derivative were raised in mice. For 4-ABA-KLH immunogen, the titer of antibody was observed to be in the range of 1:2,000 to 1:10,000. 4-ABE-KLH could induce antibody in mice with the higher titer value of 1:20,000 to 1:60,000. Therefore, the spleen from these mice were further used in fusion procedure.

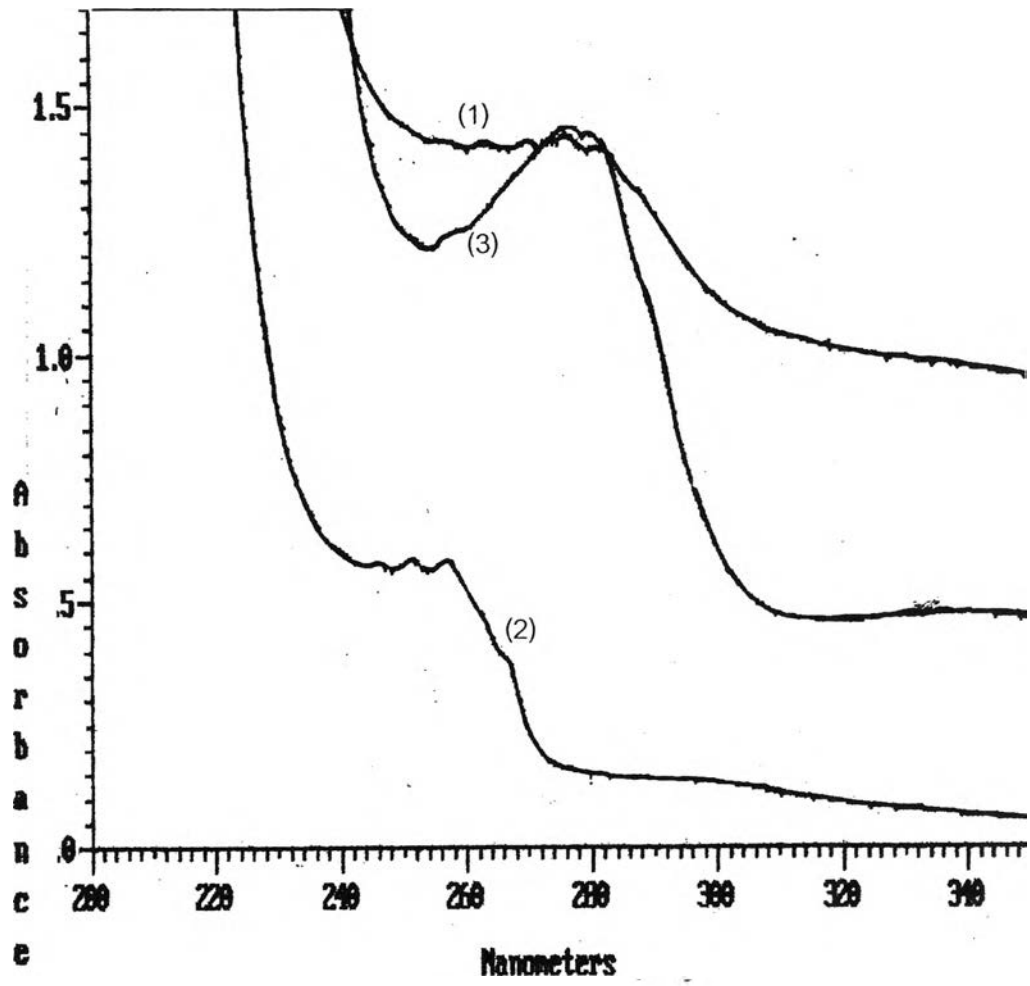


Figure 17 The UV spectra of aqueous solution of 4-ABA-KLH, 4-ABA and KLH

1 = The filtrate of 4-ABA-KLH 1.0 mg/ml

2 = 4-ABA 0.5 mg/ml (maximum absorbance at 257 nm)

3 = KLH 1.0 mg/ml (maximum absorbance at 278 nm)

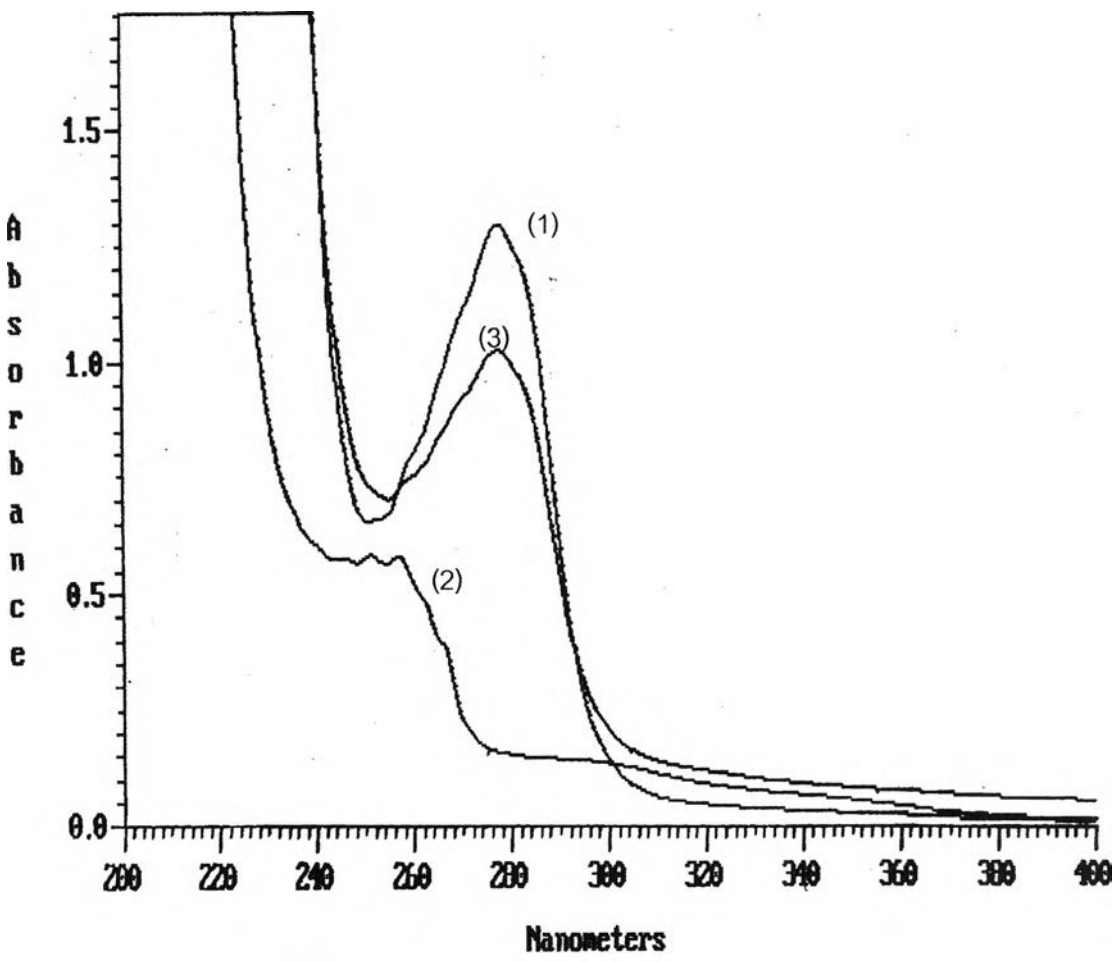


Figure 18 The UV spectra of aqueous solution of 4-ABA-BSA, 4-ABA and BSA

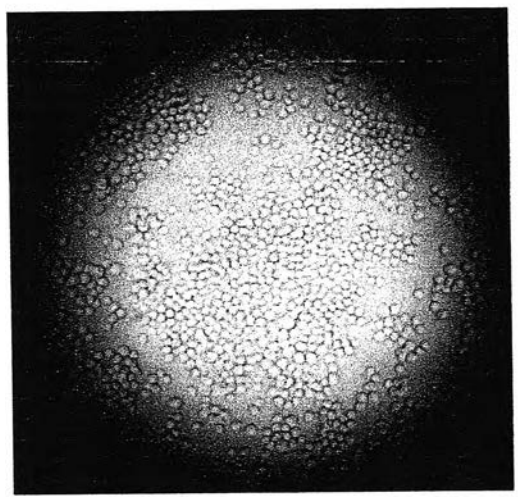
1 = 4-ABA-BSA 1.0 mg/ml

2 = 4-ABA 0.5 mg/ml (maximum absorbance at 257 nm)

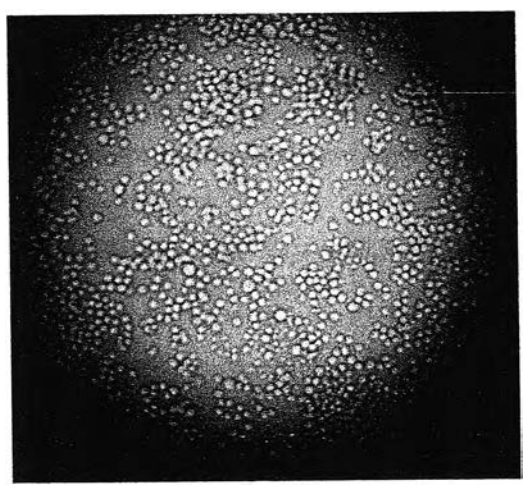
3 = BSA 1.8 mg/ml (maximum absorbance at 278 nm)

Phase 2 Preparation of myeloma cells and spleen cells

Three myeloma cell lines, P3-X63-Ag8.653, Sp2/0-Ag14 and NS-1, used in this study were magnified under the microscope as shown in Figure 19. Their growing in RPMI 1640 medium could yield approximately in the range of 10^6 to 10^7 cell/ml after four day incubation with cell viability of 90 to 99%. For their staining, they were diluted in trypan blue, a viability stain, prior to loading into the hemocytometer. Using this stain, live cells will appear clear and refratile and dead cells will be blue when viewed under the microscope.

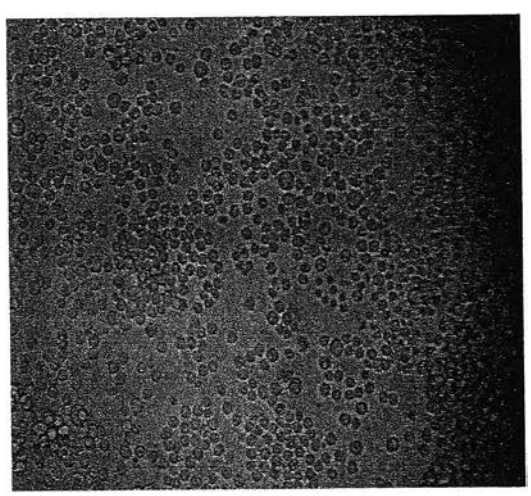


NS-1 myeloma cell line



Sp2/0-Ag14

myeloma cell line



P3-X63-Ag8.653

myeloma cell line

Figure 19 The different myeloma cell lines under inverted microscope (x100)

Each mouse provided different amount of spleen cell approximately in the range of 10^7 to 10^8 that is the typical yield of spleen cell in mouse. The spleen cell preparation method by pressing the cell through the fine sieve could be used for preparing the cell because the healthy B cell was observed from trypan blue staining with the viability about 99%. Therefore, the total healthy spleen cells were used in each fusion.

The large mass of adipose tissue around the spleen was the problem for spleen cell preparation. The adipose tissue have to be removed carefully, avoiding the overtaken of spleen cells.

Phase 3 Fusion

From all of the thirty fusions, there were only two successful fusion of 4-ABE-Ab and 4-ABA-Ab, calculated as the percentage of 6.0, from using only the NS-1 myeloma cell that was the suitable cell line for these fusions.

Phase 4 Selection of hybrid

Testing of HAT medium must be tested before using for hybridoma selection in addition to the lot of medium was changed. Myeloma cells in HAT medium should be dead in four days and hybrid cells in HAT medium was survived that indicate the proper HAT medium for selection of hybridoma cells.

The HAT medium is the complete growth medium that supplemented with hypoxanthine, aminopterin and thymidine (HAT). Aminopterin blocks the main pathway of DNA synthesis. The rescue pathway is uses when the cells are uptake this exogeneous hypoxanthine from the HAT medium, depends on presence of the hypoxanthinphosphoribosyltransferase enzyme (HGPRT). The myeloma cells that are destruct in the HGPRT enzyme, dies in HAT medium. Only hybrid between myeloma cells and spleen cells survive in HAT medium because the myeloma provides the ability

to grow in cell culture medium and the spleen cells contribute the functional HGPRT enzyme.

The growth of hybridoma after fusion as displayed in Figure 20 shown in Figure 20[A]. Three days later, spleen cells and myeloma cells died (Figure 20[B]). The fusion of a spleen cell and myeloma cell resulted in a hybridoma cell which now has a complete set of enzymes to enable it to survive in selective medium containing hypoxanthine, aminopterin and thymidine (HAT).

After seven days following fusion (Figure 20[C]-[D]), wells were visually inspected as small clusters of cells for hybridoma cells growth. The colonies were observed in 144 of 960 wells for 4-ABE-KLH as the immunogen (15% fusion efficiency) and 620 of 960 wells for 4-ABA-KLH as the immunogen (64.6 % fusion efficiency). This could be shown that the successful of fusion from using 4-ABA-Ab over the 4-ABE-Ab.

The mixtures of hybrid cells are growth in HAT medium which result in consist of the different specific antibodies. Antibodies produced from the hybrid cells were determined. The titer of the antibody is not important in this stage. The first antibody screen after fusion will be required when, by observation, the density of viable hybrid cells reach approximately one third of the well. Supernatants of good growth of hybridoma were analyzed; in this way the sample contained an optimum amount of antibody. This will usually occur between 10 and 14 days post-fusion depend on the appearance of ability of cell growth, although longer periods may be required if the initial seeding density was low.

Culture supernatants were first screened for activity against the immunogen and BSA used as plate-coated hapten. The supernatant was directly tested without dilution because antibody levels in culture supernatant rarely exceed 10 µg/ml. The result showed that all of the positive wells did not react to the coupling protein. The positive and negative control in screening test were confirmed the complete system of indirect ELISA testing. Number of the positive wells for 4-ABA-Ab and 4-ABE-Ab as

presented in Table 22 were subsequently tested for competitive reaction for amphetamine, methamphetamine and ephedrine detection.

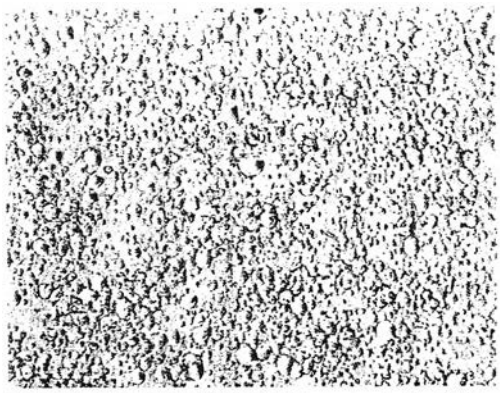
Table 22 The screening of positive hybrid cell in culture supernatant

Antibody	Number of positive wells*							
	First screen		Secondary screen		Third screen		Fourth screen	
	Day	Number	Day	Number	Day	Number	Day	Number
4-ABA-Ab	14	590	20	40	25	12	30	5
4-ABE-Ab	14	96	21	35	26	0	31	0

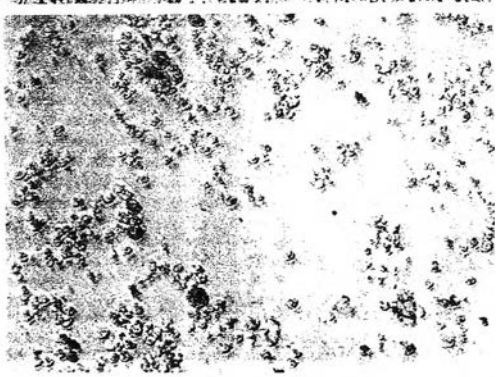
* The initial seeding was 960 wells (96x10 wells)

After changing the culture medium every 3 days, the background antibody produced by residual spleen cells was eliminated by this process of changing the medium. The decreasing of positive wells number from first to secondary screen showed that the positive result in the first screen was obtained for the residue spleen cells.

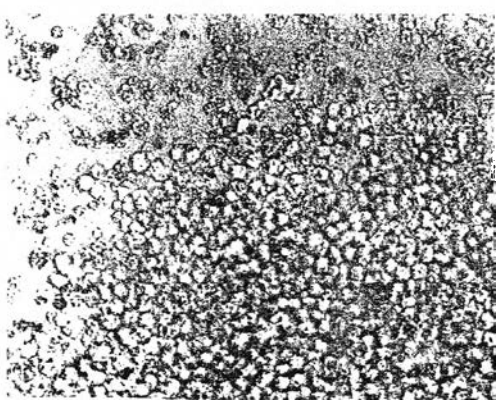
The supernatants were assayed for antibody activity approximately days 14 During this time, the hybridoma cells are almost confluent in the wells and the medium was clearly yellow. Sequential analyses of antibody activity in supernatant removed at 5 to 6 day intervals was useful in showing whether antibody production was increasing or declining in a particular culture. Therefore, the re-screened was performed in day 20-21, 25-26 and 30-31 for 4-ABA-Ab and 4-ABE-Ab, respectively.



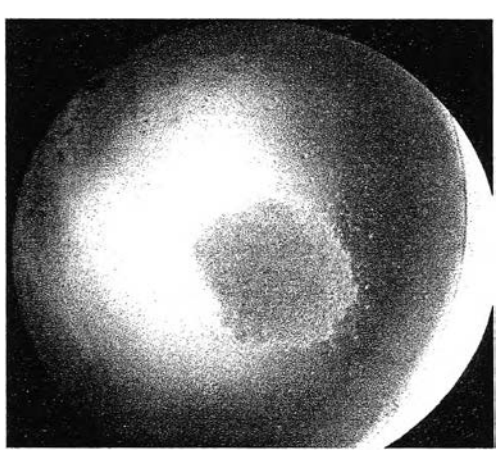
[A] Cell mixture in each well immediately after fusion



[B] Cell mixture after three days in HAT medium with the observing dead cells



[C] Cell mixture after seven days in HAT medium with the inspection for hybridoma growth



[D] A colony of the survive hybridoma cells in HAT selecting medium

Figure 20 The inspection for hybridoma growth in HAT medium (x100)

The result showed that, the 4-ABE-Ab produced cell was not found in these fusions. The growth cells from the first positive screening did not continue to produce 4-ABE-Ab because the cell was unstable or the first and second positive result from the background antibody was produced by residual spleen cells.

Therefore, only the fusion of 4-ABA-Ab production was success. As shown in Table 21, five positive wells of 4-ABA-Ab; 98, 147, 283, 296 and 408, were showed the high activity against 4-ABA hapten and giving the strong positive response in the non-competitive indirect ELISA (absorbance between 1.0 – more than 3.0). No any positive well was bound to the BSA.

These wells were tested for competitive indirect ELISA against amphetamine, methamphetamine and ephedrine. Amphetamine, methamphetamine and ephedrine were not compete with 4-ABA-BSA to bind all of the 4-ABA-Ab in five wells. Although, all of them were expanded for further cloning and rechecked with the indirect ELISA.

The expansion took about seven days but may be completed in a shorter time if the cells showed particularly vigorous growth. The cell population would then be ready for re-screening. It was advisable to re-screen at this stage because in this relatively early stage of selection the hybrid cells are particularly susceptible to chromosomal loss of antibody production or to the overgrowth by some vigorous non-secreting cells.

Phase 5 Cloning of hybridoma cell lines

Each of the positive screening well was expanded to more than one well. Therefore, there were about twelve plates from all of the five positive cells after cloning with the limit dilution method. By using the limit dilution method for cloning the hybridoma cells, the single cell was grown and increased number of cell in two weeks as shown in Figure 21. The number of wells that cell survived and grown was in the range of less than

10 to about 30 per plate that correspond to the statistical number of 30. Almost the single cell in the first cloning that survived in cloning step was grown very slowly and also dead in the end. The survive cell found in the well that contained the initial number of cell more than 5 cells. Therefore, all clusters of the survive cells were re-cloned to confirm that it was from the single cell. Each of the cloned well was re-cloned more than three times for obtaining the single cell. In the cloning step, only the strongest positive single cell was selected from each of the cloned well and defined as the MAb from that well.

During the cloning step, the well No. 147 was lost the activity in binding to 4-ABA hapten, therefore, the final wells number for further competitive ELISA were the monoclonal antibodies from well No. 98, 283, 296 and 408. All of the monoclone presented the positive in the non-competitive ELISA.

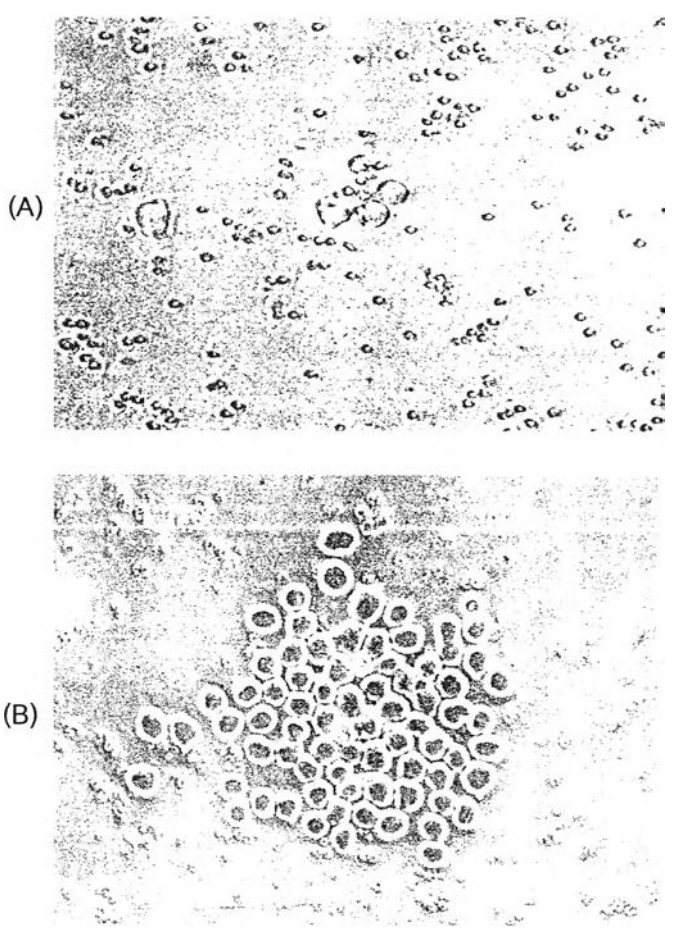


Figure 21 Cloning at one (A) and second week (B) (x100)

The competitive indirect ELISA of the MAb No. 98, 283, 296 and 408 was shown in Table 23. It was shown that only b1 and bh2 could detect amphetamine, methamphetamine and ephedrine. All of the selective results in Table 23 was observed in the concentration of 100.0 mg/L. Therefore, MAb No. 98 was expanded and the supernatant was re-screened, harvested, collected and purified. The positive cloned cell lines were frozen in liquid nitrogen for the reservation of the specific antibody.

Table 23 The competitive indirect ELISA for amphetamine, methamphetamine and ephedrine detection using MAb No.98, 283, 296 and 408

Plate coating hapten	Competitors (100.0 mg/L)											
	Amphetamine				Methamphetamine				Ephedrine			
	MAb No.				MAb No.				MAb No.			
	98	283	296	408	98	283	296	408	98	283	296	408
3-APA	NS	NS	NS	NS	S	NS	NS	NS	S	NS	NS	NS
4-ABA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3-APM	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
4-ABM	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3-APE	S	NS	NS	NS	S	NS	NS	NS	S	NS	NS	NS
4-ABE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

* the decreasing of final absorbance comparing to free drug was indicated as selective (S) and no decreasing was indicated as non-selective (NS)

Therefore, two proper combinations of b1 and bh2 were selected for further quantitative assay in the concentration range of 0 – 100.0 mg/L for amphetamine, methamphetamine and ephedrine detection.

After partial purification with ammonium sulfate method, the titration of MAb No. 98 was determined. The dilution at 50% binding of MAb No. 98 with 4-ABA-BSA was 1 : 20 and this dilution was used in for further competitive reaction. The 3-APA-BSA

and 3-APE-BSA were the proper plate coating in the competitive reaction. There were no proper combinations for detection only one analyte of amphetamine or methamphetamine or ephedrine.

B-2. Competitive indirect ELISA of amphetamine, methamphetamine and ephedrine detection using MAb No. 98

By using b1 and bh2, the competitive reaction between amphetamine, methamphetamine and ephedrine and plate coating hapten in binding to MAb No.98 was shown in Table 24 - 25 and Figure 22 - 23. The result was presented that MAb No.98 was strongly bound to plate coating hapten than the analytes. The concentration at 50% binding was not determined because the percentage of binding did not decrease to 50 in the concentration range of testing (0 - 100.0 mg/L).

The combinations of b1

The logic plot in Figure 22 showed the low sensitivity for almost every dilution used. The dilution of antibody 1 : 10 as tabulated in Table 24 that showed the competitive response for ephedrine with the r value of 0.9038 and slope of 4.2481 In contrast to the dilution of antibody 1 : 20 or 1 : 50 that might detect amphetamine, methamphetamine and ephedrine with the similar slope and r value as shown in Table 24. Therefore, the combinations of MAb No. 98 at 1 : 20 and 3-APA-BSA was selected for amphetamine, methamphetamine and ephedrine detection.

Table 24 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge heterologous combination (b1)

MAb No. 98 dilution	Linear regression parameters					
	Amphetamine		Methamphetamine		Ephedrine	
	slope	r	slope	r	slope	r
1 : 10	0.1365	0.0308	0.0145	0.0031	4.2481	0.9038
1 : 20	2.8017	0.9299	3.5209	0.9913	2.7589	0.8972
1 : 50	3.4406	0.8623	2.6175	0.8382	1.8766	0.9378

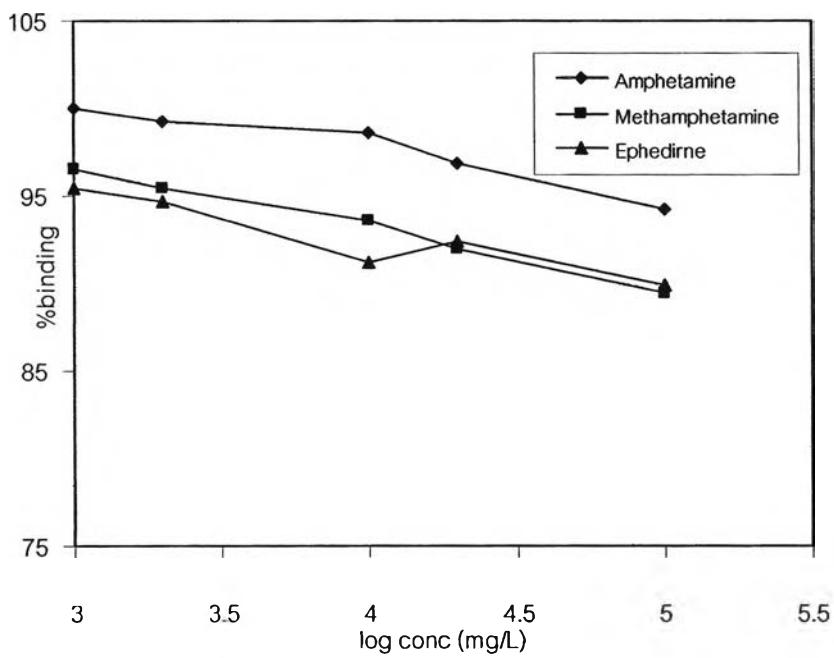


Figure 22 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge heterologous combination (b1)

The combinations of bh2

The relationship between the percentage of binding and the log concentration of analyte at different dilution of 3-APE-BSA were depicted in Figure 23. The dilutions of antibody 1 : 10 were the better proportion for amphetamine and methamphetamine detection with the r value of 0.9719 and 0.9948 as indicated in Table 25. But, the binding was not reduced to 50% in the concentration range of amphetamine, methamphetamine and ephedrine.

Table 25 Dose response relationship for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (bh2)

MAb No.98 dilution	Linear regression parameters					
	Amphetamine		Methamphetamine		Ephedrine	
	slope	r	slope	r	slope	r
1 : 10	6.7175	0.9719	8.8075	0.9948	0.5280	0.3374
1 : 20	4.374	0.9371	3.8212	0.9685	2.6828	0.7373
1 : 50	2.910	0.7740	4.3997	0.8653	4.1975	0.7735

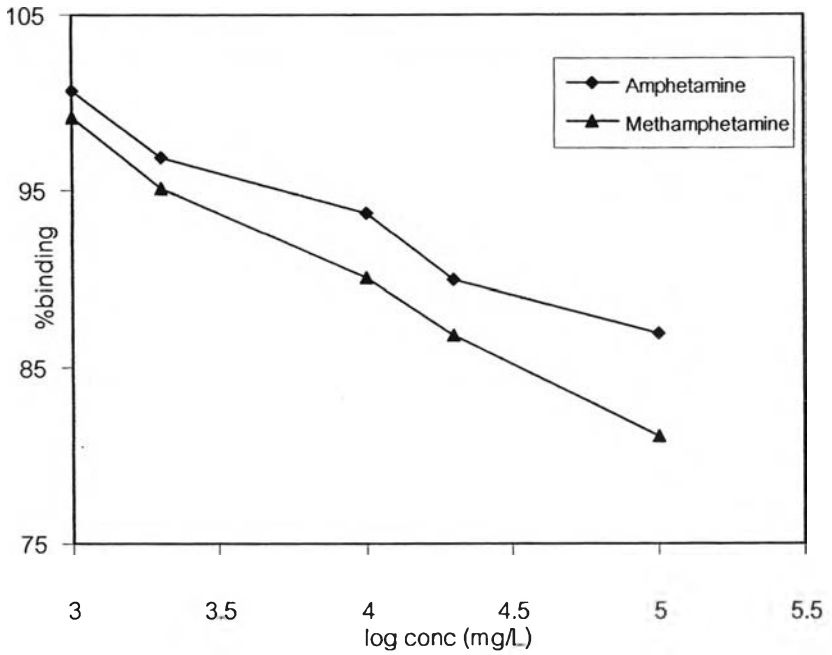


Figure 23 Dose response curve for competitive reaction of amphetamine, methamphetamine and ephedrine by bridge-hapten heterologous combination (bh2)

2.3 Cut-off determination

The cut-off value of the combinations of b1 and bh2 was determined for amphetamine with methamphetamine and amphetamine as shown in Table 26. Because of the strong binding of antibody to plate coating, the cut-off values of these combinations were very high when compare to polyclonal antibody in part A.

Table 26 Cut-off value determination of proper combinations

No.	Combinations	Analytes	Cut-off value (mg/L)
1	b1	Amphetamine	10.0
		Methamphetamine	10.0
		Ephedrine	6.7
2	bh2	Amphetamine	9.0
		Methamphetamine	9.0

The cut-off concentration of b1 and bh2 were accepted from the confirmation test. Therefore, both combinations were carried through the cross-reactivity against the same cross-reactants as part A.

2.4 Cross-reactivity

No cross-reaction against MAb No. 98 antibody was observed from the cross-reactants as shown in Table 27. This confirmed that the MAb No. 98 was bound very well to the immunogen hapten, 4-ABA, with the low cross-reactivity to all of the cross-reactants.

The overall results of this study implied that the MAb No.98 was bound very strongly to the four ethylene bridge of plate-coating derivatives, 4-ABA, 4-ABM and 4-ABE. Amphetamine, methamphetamine and ephedrine could compete to bind with MAb No. 98 when using the three ethylene bridge derivatives of amphetamine and ephedrine, 3-APA and 3-APE, as plate-coating hapten. But the competition did not occur when using the 3-APM hapten, the three ethylene bridge and tertiary amine. MAb No 98 would possibly specific for the tertiary N-alkyl moiety.

Table 27 The cross-reactivity for the combination of b1 and bh2

Compound names	b1		bh2	
	Concentration at 90% binding (mg/L)	Cross-reactivity (%)	Concentration at 90% binding (mg/L)	Cross-reactivity (%)
Amphetamine	200.0	50.0	100.0	100.0
Methamphetamine	100.0	100.0	100.0	100.0
Ephedrine	100.0	100.0	500.0	20.0
Phenylpropanolamine	>10,000	<1.0	>10,000	<1.0
Pseudoephedrine	>10,000	<1.0	>10,000	<1.0
Ranitidine	>10,000	<1.0	>10,000	<1.0
Cimetidine	>10,000	<1.0	>10,000	<1.0
Chlorpromazine	>10,000	<1.0	>10,000	<1.0
Chlorpheniramine	>10,000	<1.0	>10,000	<1.0
Ibuprofen	>10,000	<1.0	>10,000	<1.0
Diphenhydramine	>10,000	<1.0	>10,000	<1.0
Caffeine	>10,000	<1.0	>10,000	<1.0
Theophylline	>10,000	<1.0	>10,000	<1.0
Paracetamol	>10,000	<1.0	>10,000	<1.0
Aspirin	>10,000	<1.0	>10,000	<1.0
Ampicillin	>10,000	<1.0	>10,000	<1.0
Vitamin C	>10,000	<1.0	>10,000	<1.0

Part C. Detection of amphetamine, methamphetamine and ephedrine via membrane immunoassay

From the competitive ELISA for amphetamine, methamphetamine and ephedrine detection in part A and B, the proper combinations of 4-ABE-Ab and 3-APM-BSA for methamphetamine and ephedrine detection was selected for membrane immunoassay. The 4-ABE-Ab-Au and 3-APM-BSA were prepared and used in the membrane immunoassay.

C-1 Preparation of gold-labeled antibodies

A 5.0 mg/ml of 4-ABE-Ab solution was dialyzed against 2 mM borax buffer pH 9.0 to remove other electrolytes which can cause the de-stabilization of colloidal gold. Antisera solution have to be freshly prepared, because upon storage the protein may aggregate and cause the clumps formation, all antibody solution have to be centrifuged and filtered under sterile condition (0.22 μm) prior to be labeled.

Determine the appropriate antiserum concentration need for stabilizing colloidal gold

The volume variable adsorption isotherms for colloidal gold (Au) with 4-ABE antiserum was shown in Figure 24. Amount of antibody for stabilizing the colloidal gold was indicated at 0.3 ml of antibody where an absorbance value approaching to zero.

The arrow at 0.3 ml of antibody indicated the amount of antiserum that could possibly stabilize the colloidal gold against flocculation with sodium chloride. Therefore, the suitable antiserum for 4-ABE concentration was 15.0 microgram of antibody (the volume obtained from the curve which indicated the minimum value of absorbance was modified to explain as concentration.

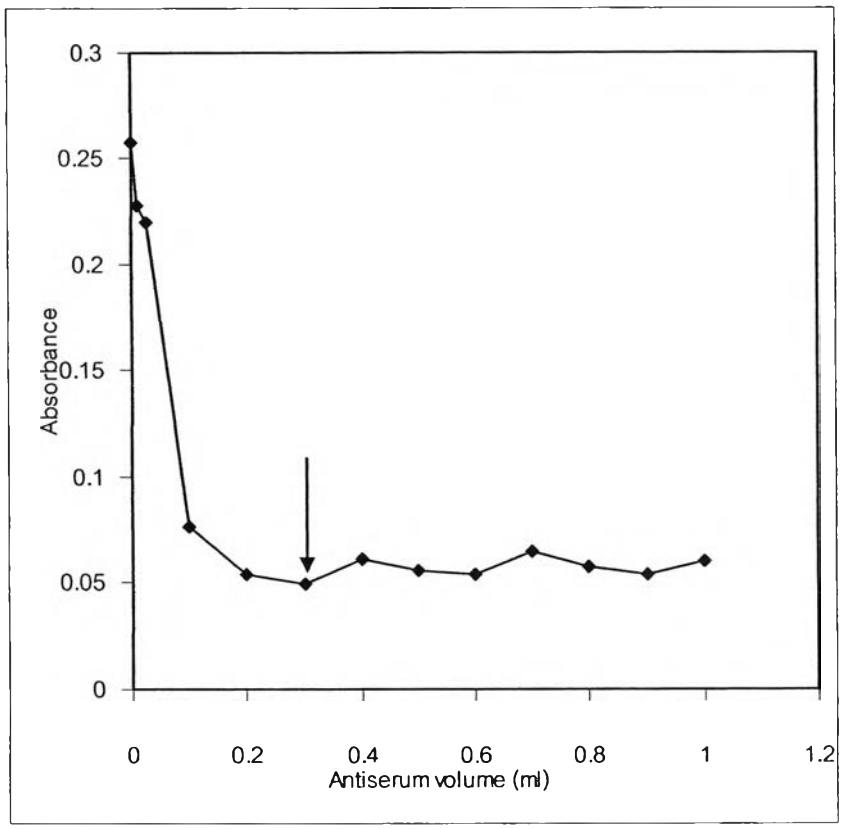


Figure 24 The relationship between absorbance and volume of antiserum solution for stabilizing colloidal gold

In the other way, the appropriate antiserum concentration need for stabilizing colloidal gold could be observed at absorption spectra by difference spectroscopy in the individual dilution of antiserum at the wavelength of 580 nm. As shown in Figure 25, curve A represented the suitable antiserum concentration need for stabilizing colloidal gold. While curve B, C, and D represented of sub-obtimal stabilize with decreasing volume of antiserum solution. These specific pattern of spectra observed coincide with the other reports in labeling gold with immunoglobulin G, concanavalin A and ovomucoid, etc. (Geoghegan et al., 1980; Horisberger, 1981; De May, 1983; Geoghegan, 1988; Martin et al., 1990).

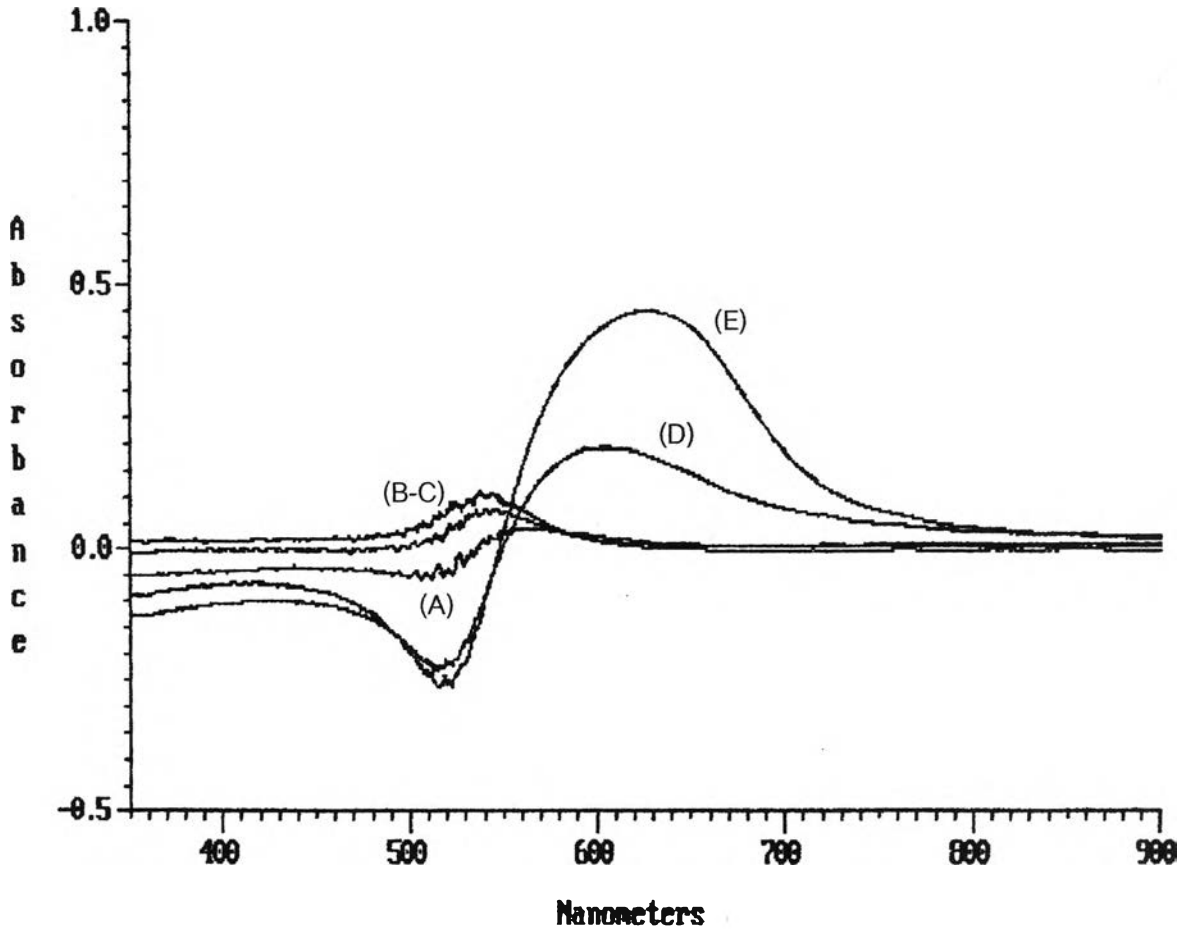


Figure 25 Visible adsorption spectra of different concentration of antisera for stabilizing colloidal gold. Instrument zeroed with an colloidal gold blank before recording

A = Optimal stabilized with sufficient antisera, the antisera at volume of 25 µl; concentration of antisera at 12.6 µg/ml

B and C = Excess antisera at volume of 100 and 200 µl; concentration of antisera at 50.4 and 100.8 µg/ml, respectively.

D = Sub-optimal stabilized with low quantities of antisera, the antisera at volume of 10 µl; concentration of antisera at 5.04 µg/ml

E = Uncoated colloidal gold, the antisera at volume of 0 µl; concentration of antisera at 0 µg/ml

Gold labeled antibodies

The color of gold-labeled antibodies solution was red as the color of colloidal gold. The product was approximately 10.0 ml and have an absorbance at 529 nm about 0.75 for gold-labeled-4-ABE-Ab. As shown in Figure 26, the maximum absorbance of 4-ABE-Ab-Au was 529 nm which represented the higher wavelength than of colloidal gold alone (520 nm). Although, there are several methods for characterizing the gold-labeled antibody such as transmission electron microscope (TEM), dynamic light scattering (DLS), but this study used only with absorption spectrophotometry (Martin et al., 1990; Roc et al., 1987).

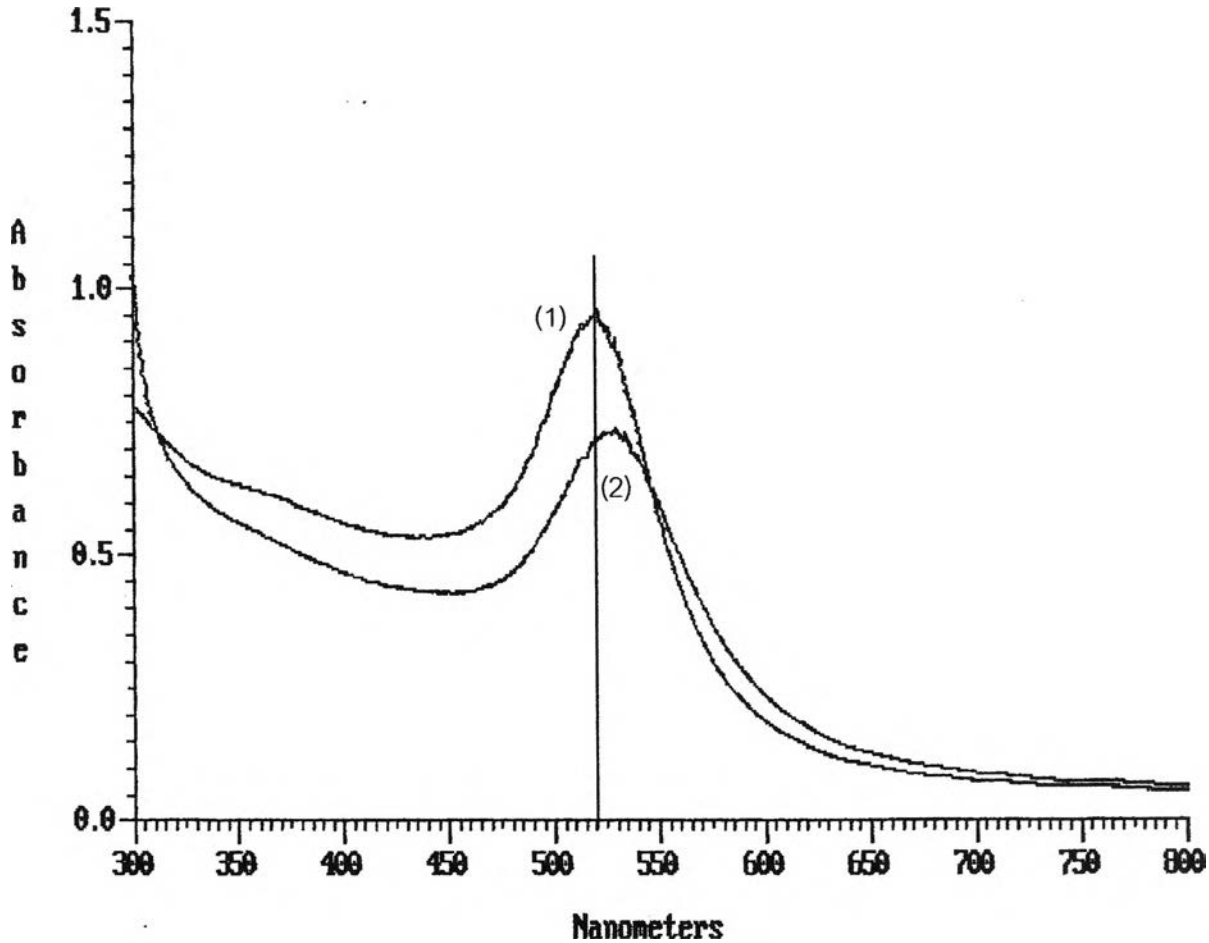


Figure 26 The UV spectra of antisera gold conjugate

1 = colloidal gold (max absorbance at 520 nm)

2 = antisera gold conjugate (max absorbance at 529 nm)

Determination of methamphetamine and ephedrine utilizing gold-labeled antibody

Methamphetamine and ephedrine could be detected by the combinations of 4-ABE-Ab-Au and 3-APM-BSA in the concentration range of 0 – 1000 µg/L (Figure 28). The saturation of response for methamphetamine and ephedrine detection was observed when the analyte concentration was more than 1,000 µg/L.

From absorbance data in Table 28, methamphetamine and ephedrine could be detected at the cut-off value of 500 µg/L with only 10% the cross-reactivity from amphetamine.

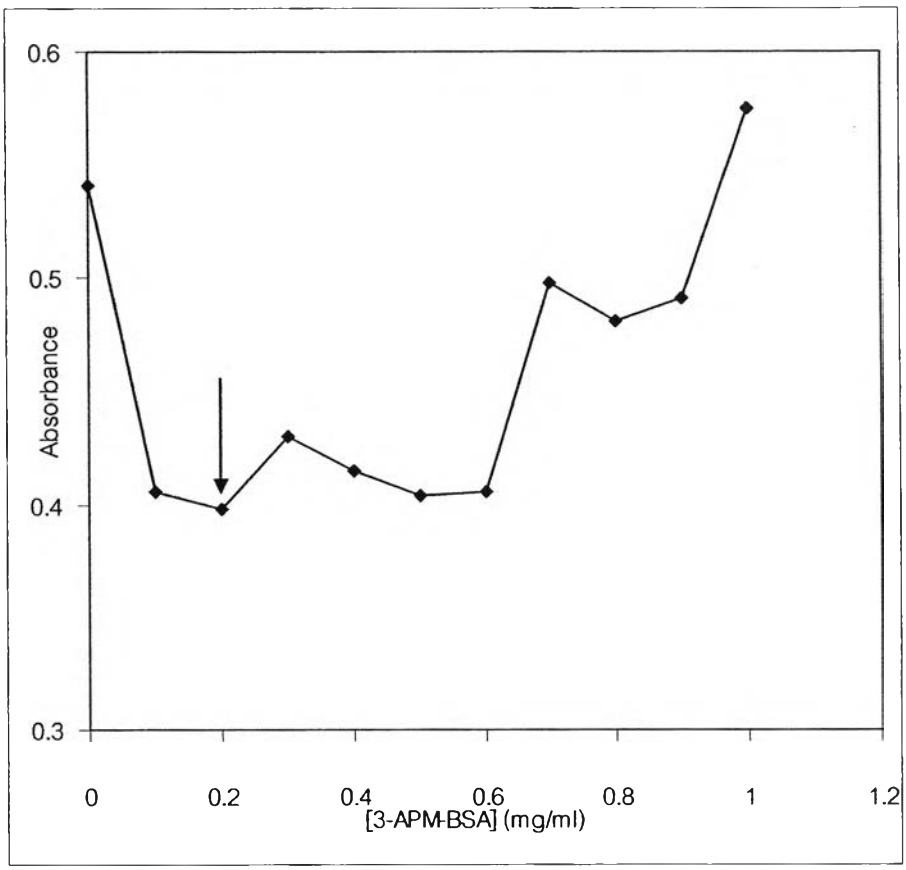


Figure 27 The relationship between absorbance and 3-APM-BSA solution for determination of the appropriate concentration of 3-APM-BSA for agglutination reaction

Table 28 The absorbance at 510 nm for agglutination detection of amphetamine, methamphetamine and ephedrine detection

Standard concentration (µg/L)	Absorbance at 510 nm		
	Amphetamine	Methamphetamine	Ephedrine
0	0.466	0.466	0.466
500	0.513	0.545	0.540
1,000	0.525	0.564	0.575
3,000	0.530	0.555	0.575
5,000	0.550	0.569	0.575
10,000	0.562	0.560	0.560

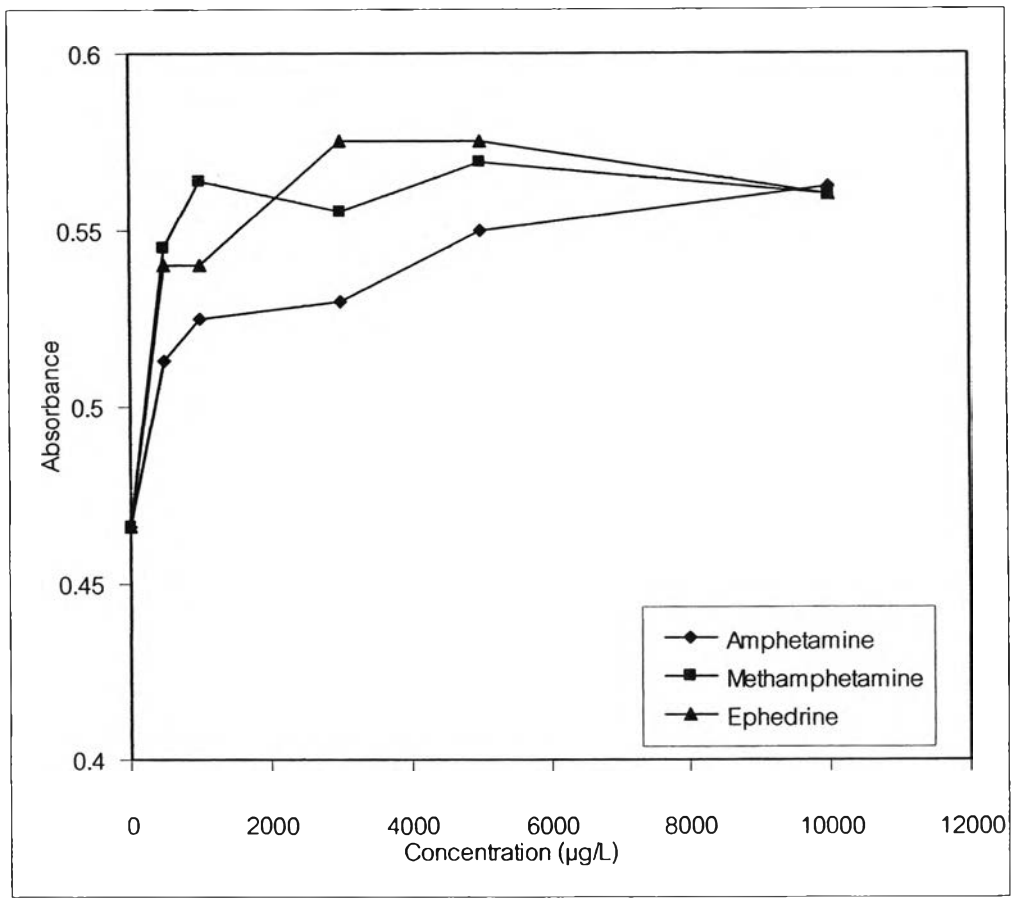


Figure 28 The plot between absorbance at 510 nm against the concentration of amphetamine, methamphetamine and ephedrine in Sol Particle Immunoassay (SPIA)

C-2 Development of membrane immunoassay

The combination of 4-ABE-Ab-Au and 3-APM-BSA that could detect methamphetamine and ephedrine at the linear concentration range of 0 – 1000 µg/L in qualitative SPIA was used in the lateral flow immunoassay.

In selection the type of membrane, no red color appeared on all seven types for non-specific binding test. It informed that all type of tested membranes could use gold as the labeling membrane immunoassay.

From the protein binding test, the red line at test band and control band were appeared on only three types of nitrocellulose membranes as shown in Figure 29. However, the PRIMA 40 nitrocellulose membrane was chosen for further lateral flow immunoassay due to it's convenience with polyester backing.

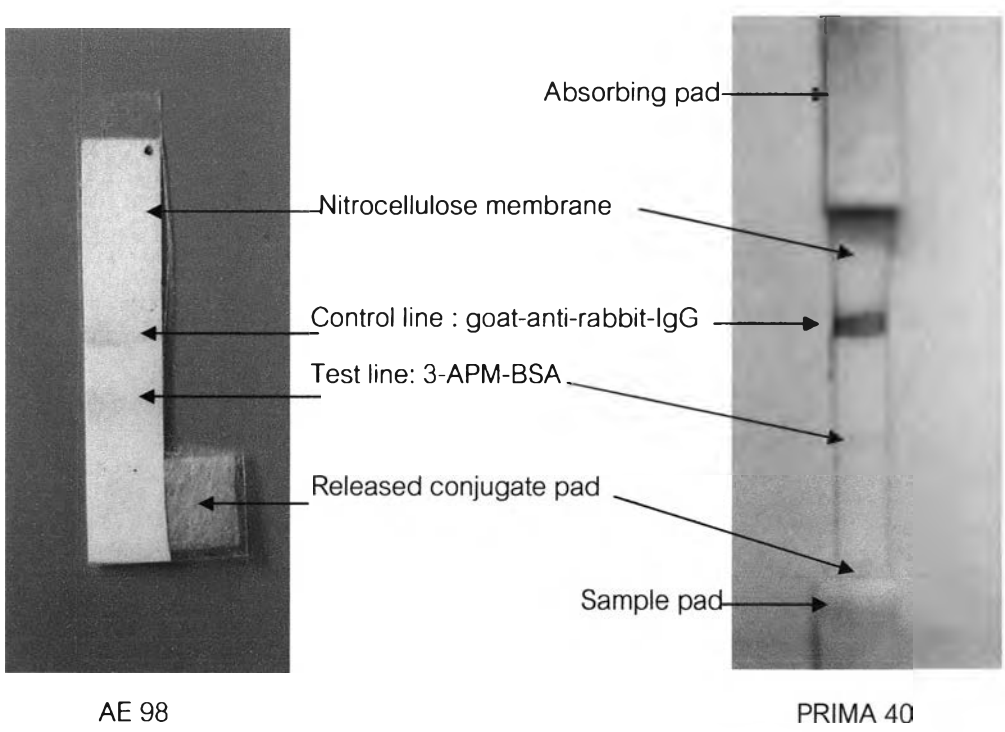


Figure 29 The red line of control line and test line on nitrocellulose membrane for protein binding testing

The 5 x 20 mm size of sample pad could absorb the 200 µl sample solution and flow through the membrane in not more than 2 minutes. Whereas the sample volume 300 and 400 µl could not absolutely adsorb on the sample pad as well as 150 µl of sample solution was insufficient for soak all of the gold conjugated from the pad. Therefore, the low intensity of control line was obtained because of the low amount of excess gold conjugated. The absorbing pad, 5 x 20 mm, also could absorb the excess solution with keeping continuous capillary flow of sample solution.

For determination of gold conjugate volume in the gold conjugated pad, the initial volume of gold conjugated was 1.0 ml. Conjugated pad in size of 9 x 20 mm was immersed in the red solution of gold label solution for 30 min. After the gold conjugate was picked up from the gold conjugate solution, the remain solution was about 0.600 ml Therefore, absorbing volume was 0.400 ml per 180 mm^2 or the gold conjugated pad that cut into 3 x 5 mm was contained 33.3 µl of gold label for apply in the membrane immunoassay which different from the 100 µl volume of gold label used in agglutination reaction test. The amount of gold label was concentrated in the volume of 33.3 µl for apply in the conjugated pad. The concentration of competitor at 10 microgram that used in agglutination detection of methamphetamine and ephedrine was insufficient to react against gold label and showed the red color line. The concentration was adjusted to the proper concentration at 200 microgram per test.

The gold conjugated pad was soaked in the other initial volume of gold conjugated and the ability of soaking was evaluated in the same as using the initial volume of 1.0 ml. Using 0.5 ml of gold conjugate solution was insufficient for soaking by the membrane size of 9x20 mm. After the constructed lateral flow immunoassay was tested, the initial volume of gold conjugated at 1.0, 1.5 and 2.0 ml was show the same intensity of test line and control line therefore using conjugated gold 33.3 µl per 3x5 mm piece was economically proper for the membrane immunoassay.

The success prepared gold conjugated pad and undesired pad with the red dot disperse in the membrane, were possibly occurred (Figure 30 - 31). After dry on

the air, the red color of the conjugate pad was stronger than the recently picked up from the gold label solution (Figure 30).

Although, the non-specific binding membrane was chosen in the previous study. The purpose of blocking membrane after application of the capture reagent is to reduce or eliminate the non-specific signal. The blocking materials selected should keep the stable and reproducible test system. They should simultaneously reduce the level of nonspecific background staining. They should also be free from interference with the interaction of antibodies and antigens at the capture line, where such interference can substantially reduce the signal intensity of the test. In this study, the blocking method of soaking the sample pad with phosphate buffer saline pH 7.4 that contain 0.05 % Tween 20 as blocking agent was used (Batteiger, Newhall and Jones, 1982).

(A) Red color gold label (B) Dark red color of gold label

Figure 30 Gold label pad A) within 24 hours
B) more than 48 hours

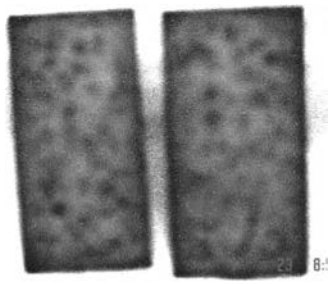


Figure 31 The undesirable conjugated pad

The white line of competitor test band and goat-anti-rabbit-IgG control band was found on the membrane after spotting by using a fine capillary tube on the surface of the membrane. The proper 20 µl of the competitor solution was presented the red color test line in contrast to the competitor solution at the volume of 10 µl could not observe that red line after testing. Whereas, the volume of 30 and 40 µl of competitor were made a scar of solid line because the too high concentration of competitor that gold label could not pass through. The amount of competitor used in SPIA and lateral flow immunoassay was different because of the principle reaction of both method was differed. Therefore, their conditions could not compare.

For the goat anti-rabbit–IgG, only 2 µl spotted on the membrane could show the strong red control line with the clear observation.

From all of the composition study, the appropriate lateral flow immunoassay was obtained as shown in Figure 32. The proper composition for lateral flow immunoassay was summarized as the following:

1. Sample pad and absorbing pad size was 5 x 20 mm
2. Conjugated pad size 3 x 5 mm from initial soaking in 4-ABE-Ab-Au 1.0 ml
3. Test line spot with 20 µl of 20 µg/ml 3-APM-BSA
4. Control line spot with 2 µl of goat anti-rabbit-IgG (1:10)

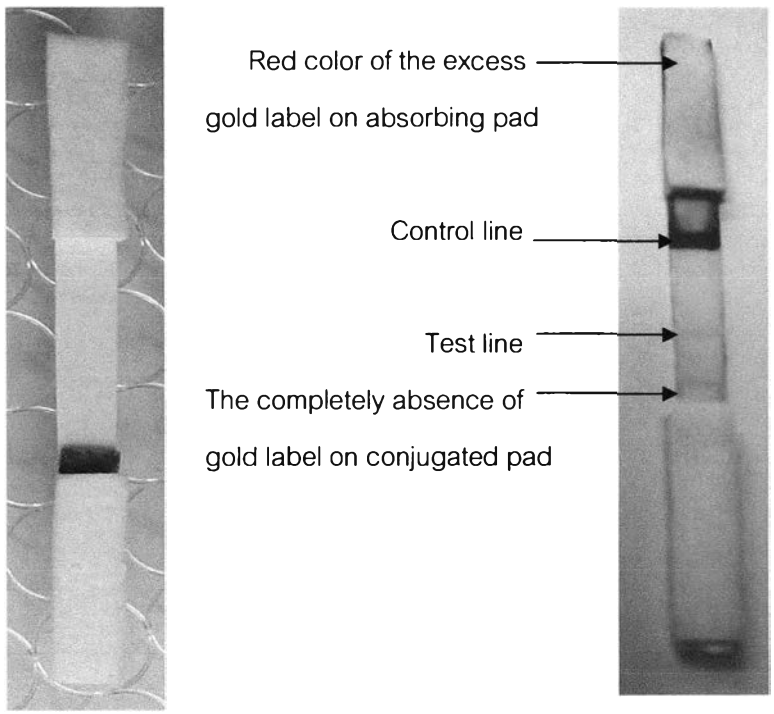


Figure 32 Lateral flow membrane immunoassay for methamphetamine and ephedrine detection

A conjugated pad containing dried conjugate was attached to the membrane strip. A sample pad was attached to the conjugate pad. When applied the aqueous solution to the sample pad (Figure 33A), the liquid sample migrates by capillary diffusion through the conjugate pad (Figure 33B), re-hydrating the gold conjugate and allowing the interaction of the sample analyte with the conjugate. The complex of gold conjugate and analyte then moves onto the membrane strip and migrates towards competitor, where it becomes immobilized and produces a distinct signal in the form of a sharp red line (Figure 33C). A second line, a control, may also be formed on the membrane by excess gold conjugate, indicating the test is complete (Figure 33D). For preparing each part of lateral flow immunoassay, the larger scale than in used size was prepared and then cut into the used size.

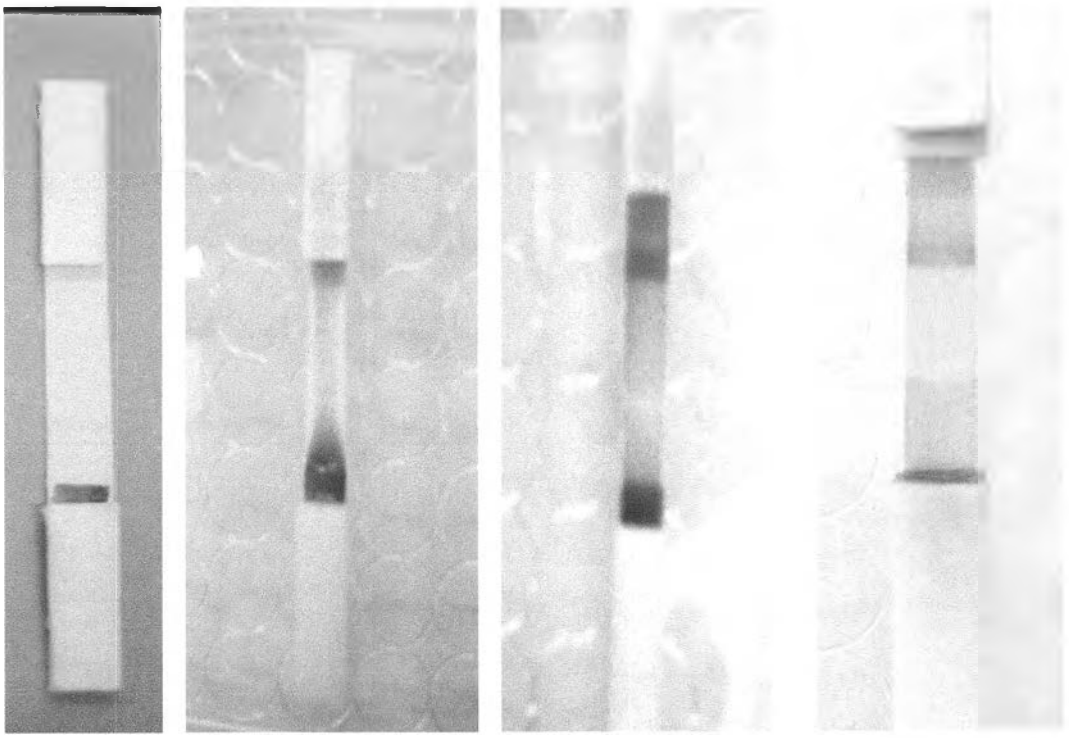


Figure 33 The running lateral flow immunoassay at 0 second (A) , 30 seconds (B) , 60 seconds (C) and 10 minutes (D)

The result of lateral flow immunoassay could determine within 20 min after that the red color may be not clear for detection. It was found that the red color of gold conjugate was stable more than 24 hours.

C-2.2 Qualitative determination of amphetamine, methamphetamine and ephedrine utilizing lateral flow immunoassay

As presented in Table 29, methamphetamine and ephedrine could be detected at the concentration of 1000 and 500 µg/L, respectively. This represented the cut-off concentration for methamphetamine and ephedrine for this membrane immunoassay. The cut-off value of methamphetamine corresponds to that prepared by

NIDA and SAMHSA. Hence, this developed membrane immunoassay would be further developed to use in real practice.

Even through, no cut-off value was proposed for ephedrine in drug-abuse screening, this result would confirm the possibility of using membrane immunoassay for ephedrine screening. In addition, as ephedrine is classified by World Anti-doping Agency (WADA) (Chester, 2003) to be doping compound, this membrane immunoassay would possibly be applicable for ephedrine screening in athlete.

Table 29 The result of amphetamine, methamphetamine and ephedrine detection by membrane immunoassay

Analyte concentration (µg/L)	The degree of detection		
	Amphetamine	Methamphetamine	Ephedrine
0	+ + +	+ + +	+ + +
250	+ + +	+ + +	+ + +
500	+ + +	+ + +	+ +
1,000	+ + +	+ +	+
5,000	+ + +	+	+
10,000	+ +	-	-

* +++ = intense red color ++ = moderated red color
+ = pale red line - = no color

From the accuracy data, this lateral flow immunoassay could detect methamphetamine at the cut-off value of 1,000 µg/L. In the case of ephedrine detection, the cut-off value of 500 µg/L in normal urine was considered as negative result and the positive result was shown in the ephedrine concentration of 625 µg/L because the color of normal urine might be interfere for the visible detection.

C-2.3 Cross-reactivity study

The cross-reactivity data was presented in Table 30. No cross-reaction against all cross-reacting compounds at the tested concentration of 1.0, 10.0 and 100.0 mg/L in artificial urine. For amphetamine, pseudoephedrine and phenylpropanolamine, the detection was positive at concentration 10.0 and 100.0 mg/L.

Amphetamine, pseudoephedrine and phenylpropanolamine could detect with lateral flow immunoassay because of the similar in phenethylamine structure to methamphetamine and ephedrine.

Table 30 Cross-reactivity data

Cross-reactants	Concentration of cross-reactants (mg/L)		
	1.0	10.0	100.0
Amphetamine	negative	positive	positive
Pseudoephedrine	negative	positive	positive
Phenylpropanolamine	negative	positive	positive
Ranitidine	negative	negative	negative
Cimetidine	negative	negative	negative
Chlorpheniramine	negative	negative	negative
Chlorpromazine	negative	negative	negative
Caffeine	negative	negative	negative
Theophylline	negative	negative	negative
Diphenhydramine	negative	negative	negative
Paracetamol	negative	negative	negative
Aspirin	negative	negative	negative
Ampicillin	negative	negative	negative
Vitamin C	negative	negative	negative
Ibuprofen	negative	negative	negative