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Simultaneous detection of amphetamine, methamphetamine and ephedrine by heterology competitive enzyme-linked immunosorbent assay

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Background: Simultaneous screening of ephedrine with amphetamine or methamphetamine in drug abusers is useful in countries, such as Thailand, that prohibit the use of ephedrine. The lack of an adequate screening test kit suitable for this purpose is a significant obstacle in the detection of ephedrine abusers. A reliable analytical method for the simultaneous detection of amphetamine, methamphetamine and ephedrine is needed.
Objective: To develop a process for the detection of amphetamine, methamphetamine and ephedrine by enzyme-linked immunosorbent assay (ELISA), based on the polyclonal antibody and heterology principle.
Methods: The 3-aminopropyl (3AP) and 4-aminobutyl (4AB) derivatives of amphetamine (A), methamphetamine (M) and ephedrine (E) were chemically synthesized. They were used for the preparations of immunogens and hapten tracers. Direct competitive ELISA of matrix combinations of antisera and hapten tracers were performed using amphetamine, methamphetamine and ephedrine as the analytes. Only the competitive reactions with specified sensitivity and specificity are selected.
Results: The study discovered three assay combinations that demonstrated concentration-dependent competition of analyte (single or multiple). They passed the confirmation test for the cut-off concentration and had no cross-reactivity with other amines or structured related compounds. The assay combinations of 4ABA-Ab with 3APA-PO and 4ABE-Ab with 3APM-PO were specific for the detection of amphetamine with ephedrine and methamphetamine with ephedrine, respectively. The third assay combination of 4ABE-Ab with 3APE-PO was highly specific to ephedrine with negligible cross-reactivity from other structure-related compounds. Direct competitive ELISA of 4ABE-Ab with 3APM-PO has been proven useful in field tests for the detection of methamphetamine in urine samples from Thai truck drivers suspected of drug abuse.
Conclusion: By using heterology, these three assay combinations could be used separately or simultaneously for drug abuse screening.

Keywords: Amphetamine, direct competitive ELISA, ephedrine, heterology, methamphetamine, screening test.

The phenylisopropylamine sympatomimetic agents are a group of compounds which have strong central nervous system (CNS) stimulant effects. These agents include amphetamine, methamphetamine (N-methyl amphetamine) and ephedrine (β-hydroxy methamphetamine) [1]. Amphetamine and methamphetamine have been classified as potent addictive stimulant drugs, the abuse of which is a well-known problem in many countries. Amphetamine and methamphetamine abuse is also associated with the use of ephedrine. Ephedrine is a crucial chemical precursor for illicit production of methamphetamine and its related drugs [2]. When used in the illicit manufacture of methamphetamine, residual ephedrine remains in the methamphetamine product. Also, ephedrine is intentionally added to illicit methamphetamine or amphetamine because it is cheaper than the other two drugs. This adulteration of illicit methamphetamine and amphetamine by ephedrine is common in Asian countries, including Thailand. Hence, the use of medical ephedrine is prohibited in these countries.

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For effective screening of amphetamine or methamphetamine in suspected drug abusers, the on-site urine test is generally used. A sample with a positive result is subjected to further laboratory analysis for confirmation. To obtain quick screening results, the analytical method used has to react promptly. Immunoassays demonstrating fast reactions are most often used for this. Some screening test kits are commercially available [3-10]. Meanwhile, a variety of immunoassays for both amphetamine and methamphetamine and some other derivatives have been reported [10-18]. However, these immunoassays have shown false negatives for ephedrine in urine, due to very low cross reactivity with ephedrine [10-15, 19]. In addition, no screening test for ephedrine in urine is available. Only one ephedrine detection in plasma with radioimmunoassay was published [20]. In Thailand and other countries where all three compounds are illegal, the nonselective amine color test is used for screening. Unfortunately, the amine color test gives positive purple color for most amine compounds, including many antihistamines and decongestants. Therefore, the confirmation analysis is flooded with too many false positives, which cause undesirable consequences. A specific immunoassay that can detect all three phenylisopropylamine agents is needed for accurate and reliable detection of illicit compounds in suspect users.

Guided by competitive immunoassay with heterology, this study proposes a direct competitive ELISA for detecting abuse of amphetamine, methamphetamine and ephedrine. Two derivatives of amphetamine, methamphetamine and ephedrine were chemically synthesized for preparing immunogens and hapten tracers. The matrix combinations of six polyclonal antibodies and six hapten tracers were explored for the assay combinations that could detect amphetamine, methamphetamine and ephedrine when present, separately or simultaneously. The selected combination has also been proven practical.

In competitive immunoassay, the analyte or hapten competes with hapten tracer for the binding site on the antibody [21]. Enzyme-linked immuno-sorbent assay (ELISA) techniques are frequently utilized for this purpose. Generally, the competitive assay formats can be divided into homologous and heterologous formats according to the molecular structure of the hapten used. In the homologous format, the same molecular structure of hapten is used in preparing immunogen and hapten tracer. Different molecular structures of hapten are used in heterologous format. The differences in hapten molecular structures for immunogen and hapten tracer have been divided into three types of heterology. Named according to their molecular differences, they are bridge, hapten and site heterologies [22]. Multiple heterology is also possible by combining more than one type of heterology, such as bridge-hapten heterology. Demonstrating sensitivity and specificity enhancement, the heterology concept has been used in several immunoassays [22-29]. However, to be useful, heterologous immunoassay results have to be empirically determined [23-25, 30].

The heterology principle is applied in this study for the determination of three analytes (amphetamine, methamphetamine and ephedrine) altogether or separately, by setting the matrix combinations of antisera and hapten tracers.

Materials and methods

Materials and solutions

All chemicals were of analytical grades and used as received. All solutions were prepared with double distilled water. D-Amphetamine sulfate d-methamphetamine hydrochloride and l-ephedrine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, USA) with Thai Food and Drug Administration (FDA) permission. Freund's complete adjuvant, Freund's incomplete adjuvant, bovine serum albumin (BSA), N-ethyl-N-(3-dimethyl aminopropyl) carbodiimide (EDC) hydrochloride were also obtained from Sigma Chemical Co. N-(3-bromopropyl) phthalimide and N-(4-bromobutyl) phthalimide were purchased from Fluka Chemie AG (Germany). Horseradish peroxidase enzyme (RZ $\geq$3) and O-phenylene diamine (OPD) were from Zymed Lab (USA).

Phosphate buffer saline (PBS; pH 7.0) was prepared by mixing appropriate proportions of 0.1 M dibasic sodium phosphate, monobasic sodium phosphate and sodium chloride solutions. Citrate-phosphate buffer, pH 5.0 was prepared by mixing 0.1 M citric acid with 0.2 M dibasic sodium phosphate dihydrate and adjusting the final pH to 5.0.

Chemical synthesis of hapten derivatives

Amphetamine, methamphetamine and ephedrine were all haptens. The N-(3-aminopropyl) and N-(4-aminobutyl) derivatives of each hapten were

chemically synthesized according to the method modified from Cheng et al. [31]. Briefly, the basic form of each hapten (10 mmol) was refluxed for 12 hours with 15 mmol of either N-(3-bromopropyl) phthalimide (4.0 g) or N-(4-bromobutyl) phthalimide (4.2 g) in 25 ml of absolute ethanol. The intermediate was recrystalized in hot water after solvent evaporation and refluxed for another two hours in absolute ethanol and excess hydrazine hydrate. The reaction mixture was acidified with 1.0 M HCl. The filtrate was separated and made alkaline with 5.0 M NaOH before extracting the N-(3-aminopropyl) or N-(4-aminobutyl) derivatives with chloroform. The pale yellow liquid products were obtained after the evaporation of the solvent.

These six synthesized hapten derivatives (**Fig. 1**) were confirmed by NMR and IR spectroscopic techniques prior to being used as follows:

N-(3-bromopropyl)amphetamine (3APA), ^{1}H NMR (DMSO-d_6) 1.05-1.10 (d, 3H, CH_3), 1.50-1.65 (q, 2H, CH_2), 2.55-2.80 (m, 6H, $3CH_2$), 2.85-2.95 (m, 1H, CH), and 7.20-7.40 (m, 5H, 5CH); IR ($CHCl_3$) 3360, 3284, 3028, 2952, 1620, 1439, 1100, 741, 699/cm.

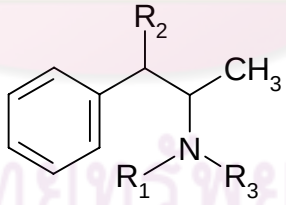
N-(4-bromobutyl)amphetamine (4ABA), ^{1}H NMR (DMSO-d_6) 1.05-1.15 (d, 3H, CH_3), 1.50-1.60 (m, 4H, $2CH_2$), 2.50-2.85 (m, 6H, $3CH_2$), 2.85-3.00 (m, 1H, CH), and 7.20-7.40 (m, 5H, 5CH); IR ($CHCl_3$) 3360, 3284, 3032, 2952, 1600, 1439, 1100, 744, 701/cm.

N-(3-bromopropyl)methamphetamine (3APM), ^{1}H NMR (DMSO-d_6) 0.95-1.05 (d, 3H, CH_3), 1.50-1.65 (q, 2H, CH_2), 2.35 (s, 3H, CH_3), 2.45-2.55 (m, 3H, CH, CH_2) 2.70-2.80 (t, 2H, CH_2), 2.95-3.05 (m, 2H, CH_2) and 7.20-7.40 (m, 5H, 5CH); IR ($CHCl_3$) 3395, 3284, 3038, 2955, 1620, 1442, 1100, 739, 700/cm.

N-(4-bromobutyl)methamphetamine (4ABM), ^{1}H NMR (DMSO-d_6) 0.95-1.00 (d, 3H, CH_3), 1.45-1.60 (m, 4H, $2CH_2$), 2.35 (s, 3H, CH_3), 2.40-2.60 (m, 3H, CH, CH_2) 2.70-2.80 (t, 2H, CH_2), 2.95-3.05 (m, 2H, CH_2) and 7.20-7.40 (m, 5H, 5CH); IR ($CHCl_3$) 3367, 3292, 3062, 2930, 1621, 1440, 1108, 753, 700/cm.

N-(3-bromopropyl)ephedrine (3APE), ^{1}H NMR (DMSO-d_6) 0.70-0.80 (d, 3H, CH_3), 1.40-1.60 (m, 2H, CH_2), 2.20 (s, 3H, CH_3), 1.60-2.20 (br, 2H, NH) 2.30-2.80 (m, 5H, CH, $2CH_2$) 4.70-4.80 (d, 1H, CH) and 7.20-7.40 (m, 5H, 5CH); IR ($CHCl_3$) 3350, 3292, 3062, 2905, 1618, 1460, 1042, 759, 702/cm.

N-(4-bromobutyl)ephedrine (4ABE), ^{1}H NMR (DMSO-d_6) 0.80-0.90 (d, 3H, CH_3), 1.30-1.60 (m, 4H, $2CH_2$), 2.30 (s, 3H, CH_3), 1.50-2.20 (br, 2H, NH) 2.40-2.90 (m, 5H, CH, $2CH_2$) 4.30-4.40 (d, 1H, CH) and 7.20-7.40 (m, 5H, 5CH); IR ($CHCl_3$) 3300, 2915, 2890, 1638, 1451, 1041, 745, 701/cm.



Compound	R1	R2	R3
Amphetamine	H	H	H
Methamphetamine	CH_3	H	H
Ephedrine	CH_3	OH	H
N-(3-aminopropyl) amphetamine (3APA)	H	H	$(CH_2)_3NH_2$
N-(4-aminobutyl) amphetamine (4ABA)	H	H	$(CH_2)_4NH_2$
N-(3-aminopropyl) methamphetamine (3APM)	CH_3	H	$(CH_2)_3NH_2$
N-(4-aminobutyl) methamphetamine (4ABM)	CH_3	H	$(CH_2)_4NH_2$
N-(3-aminopropyl) ephedrine (3APE)	CH_3	OH	$(CH_2)_3NH_2$
N-(4-aminobutyl) ephedrine (4ABE)	CH_3	OH	$(CH_2)_4NH_2$

Fig. 1 Structures of amphetamine, methamphetamine and ephedrine derivatives.

Preparation of immunogen and hapten tracer

The immunogen (3APA-BSA, 4ABA-BSA, 3APM-BSA, 4ABM-BSA, 3APE-BSA and 4ABE-BSA) of each derivative was prepared by the carbodiimide coupling method [31], using a molar ratio of hapten derivative to BSA of 100. The molar ratio of each hapten derivative covalently attached to BSA was determined, from the absorbance values of these compounds at 280 and 257 nm, to be in the range of 7 to 29.

The hapten tracer (3APA-PO, 4ABA-PO, 3APM-PO, 4ABM-PO, 3APE-PO and 4ABE-PO) of each derivative was prepared by the periodate method [32]. The starting molar ratio of hapten to enzyme was 100. The molar ratio of hapten conjugated to the enzyme was determined from their spectral characteristics at 280 and 402 nm to be in the range of 8 to 35. The enzyme activity of each hapten tracer was evaluated from the absorbance of the enzyme-substrate reaction at 492 nm, to be used for setting the proper dilution of the hapten tracer in competitive assay.

Preparation of antibodies against amphetamine, methamphetamine and ephedrine

Polyclonal antibodies against amphetamine, methamphetamine and ephedrine were raised in New Zealand white rabbits (2.0-2.5 kg body weight). For each immunogen, two rabbits were subcutaneously inoculated with 1 mg immunogen emulsified with 50 % Freund's complete adjuvant in saline. Booster immunizations were given at approximately two weeks interval, following the same procedure, by injecting 1.5 mg immunogen in Freund's incomplete adjuvant. The first antiserum was obtained by bleeding the rabbit after three weeks of primary immunization. Antisera collections were done every week for three months. The antisera IgG fractions were purified by the saturated ammonium sulfate method [32]. The potencies of antibodies were determined by the titer values to be in the range of 1:250 to 1:30,000. These titer values were utilized as the initial dilutions of antisera for competitive ELISA.

Procedure for the competitive ELISA of amphetamine, methamphetamine and ephedrine

Heterologous combinations between six antisera, raised from six different immunogens and six hapten tracers, were set up into three types. They were bridge heterology, hapten heterology and bridge-hapten heterology (**Table 1**). Also, the homology of each hapten derivative was studied as a comparison.

The direct competitive ELISA of each combination, having amphetamine, methamphetamine and ephedrine as the hapten competitor (analyte), was performed following Aoki's method with slight modifications [11].

The concentrations of antisera and hapten tracers were optimized by checkerboard titration in which a series of antiserum and hapten tracer dilutions were used. In general, unless otherwise stated, the volume of each incubation step was 100 µl and the microtiter plates were washed with PBS-T after each incubation step. Briefly, polystyrene microtiter wells were coated overnight with antisera in carbonate buffer, pH 9.6 at 4 °C. After washing, 3 % BSA in PBS-T was added. The wells had been incubated for 1 hour at 37 °C and washed before keeping at 4 °C until they were used.

Fifty µl of diluted hapten tracer was added to each well that contained 50 µl of serial dilutions (0-3.0 µg/ml) of the analyte, either amphetamine, methamphetamine or ephedrine solution. The competitive reaction between hapten tracer and analyte proceeded at 37 °C for 2 hours. After washing, a freshly prepared chromogen-substrated solution containing O-phenylene diamine in citrate-buffer and H_2O_2 (30 %) (2:1 v/v) was added. The plates were incubated at room temperature for 20 minutes in the

Table 1. Proposed matrix combinations of antisera and hapten tracers.

Antisera	Hapten tracers					
	3APA	4ABA	3APM	4ABM	3APE	4ABE
3APA	I	II	III	IV	III	IV
4ABA	II	I	IV	III	IV	III
3APM	III	IV	I	II	III	IV
4ABM	IV	III	II	I	IV	III
3APE	III	IV	III	IV	I	II
4ABE	IV	III	IV	III	II	I

I=homology ; II=bridge heterology; III=hapten heterology; IV= bridge-hapten heterology

dark. Fifty µl of 4 N H_2SO_4 were added to the wells to stop the enzyme reaction. Absorbance values were measured using a microplate reader at the wavelength of 492 nm.

The competitive reaction was indicated by the concentration of analyte that could inhibit the tracer for 50 % antibody binding site (IC50 value). Competitive curves were constructed by plotting % B/Bo values against the logarithm of analyte concentrations [33]. The % B/Bo values were determined from the absorbance values by the following equation:

$$\% \text{ B/Bo} = [(\text{A-Am})/(\text{Ao-Am})] \times 100. \quad (1)$$

where A is the absorbance of the analyte at the given concentration, Am is the absorbance at maximum dose of the analyte and Ao is the absorbance of the analyte at the zero concentration.

The performance of the analyte as the concentration-dependent competitor was determined from the linearity of the competitive curves. The value of the slope supplied the degree of competition of the assay, and the correlation coefficient (r) indicated the possible relationship between the competitive response and the concentration of the analyte.

Determination of sensitivity and specificity of competitive ELISA

The sensitivity of hapten detection was determined as the cut-off concentration of hapten at the border line between the presence (positive screening) and absence (negative screening) of the drug in the samples or specimens [34]. Ten replications of direct competitive ELISA at zero drug concentration were performed. The cut-off absorbance was determined to be the mean of absorbance at zero drug concentration minus three times standard deviation. From this cut-off absorbance, the cut-off concentration was determined and confirmed at 30 % [21, 35, 36].

The specificity of the selected combinations was also investigated. Several chemical compounds whose functional groups or structures are related to amphetamine, methamphetamine and ephedrine were tested for cross-reactivity. The concentration of cross-reactants that could inhibit the same binding as the analyte at concentration of 1 µg/ml ($IC_{1\mu g/ml}$) was determined by direct competitive ELISA. The cross-reactivity values were calculated as follows:

$$\% \text{ Cross reactivity} = [(IC_{1\mu g/ml} \text{ of analyte} / IC_{1/\mu g/ml} \text{ of cross-reactant})] \times 100. \quad (2)$$

Only the assay combinations that exhibit the minimum cross-reactivity with the tested cross- reactant would be selected.

Results

Competitive ELISA

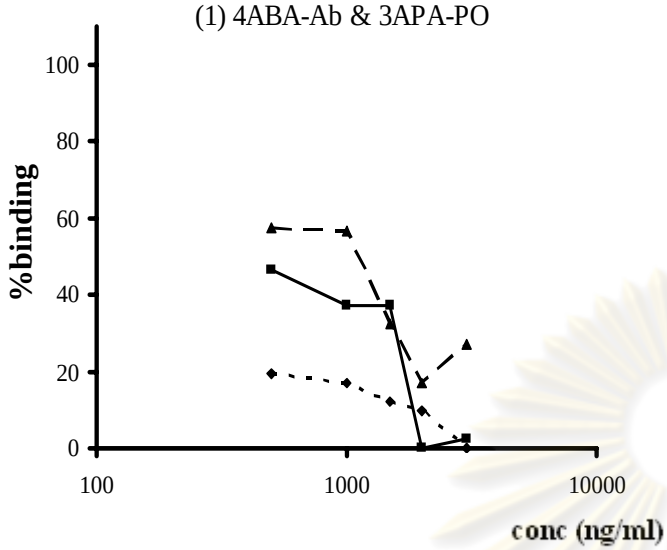
By direct competitive ELISA of 6x6 matrix combinations of antisera and hapten tracers, the patterns of competitiveness of amphetamine, methamphetamine and ephedrine independently varied as shown in **Table 2.** The results of the competitive reactions could be classified into eleven high competitive assays (assay no.11-12, 21-25, 33-36), with the IC50 value $\leq$ 1.5 µg/ml; eleven low competitive assays (assay no.1-7, 10, 15, 28-29) with the IC50>1.5 µg/ml; twelve non competitive responses (NR) (assay no.8-9, 13-14, 16, 18, 19, 26, 30, 31-32); and two concentration-independent competitions (CI) (assay no.17, 20) in which the IC50 could not be determined.

Among eleven high competitive assays, amphetamine, methamphetamine and ephedrine displayed unequal competitiveness (**Fig. 2**). Amphetamine, methamphetamine and ephedrine could exhibit competitiveness in assay no. 23 which is the combination of 4ABE-Ab and 4ABA-PO. Amphetamine and ephedrine could compete with 3APA-PO in the presence of 4ABA-Ab (assay no.11). The competitiveness of methamphetamine and ephedrine could be observed in five assay combinations (assay no.22, 24, 34-36). In addition, both ephedrine and methamphetamine exhibit competitiveness in two assays (no.12, 25 and 21, 33), respectively. The slope values which represent the competitive degrees of eleven competitive assays varied independently between 14 and 97 (**Table 3**).

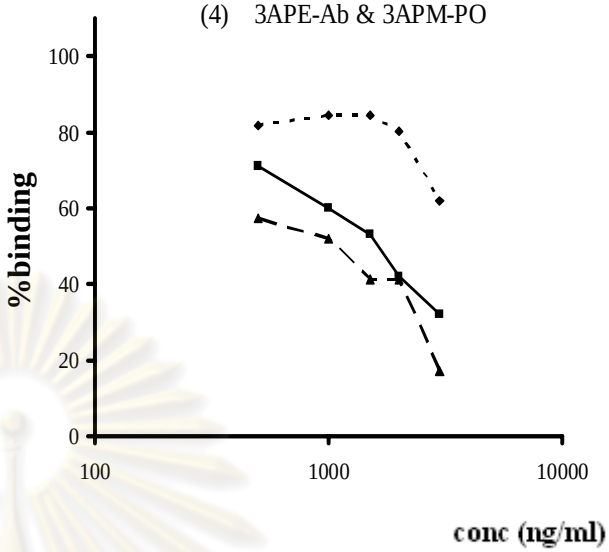
Sensitivity and specificity of the competitive assays

Further investigation of the results of the eleven high competitive assays by determination and confirmation of the cut-off concentration of analytes showed that only six competitive assays (no.11, 12, 21, 24, 34, 36) passed the cut-off confirmation test. Their cut-off concentrations were between 4-55 ng/ml (**Table 4**). The other five competitive assays that failed the confirmation test, suggesting insufficient sensitivity to detect the analyte for screening purposes, were not subjected to further investigation.

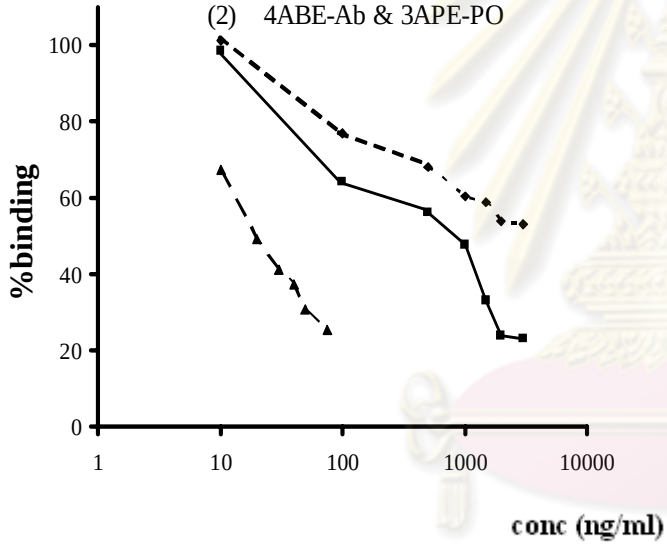
(1) 4ABA-Ab & 3APA-PO



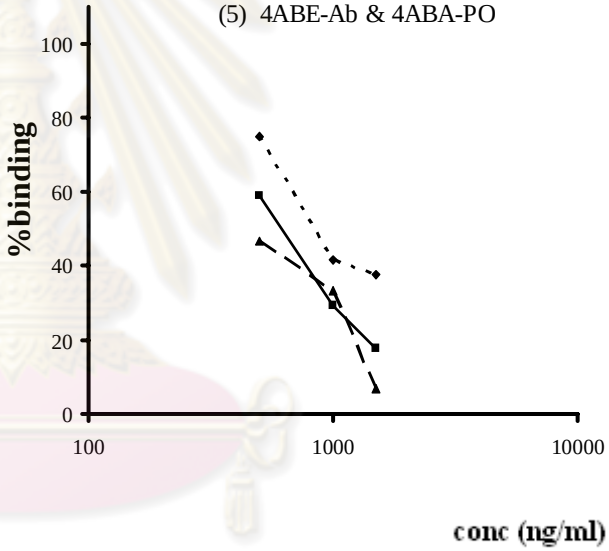
(4) 3APE-Ab & 3APM-PO



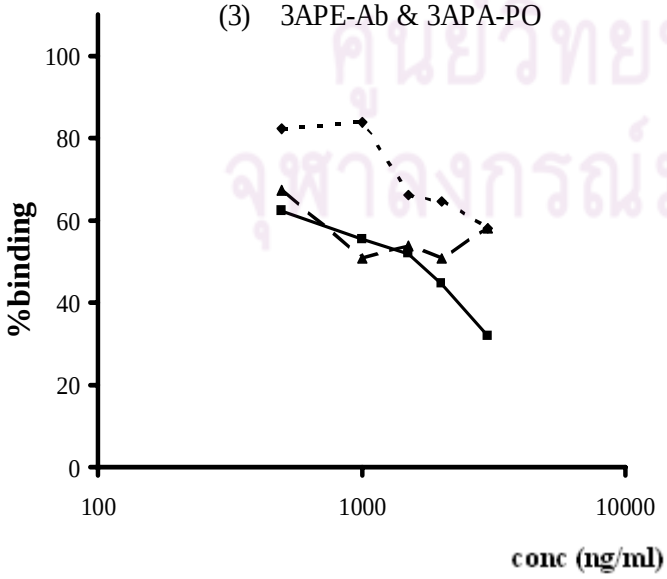
(2) 4ABE-Ab & 3APE-PO



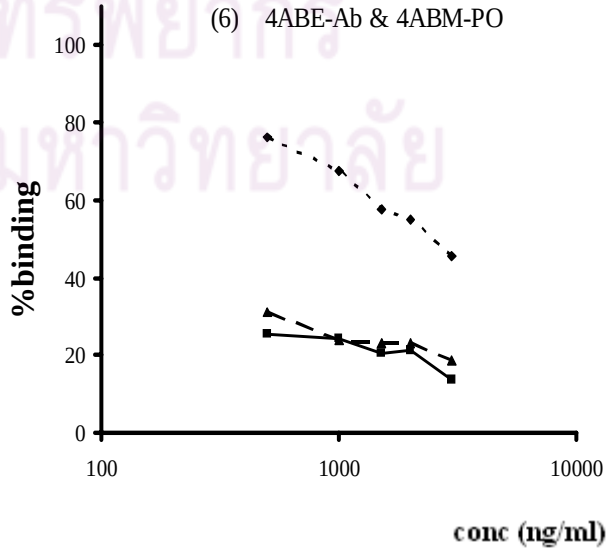
(5) 4ABE-Ab & 4ABA-PO



(3) 3APE-Ab & 3APA-PO



(6) 4ABE-Ab & 4ABM-PO



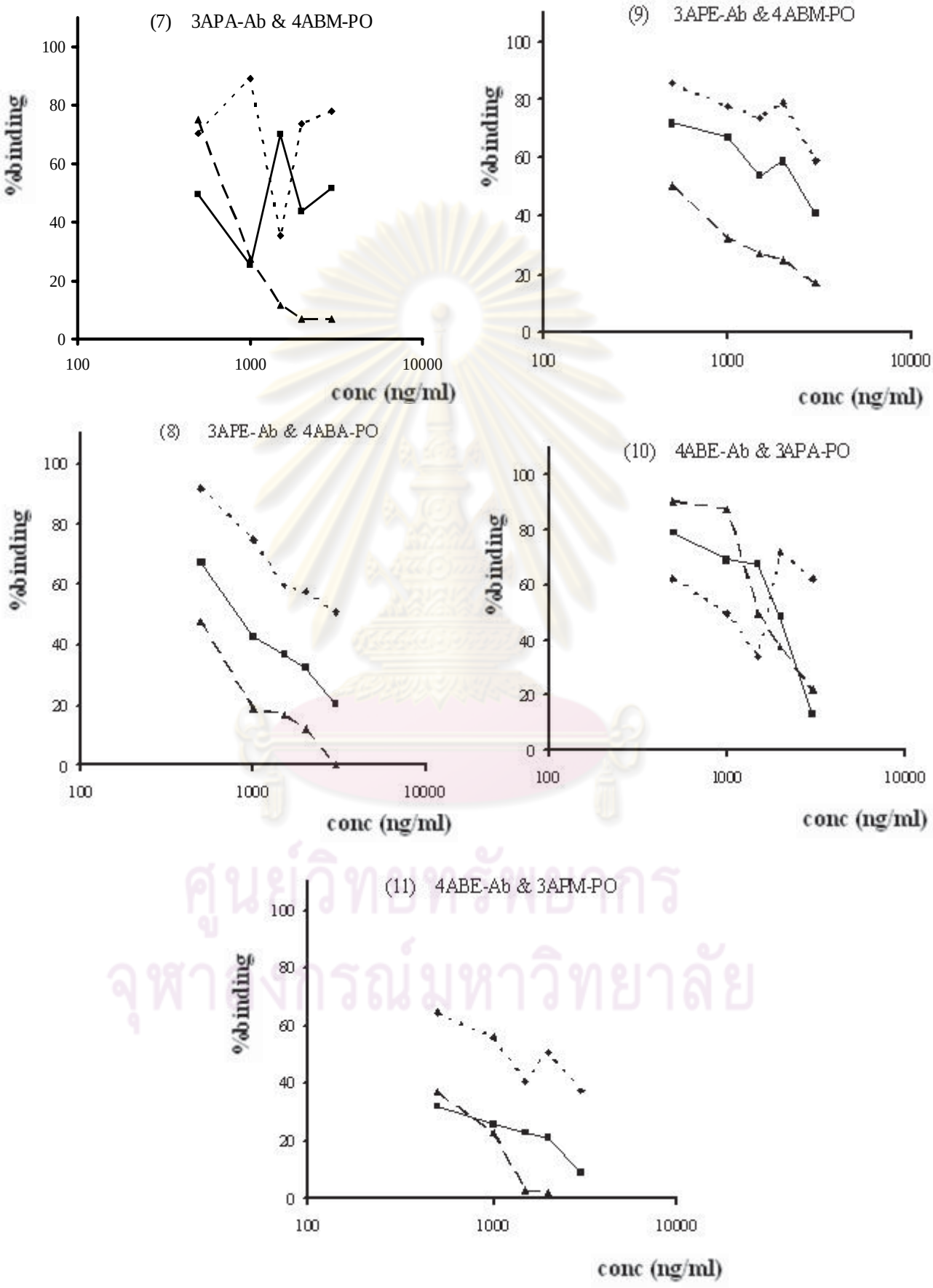


Fig. 2 Dose-response relationship of selected competitive immunoassays.
- - - - Amphetamine; ——— Methamphetamine; — — Ephedrine.

Table 2. Competitive reactions of amphetamine, methamphetamine and ephedrine.

Assay No.	Type of heterology	Hapten derivative		Competitive reaction / IC50 (μg/ml)		
		Immunogen	tracer	A[1]	M[2]	E[3]
1	NO	3APA	3APA	> 3.0	> 3.0	> 3.0
2		4ABA	4ABA	> 3.0	> 3.0	> 3.0
3		3APM	3APM	> 3.0	> 3.0	> 3.0
4		4ABM	4ABM	> 3.0	> 3.0	> 3.0
5		3APE	3APE	> 3.0	> 3.0	> 3.0
6		4ABE	4ABE	> 3.0	> 3.0	> 3.0
7	Bridge	3APA	4ABA	> 3.0	> 3.0	> 3.0
8		3APM	4ABM	NR[4]	NR	NR
9		4ABM	3APM	NR	NR	NR
10		3APE	4ABE	> 3.0	> 3.0	> 3.0
11		4ABA	3APA	0.5	> 1.5	1.5
12		4ABE	3APE	> 3.0	>3.0	< 0.5
13	Hapten	3APA	3APM	NR	NR	NR
14		3APA	3APE	NR	NR	NR
15		4ABA	4ABM	> 3.0	> 3.0	> 3.0
16		4ABA	4ABE	NR	NR	NR
17		3APM	3APA	CI[5]	CI	CI
18		3APM	3APE	NR	NR	NR
19		4ABM	4ABA	> 3.0	NR	NR
20		4ABM	4ABE	CI	CI	CI
21		3APE	3APA	CI	1.3	2.5
22		3APE	3APM	CI	1.5	1.0
23		4ABE	4ABA	0.8	0.6	0.4
24		4ABE	4ABM	2.4	0.4	0.3
25	Bridge-hapten	3APA	4ABM	3.0	> 5.0	0.7
26		3APA	4ABE	NR	NR	NR
27		4ABA	3APM	1.3	1.3	1.3
28		4ABA	3APE	> 3.0	> 3.0	> 3.0
29		3APM	4ABA	> 3.0	3.0	3.0
30		3APA	4ABE	NR	NR	NR
31		4ABM	3APA	NR	> 5.0	3.0
32		4ABM	3APE	NR	NR	NR
33		3APE	4ABA	3.0	0.7	NR
34		3APE	4ABM	NR	1.5	1.0
35		4ABE	3APA	NR	1.0	1.2
36		4ABE	3APM	> 3.0	0.3	0.3

[1]A=Amphetamine; [2]M=Methamphetamine; [3]E=Ephedrine; [4]NR=non competitive response; [5]CI=concentration independence. Three replications of each assay no. were performed according to the procedure of direct competitive ELISA. The average observed absorbance values were used for calculating the %B/Bo (eq.1); IC50 values were determined from the competitive curves.

Table 3. Dose-response relationship of selected competitive immunoassays.

Selected No (Assay No)	Type of heterology	Hapten derivative (dilutions)		Analyte[1]	Competitive curve (0-3.0 µg/ml)	
		Immunogen	tracer		Slope	r
1	Bridge	4ABA	3APA	A	24	0.9644
(11)		(1 : 1000)	(1 : 7500)	E	52	0.8479
2		4ABE	3APE	E	48	0.9868
(12)		(1 : 350)	(1 : 1500)			
3	Hapten	3APE	3APA	M	37	0.9536
(21)		(1 : 100)	(1 : 7500)			
4		3APE	3APM	M	50	0.9686
(22)		(1 : 100)	(1 : 10000)	E	47	0.8288
5		4ABE	4ABA	A	80	0.9606
(23)		(1 : 500)	(1 : 10000)	M	87	0.9930
				E	80	0.9508
6		4ABE	4ABM	M	14	0.8024
(24)		(1: 200)	(1: 5000)	E	15	0.9100
7	Bridge -	3APA	4ABM	E	90	0.8568
(25)	hapten	(1 : 100)	(1 : 2500)			
8		3APE	4ABA	M	72	0.9615
(33)		(1 : 100)	(1 : 5000)			
9		3APE	4ABM	M	37	0.8328
(34)		(1 : 100)	(1 : 2500)	E	42	0.9680
10		4ABE	3APA	M	76	0.7696
(35)		(1 : 200)	(1 : 5000)	E	97	0.8917
11		4ABE	3APM	M	26	0.9990
(36)		(1 : 200)	(1 : 7500)	E	63	0.9399

[1]A=Amphetamine; M=Methamphetamine; E=Ephedrine; Three replications of each assay no. were performed according to the procedure of direct competitive ELISA. The average observed absorbance values were used for calculating the %B/Bo (eq.1). Slope and r values were determined from the regression equation of the competitive curves (% binding *vs*. log concentration of analyte).

Table 4. Cut-off concentrations of selected competitive immunoassays.

Selected No (Assay No)	Hapten derivative		Analyte[1]	Cut-off conc. (ng/ml)
	Immunogen	Tracer		
1	4ABA	3APA	A	15.0
(11)				
2	4ABE	3APE	E	7.0
(12)				
3	3APE	3APA	M	55.0
(21)				
4	4ABE	4ABM	M	32.0
(24)			E	4.0
5	3APE	4ABM	E	30.0
(34)				
6	4ABE	3APM	M	12.0
(36)			E	5.0

[1]A=Amphetamine; M=Methamphetamine; E=Ephedrine; Ten replications of direct competitive ELISA at zero drug concentration were performed. The cut-off absorbance was determined as the mean of absorbance at zero drug concentration minus three times standard deviation. From this cut-off absorbance, the cut-off concentration was determined and confirmed at ± 30 % of the cut-off concentration.

Table 5. Cross-reactivity study.

Analyte	% Cross reactivity		
	4ABA-Ab & 3APA-PO	4ABE-Ab & 3APE-PO	4ABE-Ab & 3APM-PO
Amphetamine	100.0	< 2.0	5.0
Methamphetamine	15.0	< 5.0	100.0
Ephedrine	200.0	100.0	100.0
Phenylpropanolamine	0.1	0.1	5.0
Pseudoephedrine	10.0	0.1	0.05
Ranitidine	< 0.1	< 0.1	< 0.05
Cimetidine	0.1	< 0.1	< 0.05
Chlorpheniramine	1.0	< 0.1	< 0.05
Ibuprofen	< 0.1	< 0.1	< 0.05
Diphenhydramine	10.0	< 0.1	< 0.05
Caffeine	< 0.1	< 0.1	< 0.05
Theophylline	< 0.1	< 0.1	< 0.05
Paracetamol	0.1	< 0.1	< 0.05
Aspirin	< 0.1	< 0.1	< 0.05
Ampicillin	0.1	< 0.1	< 0.05

% Cross reactivity = [($IC_{1\mu g/ml}$ of analyte / $IC_{1/\mu g/ml}$ of cross reactant)] x 100.

The testing results for specificity of selected combinations, another indicator of screening purpose suitability, showed that only three competitive assays (no.11, 12, 36) have low % cross-reactivity of cross-reactants (**Table 5**). These three competitive assays are 4ABA-Ab with 3APA-PO for amphetamine and ephedrine detections, 4ABE-Ab with 3APE-PO for ephedrine detection and 4ABE-Ab with 3APM-PO for methamphetamine and ephedrine detections. Among the three assay combinations, negligible cross-reaction was clearly observed from all cross-reactants with the % cross-reactivity of 0.1-5.0 % for 4ABE-Ab with 3APE-PO and 4ABE-Ab with 3APM-PO assay combinations. The 4ABA-Ab with 3APA-PO combination showed slight cross-reactivity with methamphetamine, pseudoephedrine and diphenhydramine (**Table 5**). Therefore these three assay combinations were finally selected.

Discussion

The use of polyclonal antibodies and heterology could direct the detection of single and multi-analytes. The selected three final assay combinations can be used to detect amphetamine, methamphetamine and ephedrine simultaneously due to their different specificity. One competitive assay combination of 4ABE-Ab with 3APE-PO (no.12) is only highly specific to ephedrine with negligible cross-reactivity. The other two competitive assays are each specific for different analytes. The 4ABA-Ab with 3APA-PO combination (no.11) is specific for amphetamine and ephedrine but the 4ABE-Ab with 3APM-PO combination (no.36) is specific for methamphetamine and ephedrine. Consequently, the use of these three competitive assays together in one urine screening test allows all three analytes to be specifically screened. Therefore, it did not matter if ephedrine was mixed with amphetamine or methamphetamine, because these assays could be used to detect all of them. The results of this study were also empirically supported by satisfactory results, comparable to the TDx test kit, in using assay no.36 on the truck drivers suspected of drug abuse.

The success of the competitiveness of amphetamine and ephedrine in assay no.11 and 12 could be explained by the different spacer arm lengths of immunogens and hapten tracers. The competitiveness of amphetamine and ephedrine in assay no.11 and ephedrine alone in assay no.12 would be due to the arm lengths of 3APA-PO in assay no.11 and of 3APE-PO in assay no.12 being shorter than the spacer arm lengths of immunogen for antisera, 4ABA-Ab in assay no.11 and 4ABE-Ab in assay no.12, respectively. The shorter spacer arm length of the tracer reduces the affinity of the antibody, thereby giving the analyte a better chance to compete. These two competitive

assays were bridge heterology and competitive enhancement that were observed in low concentration of analyte. Therefore, this study results are consistent with other study results suggesting that bridge heterology could enhance the sensitivity of immunoassays [23-25, 27]. In contrast from these previous studies, which assay only single analyte, our study used three analytes in each competitive assay. The steric effect due to differences in the molecular structure of these three analytes, amphetamine, methamphetamine and ephedrine also influences the competitiveness. Moreover, the competitive reaction of ephedrine in assay no.12 could be due to both bridge heterology and immunogenicity of 4ABE-Ab to ephedrine. The competitiveness of amphetamine in assay no.11 under similar conditions resulted from bridge heterology and immunogenic character. For ephedrine competitiveness in the same assay (no.11), however, it could be explained that the molecular structure of ephedrine is more steric than the molecular structure of methamphetamine. Ephedrine, thus, retards the binding affinity of 4ABA-Ab to the 3APA-PO. Then, the greater competitiveness of ephedrine than methamphetamine in this assay (no.11) is observed.

For assay no.36 (4ABE-Ab with 3APM-PO) combination, it was bridge-hapten heterology and two kinds of differences between antisera and hapten tracers. First, the hapten for 3APM-PO contains a 3-aminopropyl- functional group whose spacer arm length is shorter than the spacer arm length of antisera (4ABE-Ab). Secondly, 3APM-PO is a methamphetamine derivative which is less steric than the ephedrine derivative of antisera (4ABE-Ab) (**Fig. 1**). Synergism of these effects resulted in a weakening recognition ability of 4ABE-Ab to 3APM-PO, allowing methamphetamine and ephedrine to have equivalent competitiveness. Also, other cases of insensitive competitiveness of amphetamine in this assay may be caused by the small molecular structure of amphetamine compared to the molecular structure of methamphetamine and ephedrine. With this small molecular structure, more concentration is needed for amphetamine to be able to compete (**Table 2**).

In this study, one competitive assay (no.23) found that all three analytes could exhibit a high degree of competitiveness with 4ABA-PO tracer. They demonstrated high competitive binding to 4ABE-Ab with a very low IC50 (0.4-0.8 μg/ml) and high slope values of 80-87. Unfortunately, this competitive assay failed the cut-off confirmation test. Therefore it is not suitable for detection purposes and was not selected. Further work to improve this competitive assay (no.23), for better sensitivity in detecting all three analytes simultaneously is needed. The adjustment of suitable proportion of antibody and hapten tracer for producing higher signal response and the adjustment for density optimization of hapten in the tracer conjugation should be considered.

From the results of six homologous assays (no.1-6), all three analytes (amphetamine, methamphetamine and ephedrine) as competitors were found to be insensitive. It could be explained that when the same hapten derivative is used for preparing immunogen and hapten tracer, the binding site on the antibody molecule is large enough to accommodate both the hapten and its bridge linkage to the tracer [22]. As a result, the antibody exhibits stronger binding to homologous hapten tracer than to the bridge lacking analyte. Low competitiveness of homologous immunoassays was also found in other studies [23, 27].

The possibility of using the heterology principle to enhance the competitive reaction of more than one analyte was suggested by the results of this study. Previously simultaneous immunochemical analysis of analytes has been demonstrated based on the use of two different enzymes [37], radioactive labels [38], fluorophores [39, 40], metal-labels [41] or others. However, these assays pose many complicate preparation steps. The use of one enzyme label with different hapten tracers and immunogens in this study proposes the other effective method for simultaneous analysis in the future.

Conclusion

The problem of the simultaneous determination of amphetamine with ephedrine or methamphetamine with ephedrine was successfully solved in this study via heterologous immunoassays. Also, the detection of ephedrine by immunoassay, which has never been reported previously, was demonstrated.

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