CHAPTER II

MATERIALS AND METHODS

Chemical compounds and reagents

All chemical compounds and reagents were of analytical grade and used as received. d-Amphetamine sulfate, d-methamphetamine hydrochloride and l -ephedrine hydrochloride were from Sigma (St Louis, MO, U.S.A.) and under government control. dl-phenylpropanolamine, d-pseudoephedrine hydrochloride, bovine serum albumin, colloidal gold (50 nm), dimethyl sulfoxide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), polyethylene glycol 1500, polyethylene glycol 3350, potassium dihydrogen phosphate, sodium chloride, sodium hydroxide, Tris-(hydroxymethyl)aminomethane, hypoxanthine-aminopterin-thymidine medium (HAT medium), hypoxanthine-thymidine medium (HT medium) and chloroform-d were also from Sigma (St Louis, MO, USA). Ammonium sulfate, potassium chloride, sodium acetate, sodium bicarbonate, sodium carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate, 35% hydrogen peroxide, methanol, sulfuric acid and tween 20, were obtained from Merck (Darmstadt, Germany).

Complete Freund's adjuvant and incomplete Freund's adjuvant were from Life Technologies (Grand Island, NY, USA). Glutamine, penicillin-streptomycin solution, RPMI 1640 medium and sodium pyruvate were from HyClone (Logan, Utah, USA). The other reagents were citric acid from Carlo Erba (Farmitalia, Germany), horseradish peroxidase (RZ $\geq$ 3) from Amresco (Solon, USA), keyhole limpet hemocyanin from Pierce (Rochford, USA) and o-phenylenediamine from Zymed Laboratories (Carlton Court, USA).

The membranes used were; AE 98, AE 99, PRIMA 40 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), BioTrace™ NT, BioTrace™ PVDF, 6,6 Biodyne A amphoteric Nylon (PALL Life Science, NY, USA), and UltraBind modified polyethersulfone (Gelman Science, NY, USA).

Polyclonal antisera from immunogens of N-(3-aminopropyl)amphetamine (3-APA), N-(4-aminobutyl)amphetamine (4-ABA), N-(3-aminopropyl)methamphetamine (3-APM), N-(4-aminobutyl)methamphetamine (4-ABM), N-(3-aminopropyl)ephedrine (3-APE) and N-(4-aminobutyl)ephedrine(4-ABE), and the enzyme-labeled, 3-APA-HRP, 4-ABA-HRP, 3-APM-HRP, 4-ABM-HRP, 3-APE-HRP and 4-ABE-HRP, have been prepared by W. Matapatara in the year of 1999.

Apparatus and instrument

The specific instruments used for cell culture and ELISA were autoclave (TOMY, Japan), centrifuge (Hettich, EBA 12, Germany), centrifuge(Beckman Coulter, Allegra X-12R, USA), - 20°C freezer (Hitachi, Japan), - 70°C freezer (Forma Scientific, USP), incubator (Memmert, Germany), laminar flow cabinet (Holten, Denmark), liquid nitrogen container (Forma Scientific, USA), microscope (Olympus, USP), microplate reader (ELISA plate reader Bio-Rad model 3550, Bio-Rad laboratories, USP).

The OPTIMA™ L SERIES Preparative Ultracentrifuges 0 – 40°C (Beckman Instruments, Inc., USA) was used for conjugated gold preparation.

The other apparatus utilized in the study were as following; analytical balance (METTLER® AG 245 TOLEDO, Switzerland), ultraviolet spectrophotometer (Spectronic 3000 array, Milton Roy Co. , USA), vacuum concentrator (Heto model CT 110, Denmark.), pH-meter (Consort pH meter model C231, Belgium), dialysis tubing (Sigma, USA), immuno-plate (NUNC, Denmark), micropipet (Socorex, USP) and vortex mixer (Genei Scientific Industries Inc., New York, USP).

Preparation of solution

1. Growth media

Complete growth media (GM), all reagents are HyClone, USA. Products compose of 500 ml RPMI 1640, 50 ml (10%) Fetal bovine serum, 1 ml (10 X) Penicillin-streptomycin solution, 5 ml (1%) sodium pyruvate (100 mM) and 5 ml (1%) L-glutamine (200 mM).

2. HAT and HT media.

Hypoxanthine, aminopterin and thymidine are added to GM. HAT and HT stock (50X) were diluted before used. HAT and HT media were prepared by added 10.0 ml of the stock solution of HAT and HT in 500 ml complete GM.

3. Polyethylene glycol (50% w/v mixture)

Polyethylene glycol (PEG) is the fusion-inducing agent. It should be white and odorless. It is advisable to buy specifically manufactured and pre-tested for hybridoma development, since both its toxicity and its ability to promote fusion vary from batch to batch. PEG-1450 (MW 1300-1600: Sigma Chemicals Co.) or PEG-3350 is melted in water bath, allowed to cool 37°C. Added 10 ml pre-warmed sterile RPMI-1640 and aliquot to 1.0 ml in micro-centrifuge tube. The resulting 50% w/v PEG solution can be stored frozen and melted in water bath at 37° C for each fusion.

4. Freezing media

Dimethyl sulfoxide (DMSO) is used in the cryopreservation of hybridomas by adding 10 ml of DMSO to 90 ml GM.

5. Phosphate buffer saline (PBS) pH 7.4

The composition is as following: 0.2 g. KH_2PO_4 , 2.9 g. Na_2HPO_4. 12 H_2O , 8.0 g. NaCl and 0.2 g KCl in 1000 ml of distilled water.

6. OPD substrate solution

Dissolve OPD tablet in 12.0 ml of citrate-phosphate buffer pH 5.0, compose of citric acid 7.3 g and Na_2HPO_4. 2 H_2O 11.86 g dissolved in 1 L of distilled water; adjust pH with acid or dibasic salt at the same concentration. A 12 µl of 35%v/v hydrogen peroxide is added before used.

Experimental Animals

Female BALC/c mice aged 4-6 weeks with approximate weight of 20 gm, obtained from the National Laboratory Animal Centre, Mahidol University, were used for monoclonal antibody induction.

Methods

This study composed of 3 parts. They were

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

Part B. Immunoanalytical method for amphetamine, methamphetamine and ephedrine detection using monoclonal antibody

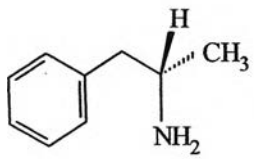
Part C. Detection of amphetamine, methamphetamine and ephedrine via membrane immunoassay

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

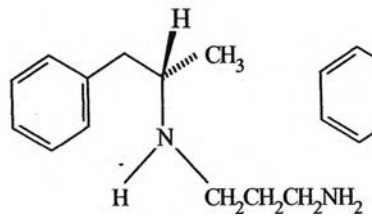
Determination of amphetamine, methamphetamine and ephedrine based on polyclonal antibodies was performed by using the direct competitive ELISA that the analytes were competed with the enzyme-labeled hapten in binding to antibodies. The heterologous combinations were used in this study. The heterologous combinations the immunizing hapten and enzyme-labeled hapten differed in their molecular structures. The selectivity and sensitivity for detection of amphetamine, methamphetamine and ephedrine by using homologous and heterologous combinations were studied and the appropriate combinations were selected for further monoclonal antibody studies.

A-1 Promptness of antisera and peroxidased-labeled derivatives

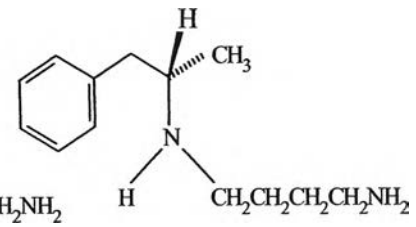
Six antisera and peroxidase-labeled derivatives (Figure 3) have been prepared since 1999 (Sirikatitham, 1999; Matapatara, 1999). It is necessary to recheck the titer of antisera as well as the activity of peroxidase-labeled derivatives before setting the immuno-analytical methods for amphetamine, methamphetamine and ephedrine detection.



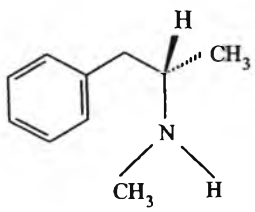
d-Amphetamine



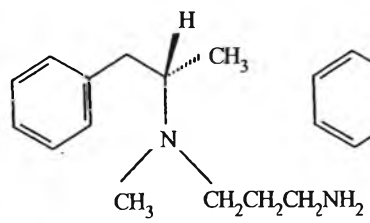
3-APA



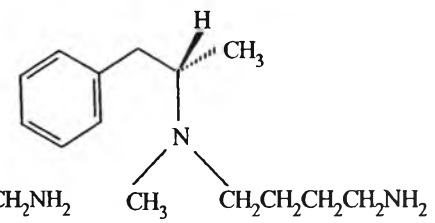
4-ABA



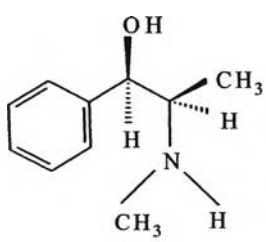
d-Methamphetamine



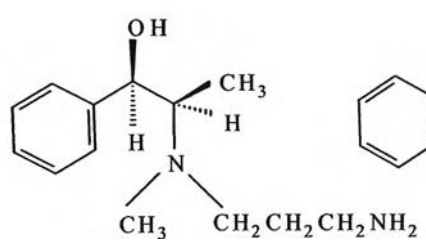
3-APM



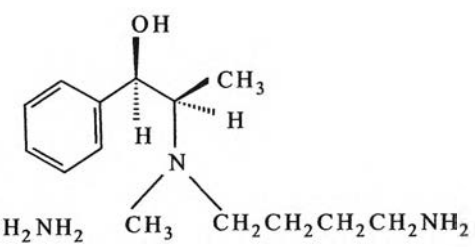
4-ABM



l-Ephedrine



3-APE



4-ABE

Derivatives of amphetamine

3-APA = N-(3-aminopropyl)amphetamine, 4-ABA = N-(4-aminobutyl)amphetamine

Derivatives of methamphetamine

3-APM = N-(3-aminopropyl)methamphetamine

4-ABM = N-(4-aminobutyl)methamphetamine

Derivatives of ephedrine

3-APE = N-(3-aminobutyl)ephedrine, 4-ABE = N-(4-aminobutyl)ephedrine

Figure 3 Chemical structures of amphetamine, methamphetamine ephedrine and their derivatives

A-1.1 Determination of the activity of peroxidase enzyme in peroxidase-labeled hapten

Procedure

The serial dilutions of the peroxidase, based on the activity of conjugated-peroxidase enzyme, and conjugated-peroxidase enzyme were prepared. Either diluted enzyme or conjugated enzyme was mixed with OPD substrate (volume ratio of 1:1) and the mixture was incubated at room temperature for 20 min before stopping the reaction with 50 µl of 4N sulfuric acid. The absorbance of the mixture solution was measured at 490 nm using a 96-well plate reader.

The dilution of conjugated-peroxidase enzyme that presented the similar absorbance to peroxidase was observed. The percentage of remained activity of conjugated-peroxidase enzyme was calculated by comparison the dilution at the similar response of conjugated-peroxidase enzyme to peroxidase enzyme. The remained activity of conjugated-peroxidase enzyme was obtained and the percentage of decreasing activity per year could be calculated. The proper dilution of each labeling enzyme was used for determining titer of the antisera.

A-1.2 Antisera's titer determination

Polyclonal antisera titer from these haptens , 3-APA, 4-ABA, 3-APM, 4-ABM, 3-APE and 4-ABE, were determined by using the microtiter plate technique. The titer of antiserum is defined as the dilution at which 50% of HRP-labeled hapten bind to the antibody (Yalow and Berson, 1964; Ciabatton, 1987). The titer of antiserum was determined as the following procedure:

Procedure

Antisera purification

Each antiserum was purified using ammonium sulfate method (Tijssen, 1985, Walker, 1996). Saturated ammonium sulfate was prepared at least 24 hrs before used and stored in refrigerator at 4°C. The antiserum was incubated at 4°C in the ice bath with gently stirring, while adding dropwise of saturated ammonium sulfate solution in the equal volume. The incubation was proceeded for 1 hr. The mixture was centrifuged at 2000 g for 30 min. The supernatant was discarded and the precipitate was re-dissolved in PBS. The procedure was repeated for the other two times then the purified antiserum solution was dialyzed against PBS pH 7.4 at 4°C until ammonium sulfate could not found in the dialyzed buffer. The final purified antiserum was lyophilized and kept frozen at -48°C.

Protein concentration of each purified antiserum was determined by measuring spectrophotometric absorbance at 260 and 280 nm (Johnstone and Thorpe, 1982). Purified antiserum at the concentration of 1.0 mg/ml in PBS pH 7.4 was prepared and measured the absorbance at 260 and 280 nm, using PBS pH 7.4 as the blank reagent. The concentration of protein in purified antiserum was calculated as the followed equation:

Protein concentration = (1.55 x absorbance at 280 nm) – (0.77 x absorbance at 260 nm)
(mg/ml) .....(1)

Titer determination

The ten fold serial dilutions of antibodies, from 1:10 to $1:10^7$, were prepared from purified antisera in the concentration of 1.0 mg/ml in 0.05 M sodium carbonate buffer pH 9.6. These antisera solution was pipetted into the wells of microplate in the volume of 100 µl, 3 wells for each antibody dilution. The plate was

incubated overnight at 4°C in the refrigerator. Then, it was washed three times with PBS-T pH 7.4 before adding a 100 µl of 2% BSA in PBS pH 7.4. The incubation was proceeded at 37° C for 1 hour. The excess BSA solution was washed out with PBS-T pH 7.4 three times. A 100 µl conjugated enzyme was added and the plate was incubated at 37°C for 2 hours before washing out with PBS-T pH 7.4 for three times. A 100 µl of freshly prepared OPD substrate solution was added to each well. The enzyme substrate mixture was kept in the dark at room temperature for 20 minutes before stopping the reaction with 50 µl of 4N sulfuric acid. The absorbance of the final solution was determined at 490 nm using microplate reader. The percentage of enzyme binding to antiserum was calculated and the titer of antibody was determined from the plot of %binding against dilution of antibody.

$$\%\text{binding} = \frac{\text{Absorbance of each diluted antiserum}}{\text{Maximum absorbance of diluted antiserum}} \times 100 \quad \text{.....(2)}$$

A-2 Heterology study

The competitive immunoassay of amphetamine, methamphetamine and ephedrine by homologous combinations, have already been studied using the direct ELISA (Sirikatitham, 1999; Matapatara, 1999). The unsatisfied of homologous immunoassay of these compounds on their lower sensitivity and specificity was clearly observed. Therefore, only the heterologous immunoassay of amphetamine, methamphetamine and ephedrine were more examined in detail in this study.

A-2.1 Selection of proper heterologous combinations

The heterologous competitive reaction between amphetamine, methamphetamine and ephedrine and enzyme-labeled hapten in binding to their antibodies were studied by using direct ELISA. The combinations were shown in Table 1.

Procedure

Based on their titer value, two or three dilutions of antisera in 0.05 M sodium carbonate buffer pH 9.6 and based on the activity of enzyme labeled, two or three dilutions of HRP-labeled hapten in PBS pH 7.4 were used in competitive reaction. Amphetamine, methamphetamine and ephedrine were analyzed for each combination in Table 1. Each diluted antisera solution was pipetted into the wells of microplate in the volume of 100 µl, 2 wells for each antibody dilution and the plate was incubated overnight at 4°C in the refrigerator. Then, the microtiter plate technique as already described on A-1.2 was followed. The series of amphetamine, methamphetamine and ephedrine aqueous solution in PBS pH 7.4 in the range of 0 - 3,000 µg/L were added to compete with the enzyme labeled in binding to antibody. The competitive reaction between the analyte and enzyme labeled in binding to antibody was determined using regression analysis such that the value of correlation coefficient (r) would indicate the proper combination of the reaction and the slope of each logic plot could imply the sensitivity of immuno-detection.

Table 1 The studied heterologous combinations

Antibody	Enzyme-labeled					
	Bridge (B)		Hapten (H)		Bridge-hapten (BH)	
	Hapten	Code	Hapten	Code	Hapten	Code
3-APA	4-ABA	B1	3-APM	H1	4-ABM	BH1
			3-APE	H2	4-ABE	BH2
4-ABA	3-APA	B2	4-ABM	H3	3-APM	BH3
			4-ABE	H4	3-APE	BH4
3-APM	4-ABM	B3	3-APA	H5	4-ABA	BH5
			3-APE	H6	4-ABE	BH6
4-ABM	3-APM	B4	4-ABA	H7	3-APA	BH7
			4-ABE	H8	3-APE	BH8
3-APE	4-ABE	B5	3-APA	H9	4-ABA	BH9
			3-APM	H10	4-ABM	BH10
4-ABE	3-APE	B6	4-APA	H11	3-APA	BH11
			4-ABM	H12	3-APM	BH12

The percentage of enzyme labeled binding to antiserum was calculated as the percentage bound by comparing the binding on the concentration range of analyte to the binding when absence of the analyte. Then, %binding was plotted against the log concentration of the analyte (the logic plot). The relationship between the %binding and the log of amphetamine or methamphetamine or ephedrine concentration was determined from the logic plot of each possible combination studied. The regression equation of the proportion reaction was generated and the r and slope value were determined. The no response (NR) was defined for the combinations that showed the scatter data or no reaction in competition. The competitive reaction with the highest value of r and slope would indicate the appropriateness of the combinations (Standefer and Saunders, 1978). The best competitive reaction with the highest value of r from each

studied would be selected. Finally, the appropriate competitive reactions with the high value of r and slope were selected for further the qualitative determination and cross-reaction study.

A-2.2 Cut-off determination

A microtiter plate technique was used for cut-off determination of amphetamine, methamphetamine and ephedrine in sample. The cut-off concentration is the concentration at borderline between the presence (positive) and absence (negative) of drug in sample (Tijssen, 1985; Wild, 1994).

The cut-off value can be determined in various definitions. The cut-off value is sometimes set at two or three times the mean of the results from the negative group of tested sample (Malvano et al., 1982) or set at the mean of the response at zero drug concentration plus or minus two or three times of standard deviation (S.D.) (Cremer et al., 1982; Richardson et al., 1983; Eremin et al., 1987; Needleman and Romberg, 1990; Wild, 1994; Badia et al., 1998). The cut-off value sometimes set at 0.15 or 0.20 absorbance of response unit (Halbert et al., 1983).

In this study, the cut-off value was determined as the mean of the response at zero drug concentration minus 3 S.D. The appropriate combinations in which the highest r from section A-2.1 were selected for cut-off determination. A direct ELISA was used in qualitative detection of amphetamine and/or methamphetamine and/or ephedrine. The presence of these drugs in sample could be qualified based on the cut-off concentration determination.

Procedure

The standard solution of amphetamine, methamphetamine and ephedrine in the concentration range of 0 - 3000 ug/L were prepared in PBS pH 7.4 and analyzed in duplicate as well as the six replicate of zero drug samples. The competitive direct ELISA

as section A-2.1 was followed. The plot of absorbance and the concentration of standard solution was performed. The mean and S.D. was determined from observed absorbance (n=6). The absorbance at cut-off was calculated as the mean of the absorbance at zero drug concentration minus 3 S.D. as the following equation:

Cut-off (absorbance) = mean of zero drug absorbance - 3 S.D. of zero drug absorbance

From the plot of each standard curve that plotted between absorbance and the concentration of the standard, the concentration at cut-off absorbance was evaluated. The accuracy of the cut-off value was also confirmed.

Confirmation of cut-off determination

In order to confirm that the determined cut-off concentration is accurate enough to detect the analyte in sample, assay response (absorbance) at the concentration more than cut-off concentration (+30%) and less than cut-off concentration (-30%) were determined (Wortberg et al., 1995; Badia et al., 1998).

Procedure

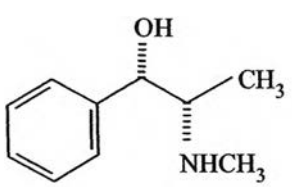
The cut-off concentration of the each analyte, 30% more than cut-off concentration and 30% less than cut-off concentration in PBS pH 7.4 were prepared. The triplicate of each concentration was analyzed with the same competitive direct ELISA procedure described in section A-2.1.

The most suitable cut-off concentration of each individual would be the concentration which showed the lesser absorbance value at concentration more than cut-off concentration and the higher absorbance value at concentration less than cut-off concentration. The combinations that showed the low cut-off value and passed the confirmation test would be carried through the cross-reaction study.

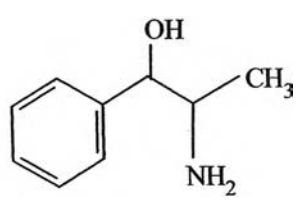
A-3 Cross-reactivity study

Cross-reactivity is the measurement of antibody response to substances other than the analytes. The National Institute on Drug Abuse (NIDA) and the Substance Abuse and Mental Health Services Administration (SAMHSA) set up the cut-off value for routine screening of amphetamine or methamphetamine. The cross-reactivity was defined in this study as the concentration of the cross-reactant that could be decreased or gave the equivalent response at 1 mg/L of the analyte (Cleeland et al., 1976; Bastos and Hoffman, 1986; Ruangyuttikarn and Moody, 1988; D'Nicuola et al., 1992).

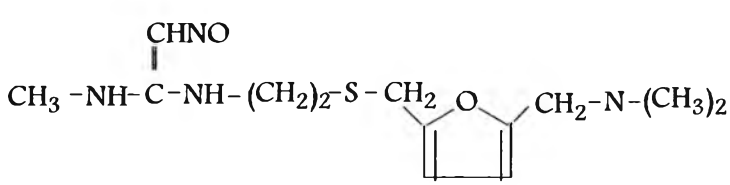
Many compounds with similar in the functional group or chemical structure to amphetamine, methamphetamine and ephedrine were used for this cross-reaction study. They were pseudoephedrine and phenylpropanolamine (phenylethylamine structure like); chlorpheniramine, ranitidine, cimetidine, chlorpromazine, and diphenhydramine (alkyl amine drugs); ibuprofen, caffeine, theophylline, paracetamol, aspirin, ampicillin and vitamin C (common medication drugs).



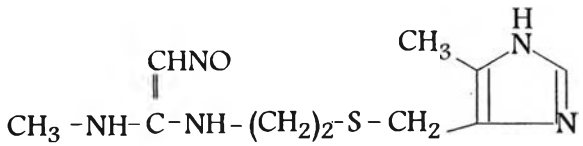
d-Pseudoephedrine



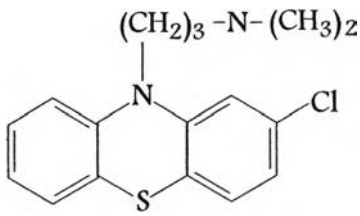
dl-Phenylpropanolamine



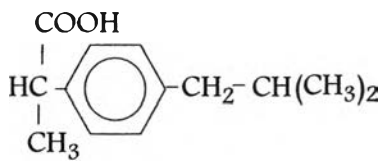
Ranitidine



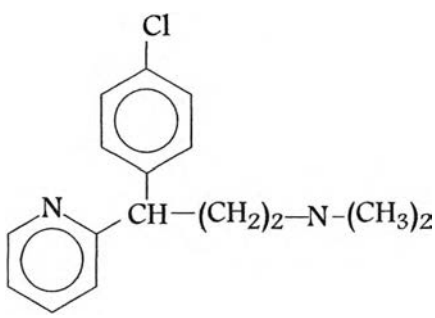
Cimetidine



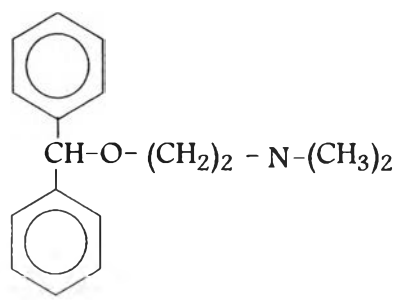
Chlorpromazine



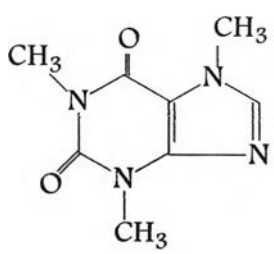
Ibuprofen



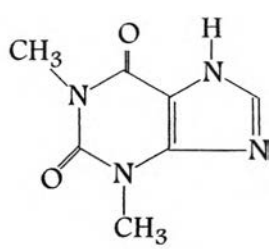
Chlorpheniramine



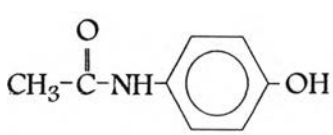
Diphenhydramine



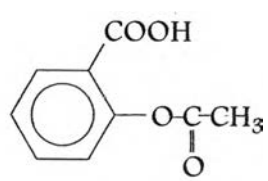
Caffeine



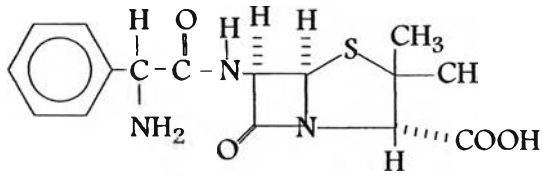
Theophylline



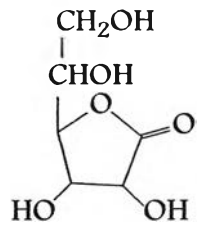
Paracetamol



Aspirin



Ampicillin



Vitamin C

Procedure

The cross-reaction of the selected reaction was undertaken by analyzing the cross reactants along with the analyte itself. The 1.0 mg/ml standard solution of amphetamine or methamphetamine or ephedrine as well as the cross-reactants in the concentration range of 0 – 1000 mg/L were prepared in PBS pH 7.4 For each cross-reactivity study, the competitive direct ELISA as described in section A-2.1 (n = 2) was followed but the cross-reactants in the concentration range of 0 – 1000 mg/L were added instead of the analyte. The amount of cross reactants that could compete with enzyme-labeled in binding to antibody equivalent to 1.0 mg/L of analyte was determined to express the cross-reactivity and calculated as followed:

$$\text{percent cross-reactivity} = \frac{\text{1.0 mg/L of analyte}}{\text{conc. of cross-reactant that produced equivalent response to 1.0 mg/L of analyte}} \times 100 \quad \text{.....(3)}$$

Part B. Immunoanalytical method for amphetamine, methamphetamine and ephedrine detection using monoclonal antibody

The selected combinations from part A were the combinations of 4-ABA-Ab and 3-APA-HRP (B2) for amphetamine detection, the combinations of 4-ABE-Ab and 3-APE-HRP (B6) for ephedrine detection and the combinations of 4-ABE-Ab and 3-APM-HRP (BH12) for methamphetamine and ephedrine detection. In monoclonal antibody study, both haptens, 4-ABA and 4-ABE, were selected for preparing the immunogens for production of monoclonal antibody in the detection of amphetamine, methamphetamine and ephedrine.

Determination of amphetamine, methamphetamine and ephedrine by using monoclonal antibody was divided into 2 steps.

1. Monoclonal antibody production
2. Competitive indirect ELISA for amphetamine, methamphetamine and ephedrine detection

B-1. Monoclonal antibody production

In producing monoclonal antibodies, the whole process composed of 5 phases as shown in Figure 4 (Liddell and Cryer, 1991; Goding, 1993; Malik and Lillehoj, 1994).

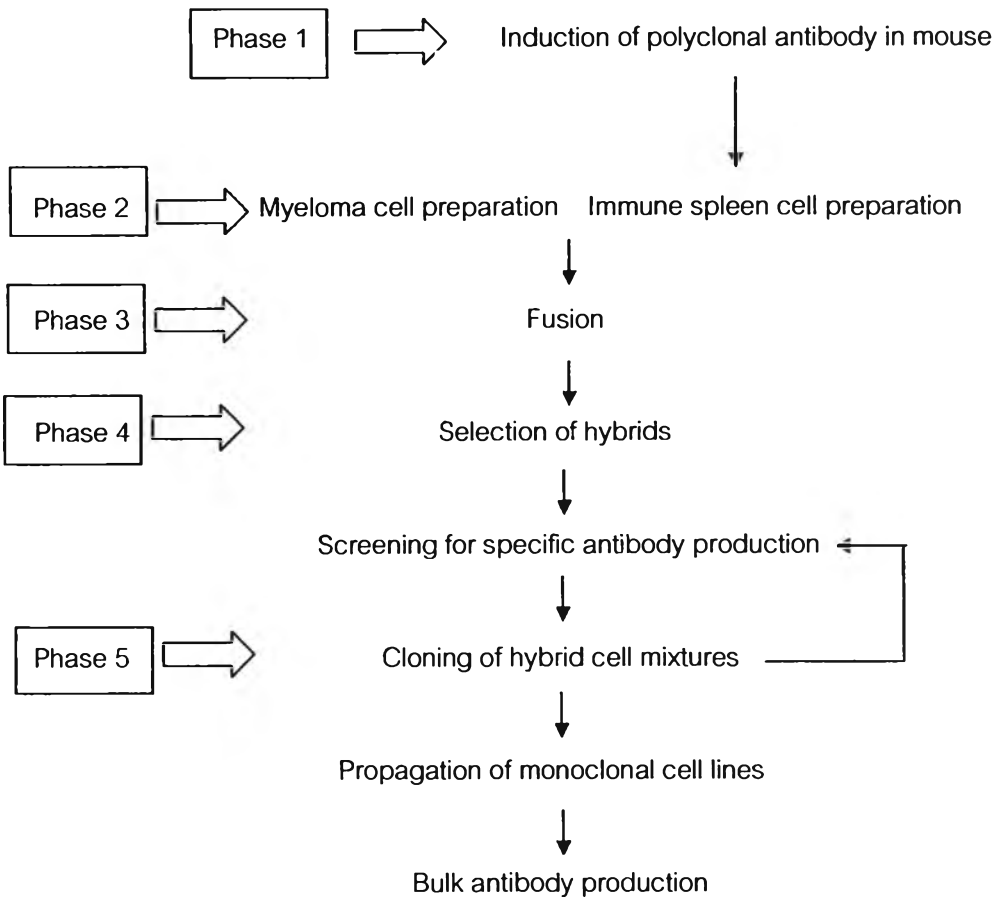


Figure 4 Process for monoclonal antibodies production

Phase 1 Induction of polyclonal antibody in mouse

1.1 Preparation of immunogen

Immunogen of N-(4-aminobutyl) amphetamine (4-ABA) and N-(4-aminobutyl)ephedrine (4-ABE) were prepared by conjugating to KLH utilizing the modified method of Cheng et al, 1973 and Mongkolsirichaikul et al., 1993.

KLH was used as carrier protein for immunogens to differentiate from BSA that was used as carrier protein for screening of test bleeds and supernatant (Danilova and Vasilov, 1991; Kitjaroentham et al., 1998).

Procedure

Dissolved 1.0 mmol of 4-ABA or 4-ABE and 1.5 mmol of N-ethyl-N-(3-dimethylaminopropyl)carbodiimide (EDCI) hydrochloride in 1.0 ml distilled water. Slowly added 0.01 μmol of KLH and stirred for 4 hours at room temperature (25°C), expecting the conjugation should be completely formed. The conjugate was purified by dialyzing against double distilled water until the excess 4-ABA or 4-ABE and EDCI could not found in the dialyzed water that could be detected by using ultraviolet spectrophotometer. The final product solution was lyophilized and kept frozen at -48°C until used.

The mole ratio of conjugation between hapten and KLH was determined by using the UV absorptivity (Erlanger et al., 1957) at 257 nm and 280 nm of hapten and KLH, respectively. The absorbance at the maximum wavelength 257 nm of 4-ABA and 4-ABE moiety in 4-ABA-KLH and 4-ABE-KLH, respectively, were determined by subtraction the absorbance of KLH from that of 4-ABA-KLH and 4-ABE-KLH. The extinction coefficient of 4-ABA, 4-ABE and KLH was measured and used for calculation the number of 4-ABA, 4-ABE and KLH mole in the immunogens.

The immunogens, 4-ABA-KLH and 4-ABE-KLH 0.1 mg were individually dissolved in 0.2 ml normal saline and then incorporated into 0.2 ml of complete Freund adjuvant (CFA) for the first immunization or 0.2 ml of incomplete Freund adjuvant (IFA) for the subsequence boosted dose.

1.2 Immunization schedule

Procedure

The female BALB/c mice aged range from 8 and 12 weeks were used. Two mice were immunized per immunogen. The immunization was proceeding with intra-peritoneal injection of 0.2 ml 4-ABA-KLH or 4-ABE-KLH in CFA through the abdominal site. Blood approximately 0.1 ml was withdrawn at the 7^{th} day after the second dose by

cutting the tail-end. The serum was separated, checking for antibody titer. If the antibody was raised in the mice, at the titer value of not less than 1:1,000 then mice were last boosted three days prior fusion. After four weeks of the first immunization, bleeding serum samples from mice were performed and preliminary assay was operated to prove the presence of polyclonal antibodies to the antigen.

Titer determination

The titer of antiserum is defined as the dilution at which 50% binding of the antibody bind to the plate-coating hapten (Yalow and Berson, 1964: Ciabattoni, 1987). Two different plate-coating haptens, 4-ABA-BSA and 4-ABE-BSA, were used for 4-ABA-Ab and 4-ABE-Ab, respectively. The non-competitive indirect ELISA was used.

Microplate coating procedure

Dissolved 0.015 mmol of 4-ABA or 4-ABE and 0.015 mmol of N-ethyl-N-(3-dimethylaminopropyl)carbodiimide (EDCI) hydrochloride in 1.0 ml distilled water. Slowly added 0.15 µmol of BSA and stirred for 4 hours at room temperature (25°C), expecting the conjugate should be completely formed. The conjugate was purified by dialyzing against double distilled water until the excess 4-ABA or 4-ABE and EDCI could not found in the dialyzed water that could be detected by using ultraviolet spectrophotometer. The final product solution was lyophilized and kept frozen at -48°C until used.

The mole ratio of conjugation between hapten and BSA was determined by using the UV absorptivity (Erlanger, 1957) at 257 nm and 280 nm of hapten and BSA, respectively. The absorbance at the maximum wavelength 257 nm of 4-ABA and 4-ABE moiety in 4-ABA-BSA and 4-ABE-BSA, respectively, were determined by subtraction the absorbance of BSA from that of 4-ABA-BSA and 4-ABE-BSA. The extinction coefficient of 4-ABA, 4-ABE and BSA was measured and used for calculation the number of 4-ABA, 4-ABE and BSA mole in the microplate coatings.

Non-competitive indirect ELISA

Since no preliminary test for the raised immune in mice was performed. This titer determination was then expanded for covering the wide range of titer.

Three dilutions of coating hapten, 1.0, 0.1 and 0.01 mg/ml, and three dilutions of goat anti-mouse-HRP labeled, 1:4000, 1:7500 and 1:10000, were used and the proper dilution of coating hapten and goat anti-mouse-HRP labeled was determined.

The sequential 10-fold dilutions of antisera, from 1 : 10 to 1 : 10^7, were prepared with PBS pH 7.4 as solvent. The well of microtiter plate were coated with 100 µl of serially diluted coating hapten, 4-ABA-BSA or 4-ABE-BSA for the 4-ABA-Ab or 4-ABE-Ab, respectively, three wells for each sera dilution and incubated overnight at 4°C. The plate was washed three times with PBS-T pH 7.4, then a 100 µl of 5% skim milk in PBS pH 7.4 was added and incubated for 1 hr at 37°C. The plate was again washed three times with PBS-T pH 7.4, before adding 100 µl of serial dilution of serum. For complete hapten-antibody interaction, the plate was incubated for 2 hr at 37°C. The excess antibody in sera was eliminated by washing three times with PBS-T pH 7.4, a 100 µl of goat anti-mouse IgG-HRP labeled was added and incubated at 37°C for 2 hr. The plate was washed three times with PBS-T pH 7.4 A 100 µl of freshly prepared OPD substrate solution was added to each well. The enzyme substrate reaction in the dark took 20 min at room temperature. The reaction was stopped by adding with 50 µl of 4N sulfuric acid. The absorbance was determined at 490 nm using microplate reader. The percentage of plate coating hapten binding to antiserum was calculated and the titer of antibody was determined from the plot of %binding against dilution of antibody.

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

Phase 2 Preparation of myeloma cells and spleen cells

2.1 Myeloma cells preparation

Myeloma cell or plasmacytoma is a neoplasm of antibody producing cells. Most of the useful rodent-derived myeloma cell lines are commercial available, for example, P3-X63-Ag8, P3-X63-Ag8.653, NS-1 and Sp2/0-Ag14.

Three myeloma cell lines, P3-X63-Ag8.653, Sp2/0-Ag14 and NS-1 were used in this study. Only the best fusion that gave the high percentage of service hybrids was selected. RPMI 1640, containing antibiotics (penicillin at 200 U/ml and streptomycin at 100 mg/ml) and fetal bovine serum is the generally basic medium for cell culture in monoclonal antibody production. In addition the percentage of fetal bovine serum added was varied depend on the objective in monoclonal antibody production.

Procedure

The whole process have to operate under the sterile condition in a class II laminar flow cabinet. About 10^4 cells of each type of cell line were seeded in a 5 ml RPMI culture medium with 10 % fetal bovine serum as the growth medium. They were incubated under humidified 10% CO_2–in-air atmosphere at 37°C in 25 cm^2 or 75 cm^2 plastic tissue culture flasks. Cell extension was observed at the third to fourth day following seeding, the optimal growth was obtained at cell numbers between 10^5 and $5x10^5$ cells/ml. Prior to fusion, it was essential that the myeloma cells were in the log phase of growth and maintenance at a cell density of not greater than 10^6 cells per ml. The cell viability was greater 96% and at least 10^7 per ml were used for each fusion. The cell viability was checked by trypan blue staining method with haemocytometer counting.

For viability test with trypan blue staining, the equal volume about 50 µl of cell suspension and trypan blue reagent were mixed, then immediately pipetted about 50 µl under the cover slip of the haemocytometer. Counting under the microscope, the

viable cell that could not take up trypan blue dye, would be bright and shiny. When cells dead, their cells membrane became permeable, the dark blue color would be observed. The bright cells were counted comparing to the total cell counted. The viability of cell culture could then be calculated.

For maintenance of myeloma cell, the cell line would be stored in freezing medium for further expansion. For keeping the cell in freezing medium, the suspension cell line was centrifuged, the supernatant was discarded. The freezing medium was added, mixed and transferred to the cryotube as the low temperature resistant vial. For long term storage, the stock of cell line should be storage in liquid nitrogen chamber.

2.2 Spleen cells preparation

In rodent fusion, the preferred lymphocyte donor is the spleen, in which approximate 20% of cells were B-lymphocytes. Spleen cells from the immunized mouse were fused with the previously prepared myeloma cells. The spleen was removed from the mouse that expressed antibody titer value of more than 1:1,000 was removed. The whole spleen was pressed through a fine sieve. The viability of cell was counted and calculated for the number of the myeloma cells for fusion. One mouse spleen yields about 10^8 nucleated cells. Therefore, myeloma cells about 10^7 was used for fusion with the desired ratio of spleen cell to myeloma cell was 10 : 1

Procedure

The experiment was performed in a sterile cabinet under the practical cell culture. Mice were sacrificed under ether anesthesia. The spleen was aseptically removed and transferred into a plastic Petri-dish containing 10 ml free serum RPMI 1640 medium for washing. The adipose tissue around spleen was dissected before cutting the spleen into a small pieces and pressing through a fine sieve to the other Petri-dish containing 10 ml free serum RPMI 1640 medium. The remaining cells on the sieve were

collected by washing out through the sieve with the free serum RPMI 1640 medium. All of the medium containing dispersed cells were transferred to a sterile centrifuge tube. The cell suspension was centrifuged at 400 g at room temperature for 10 minutes. The supernatant was separated and re-dispersed into a 10 ml free-serum RPMI 1640 medium. An aliquot portion of 10 µl was counted for the number of lymphocytes and checked for cell viability by trypan blue staining. The whole prepared spleen cells were used for one fusion.

Phase 3 Fusion of myeloma cells with immune spleen cells

Fusion is the process for merging of two cell types together by using the fusion reagent to making the cell membrane more permeable, resulting in hybrid cell that inherit both of these cells properties. The fused hybrid cells of myeloma and spleen cells are called hybridoma cells.

Procedure

Myeloma cell (10^7 – 10^8 cells) and immune spleen cells (10^8), were centrifuged at 400 g at room temperature for 5 minutes. The supernatant of each individual was separated and re-suspended in 10 ml serum-free RPMI medium before mixing. The cells mixture were centrifuged again at 400 g for 5 minutes, discarding the supernatants. The 50% PEG 1500 or PEG 3350 in serum-free RPMI medium was incubated at 37°C prior use. A 1.0 ml of this solution was slowly added over 1 minute into the cells mixture. The cell mixture was incubated at room temperature for 1 minute. Serum-free RPMI medium was subsequently transferred with very slow speed (approximately 15 minutes) into this mixture in the volume of 2x10 ml. The fusion mixture was carefully mixed and then centrifuged at 300 g for 10 minutes. The supernatant was again discarded and the mixture of cells was gently re-suspended in 10 ml of HAT selective medium. The 100 µl of cell mixture was then pipetted into 10 x 96 well plates, approximately 10^4 – 10^5 cells per well. A 100 µl HAT medium was then added to the top layer of each well. The plate was incubated in 5% CO_2 at 37°C incubator.

Phase 4 Selection of hybrid

Hybrid cell is the cell derived from the fusions between myeloma and spleen cell. Only hybrid cells can grow in HAT medium.

4.1 HAT selection

Procedure

After fusion, the HAT medium in 96 well plates was changed, using the HAT medium, on day 3, 6 and every two or three days or appearance of yellow color medium afterwards until the first screen. Within three to four days all myeloma cells should be dead that could be observed from the dark spot on the well and density of the cell was decreased in addition the survive hybrid cells were subsequently growth. From about the fifth day, hybrids should start to become visible then the cluster of cell was presented and expected to secrete the antibodies.

4.2 Screening and testing of antibody from hybridoma cells

Procedure

The primary screening of supernatant from the hybrid cells growth medium was performed by using non-competitive indirect ELISA. The harvested supernatants were used without dilution. About 100 µl supernatant of each fusion well was harvested from the culture plates and added into 96 well microtiter plate coated with 4-ABA-BSA or 4-ABE-BSA for screening the presence of antibodies for 4-ABA or 4-ABE. The proper dilution of coating hapten in 0.05 M sodium carbonate buffer pH 9.6 and goat anti-mouse IgG-HRP in PBS pH 7.4, which was determined in phase 1 section 1.2 were used. BSA coated plate was included for the validation of procedure. The microtiter plate technique as already described in phase 1 section 1.2 was followed.

At least one well per plate should contain fresh culture medium or culture supernatant that contain unrelated specificity antibody as negative control. For positive control, serial dilutions of 4-ABA or 4-ABE polyclonal antisera for 4-ABA or 4-ABE MAbs production, respectively, from the immunization step should also be included together with the similar dilutions of non-immune serum.

From primary screening, the positive cells were expanded under aseptic technique. The positive cells in each well of the 96 well plate was individually mixed and transferred about half of the medium volume to 24 well plate that contained about 500 µl of the new HAT medium. The plate was incubated in 5% CO_2, 37°C. The original cells in 96 well tissue culture plated was added up with the HAT medium for maintenance the cells and also re-screened as well as the passage positive cells. Type of medium could be change, on the way of expansion and ensure that un-fused myeloma cells died, to HT and subsequent complete medium by harvesting the old supernatant and added up with new medium. The supernatants of the positives were harvested for competitive reaction and the medium was added for maintenance the cells in the wells.

The competitive reaction for amphetamine, methamphetamine and ephedrine detection was subsequently performed after the positive cells were expanded. The enough supernatant for competitive reaction was harvested and could be stored in refrigerator at 4°C with adding 0.1% sodium azide until used. Amphetamine, methamphetamine and ephedrine in the concentration of 10.0 mg/L was individually prepared for competing against 4-ABA-BSA or 4-ABE-BSA to binding to 4-ABA-Ab or 4-ABE-Ab, respectively. The tested supernatants were harvested from only the positive screening cells in non-competitive reaction and 100 µl the standard solution at 10.0 mg/ml of amphetamine or methamphetamine or ephedrine was added to the same well of supernatant. The competitive ELISA was performed by using the microtiter plate technique as already described in non-competitive ELISA screening. In addition, the BSA coated plate was not used in this testing.

Interpretation

Primary screening of supernatants from hybrids cell, most of the wells screened will be negative for specific antibody so the mean value of background absorbance was defined as negative well. Meanwhile, the positive well should show at least double or even triple the background absorbance dependent upon the activity of antibodies produced from the fusion cells.

In the event of no specific antibody-secreting cells being detected, a positive control was assessed whether it was a true result or due to assay failure. The protein carrier, BSA, also checked for their cross-reactivity.

In competitive reaction, the decreasing of final absorbance comparing to free drug was indicated the production of specific antibodies for amphetamine, methamphetamine and ephedrine of the cells. These positive cells were all continually expanded for the following cloning step.

Phase 5 Cloning of hybridoma cell lines

The cloning method used, was the limiting dilution method that is the most commonly method used.

Limit dilution method

The limiting dilution method is the simplest and most popular method of hybridoma cloning. The purposed of cloning is to isolate specific antibody-secreting cell from hundreds of other cells in the culture so that a monoclonal cell line can be established. The hundreds of cells were diluted to the limit number of 5, 2, 1 and approximately 0.5 cell per well. The phase takes 3-4 weeks. This cloning facilitated a homologous antibody population.

Procedure

The expanded positive hybrid cells for cloning were re-suspended, and the cells were counted by trypan blue staining before diluting the cloning sample in serum free-RPMI medium as the following procedure:

From the initial counting number of cloning cell, a 100 µl of the cell suspension was removed to 15-ml centrifuge tube for cloning, added the serum free-RPMI medium for the dilution of 2000 cells per ml. Pipetted a 100-µl of a diluted cloning cells into the cloning medium, completed growth medium with 20% fetal bovine serum, to make the volume of 4.0 ml (approximately 200 cells). In each step of seeding the cell, the cell suspension should be carefully mixed to obtained homogenous suspension. Accurately pipette the diluted cloning cell 100 µl of this mixture into each well of row A and B of 96 well cell culture plate, the 2.4 ml of the diluted cloning cell was used and the 1.6 ml of the diluted cloning cell was remained. A 2.4 ml of cloning medium was added to the remaining 1.6 ml of cell suspension, this step contained 2 cells per 100 µl. The dilution was proceeded in this pattern such that one 96 well plate was performed for each cloning. Top up each well with cloning medium and two drops of cloning medium was also fed at the fifth and twelfth day.

The appearance of monoclone cell should be observed within two weeks after cloning and cell supernatants were screened for antibody activity, using non-competitive indirect ELISA. The process of re-cloning was possible until the monoclone cell was confirmed. Aliquots of formally cloned cell lines were frozen in liquid nitrogen to insure a future supply of specific antibody.

To obtain optimal amounts of antibody in supernatants, the hybridoma cells were grown in tissue culture flask 75 cm^2 or in the Petri-dish plastic plate with complete growth medium. The cell-free supernatants from multiple flasks of one hybridoma line were pooled. Each batch of supernatant containing 0.1 % sodium azide was stored in refrigerator at $4^{\circ}C$.

In phase 4, the positive wells, non-cloning cells, have been determined in competitive indirect ELISA for detection of amphetamine, methamphetamine and ephedrine with the homologous combinations. A good indication of monoclonality was that in the final clone plate, all wells displaying cell growth should also show the similar selectivity and sensitivity for detection of amphetamine, methamphetamine and ephedrine. Therefore, the competitive indirect ELISA for amphetamine, methamphetamine and ephedrine detection was determined as the following:

The cloning monoclonal antibodies were utilized in the detection of amphetamine, methamphetamine and ephedrine via homologous combinations and heterologous combinations as shown in Table 2. The proper combinations were selected for further quantitative and qualitative detection of amphetamine, methamphetamine and ephedrine.

Table 2 Homologous and heterologous combinations with monoclonal antibody

Antibody	Plate-coating hapten							
	Homologous		Bridge (b)		Hapten (h)		Bridge-hapten (bh)	
	Code	Hapten	Code	Hapten	Code	Hapten	Code	Hapten
4-ABA	homo1	4-ABA	b1	3-APA	h1	4-ABM	bh1	3-APM
					h2	4-ABE	bh2	3-APE
4-ABE	homo2	4-ABE	b2	3-APE	h3	4-APA	bh3	3-APA
					h4	4-ABM	bh4	3-APM

Homologous combinations

The homologous competitive reaction between amphetamine, methamphetamine and ephedrine and the plate-coating hapten in binding to their antibodies were studied with the similar combinations as described in section 4.2.

Heterologous combinations

The heterologous competitive reaction between amphetamine, methamphetamine and ephedrine and the plate-coating hapten in binding to MAbs were studied. The six derivatives of amphetamine, methamphetamine and ephedrine, N-(3-aminopropyl)amphetamine (3-APA), N-(4-aminobutyl)amphetamine (4-ABA), N-(3-aminopropyl)methamphetamine (3-APM), N-(4-aminobutyl)methamphetamine (4-ABM), N-(3-aminopropyl)ephedrine (3-APE) and N-(4-aminobutyl)ephedrine (4-ABE), that have been prepared since 1999 were used as the plate-coated hapten. The possible heterologous combinations as shown in Table 2, bridge, hapten and bridge-hapten combinations, were investigated utilizing competitive reaction for amphetamine, methamphetamine and ephedrine detection.

Procedure

The 2 x 100 µl of expansion MAbs supernatant was used in each competitive reaction as shown in Table 2. The proper dilution of goat anti-mouse IgG-HRP in PBS pH 7.4 and plate coating hapten, 3-APA-BSA, 4-ABA-BSA, 3-APM-BSA, 4-ABM-BSA, 3-APE-BSA and 4-ABE-BSA, were prepared. Amphetamine, methamphetamine and ephedrine in the concentration of 1.0, 10.0 and 100.0 mg/L in PBS pH 7.4 were prepared for competing to 4-ABA-Ab and 4-ABE-Ab in each combination in Table 2. The microtiter plate technique as already described in section 4.2 was followed.

The selectivity of monoclonal antibodies was defined as the ability of standard solution, amphetamine, methamphetamine and ephedrine at 1.0, 10.0 or 100.0 µg/L, that could compete against the plate coating hapten in binding to antibody. The decreasing of absorbance comparative to no drug was defined as selective for amphetamine, methamphetamine and ephedrine detection. The proper combinations in which amphetamine, methamphetamine and ephedrine could be detected, were selected for further examinations. The expansion MAbs should be purified prior to further assay.

Monoclonal antibody partial purification

Ammonium sulfate method for partial purification of hybridoma supernatants was the simplest antibody partial purification methods that do not require expensive hardware (Tijssen, 1985).

Procedure

Whole supernatant of each selected monoclonal antibody in ice was added dropwise with stirring sufficient saturated ammonium sulfate solution at 4°C to achieve a 33% saturated final concentration. The purification procedure as described in section A-1.2 was followed.

IgG content as protein concentration in partial purified monoclonal antibody was determined by using ultraviolet spectrophotometric method as described in section A-1.2.

The titer at 50% binding of monoclonal antibody to the plate coating hapten, 4-ABA-BSA and 4-ABE-BSA for 4-ABA-Ab and 4-ABE-Ab, respectively, was determined. The proper dilution of plate coating in 0.05 M sodium carbonate buffer pH 9.6 and the enzyme labeled in PBS pH 7.4 were used. The 10-fold serial dilution of 1.0 mg/ml partial purified antibody in PBS pH 7.4 was prepared and used in microtiter plate technique as described in section 1.2. The percentage of plate coating hapten binding to antiserum was calculated and the titer of antibody was determined from the plot of %binding against dilution of antibody. The titer dilution was used in the further competitive reaction.

B-2 Competitive indirect ELISA for amphetamine, methamphetamine and ephedrine detection

B-2.1 Quantitative detection

Procedure

The selected combinations from phase 5 were used in the competitive reaction. Two or three dilutions of the partial purified MAbs in PBS pH 7.4 based on the titer value were used in competitive reaction. Amphetamine, methamphetamine and ephedrine in the concentration range of 0 – 100 mg/L in PBS pH 7.4 were individually prepared for competing against the plate coating in binding to 4-ABA-Ab or 4-ABE-Ab, respectively. The proper dilution of goat anti-mouse IgG-HRP in PBS pH 7.4 and plate coating hapten in 0.05 M sodium carbonate buffer pH 9.6 were prepared. Each dilution of antibody was performed in duplicate. The microtiter plate technique as already described in section 4.2 was followed. The result of the reaction was determined using regression analysis such that the value of r indicated the appropriate reaction and the slope of each logic plot determined the sensitivity of detection.

The relationship between the %binding and the log of standard concentration was determined. From the logic plot, the combinations that showed the scatter data or no reaction in competition could be defined as no response (NR). The value of correlation coefficient (r) from the regression analysis of each logic plot was determined for the proportion reaction in binding to antibodies. The r value informed the appropriate dilution of antibody and plate-coated hapten in competitive reaction. The highest value of correlation coefficient (r) of the competitive reaction indicate the appropriateness of the system (Standefer and Saunders, 1978) and chosen for further qualitative detection. In addition, the proper dilution of antibody from each combination in which the highest value of r was selected for further qualitative detection.

B-2.2 Qualitative detection

The appropriate combinations in which the highest r value from quantitative detection were selected for cut-off value determination and cross-reactivity with the similar principle to part A.

Cut-off determination

For qualitative determination, cut-off value was defined as the value indicating the presence or absence of the suspected compound in tested sample. The cut-off value determination from part A was applied such that the cut-off value was calculated from the mean of the response at zero drug concentration minus 3 of the standard deviation of the six replicates of the zero drug response.

Procedure

The proper combinations of MAbs and plate coating from quantitative assay were used. The proper dilution of MAbs from section B-2.1 was prepared in PBS pH 7.4. The standard solution of amphetamine, methamphetamine and ephedrine in the concentration range of 0 – 10,000 µg/L were prepared in PBS pH 7.4 and determined in two replicates. The six replicates of zero drug (only PBS pH 7.4) was analyze. The competitive indirect ELISA as described in section B-2.1 was followed. The plot of absorbance and the concentration of standard solution was performed. The absorbance at cut-off was calculated as the mean of the absorbance at zero drug concentration minus 3 S.D. The concentration at cut-off was determined from the plot of standard absorbance and standard concentration. The concentration at cut-off value was confirmed followed the procedure in part A.

B-2.3 Cross-reaction determination

To determine the selectivity of monoclonal antibody induced in this study, the similar principle was followed as described in part A. The whole process for cross-reaction study in polyclonal antibody from part A was repeated with substitution of polyclonal antibody to monoclonal antibody.

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

From the polyclonal and monoclonal antibody studies, the proper combination with the high correlation coefficient in competitive ELISA was the heterologous combinations of anti-4-ABE polyclonal antibody and 3-APM with the cut-off value of 12.0 and 5.0 µg/L for methamphetamine and ephedrine detection, respectively. Therefore, the membrane immunoassay was developed based on this combination.

The procedure for development of lateral flow immunoassay was composed of two main steps.

1. Preparation of gold-labeled antibody
2. Development of membrane immunoassay

C-1 Preparation of gold-labeled antibody

C-1.1 Prepare the solution of antisera

Procedure

Antisera with IgG concentration 5.0 mg/ml in tris buffer saline (TBS) pH 8.2 was prepared and dialyzed overnight against 2 mM borax buffer pH 9.0 at 4°C. The dialyzed bag was immediately centrifuged at 40,000 rpm for 1 hour at 4°C. A series of antibody solution in the concentration range of 0 – 5.0 mg/ml in TBS pH 8.2 was prepared to a net volume of 1.0 ml.

C-1.2 Conjugation of colloidal gold with antibody

Procedure

Added 0.5 ml aliquot of each freshly prepared antibody dilution to 5.0 ml of pH-adjusted colloidal gold in the plastic tube, mixed thoroughly for 5 min. A 0.5 ml of a 10 % sodium chloride solution was added, mixed and stand for 5 min before adding 0.5 ml of 1 % carbowax aqueous solution. Measure the absorbance at 580 nm, using 5.0 ml of pH-adjusted colloidal gold diluted to 6.5 ml with distilled water as blank. The absorbance values were plotted against the volume of antisera solution. The appropriate antisera concentration need for stabilized colloidal gold should be the point at the first minimum observed absorbance.

Adsorbing colloidal gold with antibodies

Procedure

The diluted antibody in TBS pH 8.2 was prepared based on the proper concentration of IgG from the above section. A 0.5 ml of the diluted antibody in TBS was placed in polycarbonate reagent tube which had been presoaked overnight in 1%BSA and washed 10 times with distilled water before used. Immediately added 10.0 ml of the colloidal gold with gently mixing for 5 min and then incubated for 1 hour at room temperature. The complexes were then centrifuged in fix angle rotor at 15,000 rpm for 1 hour at 4°C. The supernatant was carefully aspirated and the remained dark red pellet of gold-labeled antibody was re-dispersed in 10 ml of TBS containing 10%BSA. The process of centrifugation and re-dispersed was repeated for the other two times. The absorbance of final gold-labeled antibody solution was then observed at 520 nm before stored at refrigerator temperature (4 - 8°C).

C-1.3 Determination of methamphetamine and ephedrine utilizing gold-labeled antibody

The gold-labeled antibody was used in detection of methamphetamine and ephedrine for preliminary study prior to developed membrane immunoassay by sol particle immunoassay (SPIA) which based on the agglutination inhibition principle.

Determine the optimum concentration of 3-APM-BSA in agglutinating 4-ABE-Ab-Au

Procedure

3-APM-BSA prepared from the conjugation of 3-APM to BSA toward carbodiimide method, have been prepared in part B. The stock of 3-APM-BSA solution 1.0 mg/ml. was prepared in TBS pH 8.2 containing 10 mg/ml BSA. The series dilution of 3-APM-BSA solutions in the concentration range of 0 – 1.0 mg/ml was prepared to the net volume of 1.0 ml.

An individually 50 µl of BSA conjugating hapten dilution was incubated with a 50 µl of gold-labeled antibody at 37°C for 2 hr. The absorbance was then measured at 510 nm. The dose-response curves were generated from the absorbance observed and the BSA conjugating hapten dilution. The minimum absorbance observed, would be the optimum concentration of 3-APM-BSA that agglutinate gold-labeled antibody. Finally, the volume from the curve which indicated the minimum value of absorbance was calculated to explain as concentration unit; microgram.

Quantitative determination of methamphetamine and ephedrine using SPIA

Procedure

The 50 µl of standard amphetamine, methamphetamine and ephedrine solution in the concentration range 0 – 10,000 µg/L in TBS pH 8.2 were added into the wells of a non-coated microplate, 3 well per each concentration. Then, 100 µl of gold-labeled antibody were added into each well and mixed. The plate was incubated at 37°C for 2 hr followed by addition of 50 µl of BSA conjugating hapten solution at the optimum concentration. The solution was incubated at 37°C for 2 hr before measuring the absorbance at 510 nm. The measured absorbances were plotted against the concentration of methamphetamine and ephedrine, in which the linear range of the response was determined. The values of correlation coefficient (r) from the regression analysis of the proportion reaction and the slope were determined. The competitive reaction with the high value of r indicated the appropriateness of reactions (Standefer and Saunders, 1978) for quantitative detection of methamphetamine and ephedrine. The proper condition was used in development of membrane immunoassay. The cut-off of qualitative detection was also determined based on the different absorbance from the zero drug observed.

C-2 Development of membrane immunoassay

In developing lateral flow membrane immunoassay, four pad types were involved as shown in Figure 5

1. sample pad: for loading the sample
2. gold conjugate pad: for loading 4-ABE-Ab-Au
3. reaction pad: for loading test line and control line
   - test line: 3-APM-BSA coated as the line on the membrane
   - control line: goat-anti-rabbit IgG coated as the line on the membrane.

4. absorbing pad: for keeping continuous capillary flow of sample solution and absorb the excess reaction solution.

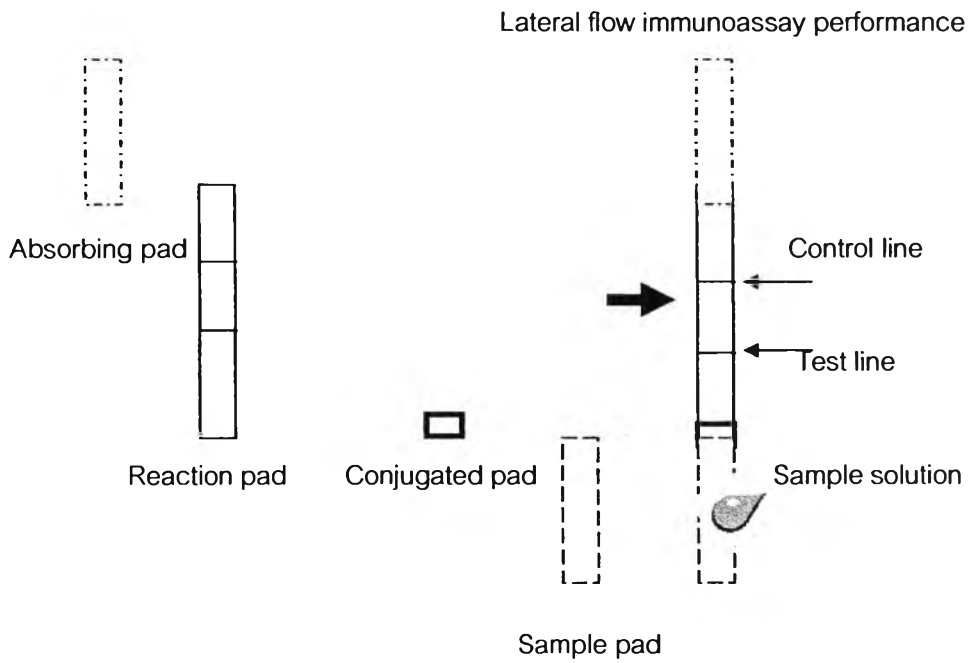


Figure 5 The completed lateral flow immunoassay performance

C-2.1 Determination of appropriate condition for membrane immunoassay

Each composition of membrane immunoassay was also studied for creating the proper reaction in the lateral flow immunoassay. There was a variety of membrane matrices used as the reaction pad in membrane immunoassay. Therefore, the proper type should be determined prior to use in membrane immunoassay.

Selection the type of membrane

The following seven different types of membrane were tested for the non-specific capture of colloidal gold and the capability of protein binding on the membrane. The proper membrane were used as the reaction pad.

1. Nitrocellulose membrane AE 98 (pore size 5.0 micron)

2. Nitrocellulose membrane AE 99 (pore size 8.0 micron)

3. PRIMA 40: PRIMA series of membranes consist of large pore nitrocellulose media cast directly upon polyester backing.

4. Nitrocellulose BioTrace™ NT (pore size 0.2 micron)

5. Polyvinylidene fluoride BioTrace™ PVDF (pore size 0.45 micron)

6. Amphoteric Nylon 6,6 Biodyne A (pore size 0.45 micron)

7. Modified polyethersulfone UltraBind (0.45 micron)

The 3-APM-BSA binding, the goat anti-rabbit-IgG binding and the non-specific binding signal, in seven type of membranes were tested as the following:

Procedure

For testing of non-specific binding, these membranes were cut into two pieces in the size of 3 x 5 mm. One piece of each membrane type was soaked in 5.0% skim milk as the blocking reagent and incubated at room temperature for 1 hr and washed three times by soaking in the PBS pH 7.4. Then, the blocked and un-blocked membranes were examined for their staining capability by immersing the membranes in the colloidal gold. After incubating for 30 min at room temperature, the color intensity was measured by the observation with naked eyes. The non-specific binding membrane, minimum or absent of the red color observed on the membrane, was selected for further testing in protein binding.

For 3-APM-BSA binding property, 3-APM-BSA and the diluted goat anti-rabbit-IgG solution (1:10) was spotted as the line by using the fine capillary tube on each membrane in the size of 5 x 35 mm. A gold conjugated pad containing dried gold-labeled antibody was prepared. The undiluted gold conjugate solution from section C-1.2 was used. Each conjugated pad in size of 9 x 20 mm was prepared and cut into the size of 3 x 5 mm and subsequently attached to the membrane strip. A sample pad in the size of 5 x 15 mm was attached onto the conjugate pad.

The construction of lateral flow format as shown in Figure 4 was performed in testing of 3-APM-BSA and goat anti-rabbit-IgG binding. When applied 300 µl of artificial urine (Mathoera et al., 2002) to the sample pad, the liquid sample migrates by capillary diffusion through the gold-labeled antibody conjugate pad, caused the re-hydrating of the gold conjugate. The complex of gold-labeled antibody conjugate and artificial urine then moves onto the membrane strip and migrates towards BSA conjugating hapten, where it becomes immobilized and produces a distinct signal in the form of a red line, test line. A second line, a control line, may also be formed on the membrane by excess gold conjugate, indicating the test is complete.

The red color at test line as well as the control line must be readily visible in the membrane, whereas the color due to the non-specific binding must be kept at a minimum. Thus, the most suitable type of membrane was selected for further lateral flow immunoassay.

For the lateral flow immunoassay, the proper type membrane from the previous selection was used. The conjugated pad was prepared by using gold-labeled antibody from section C-1.2. The size of membrane was based on the available lateral flow immunoassay. Therefore, sample pad and absorbing pad were about 5 x 20 mm and conjugated pad size was 3 x 5 mm. The sample pad and absorbing pad were tested for the capacity volume of solution that could contain in the pad. The complete lateral flow immunoassay performance used for testing this design was shown in Figure 4. The proper reaction condition was studied.

The competitive reaction was depending on amount of 4-ABE-Ab-Au and 3-APM-BSA on test line. Therefore, the appropriate size of conjugated pad as well as the amount of gold-labeled antibody were determined. The proper concentration of BSA conjugating hapten from section C-1.3 was used, then the concentration of gold-labeled antibody was adjusted. In addition, the other compositions for construction of lateral flow immunoassay were also tested.

The lateral flow immunoassay format as shown in Figure 4 was performed for determination of proper condition lateral flow immunoassay. The following components of membrane were prepared for each condition of lateral flow immunoassay that composed of each preparation as summarized in Table 3. The artificial urine was used as the sample in this determination.

Sample pad and absorbing pad were set up at the opposite site of the membrane, start point and the end, respectively. These pads were used the same material and same size 5 x 20 mm. This size of sample pad was tested for the capability of absorbing 150, 200, 300 and 400 µl of the sample solution and the absorbing pad was examined on the ability to absorb the excess solution with keeping continuous capillary flow of sample solution. Before sitting on the membrane, the sample pad was pretreated by soaking in PBS-T pH 7.4 and dried in the air.

The undiluted gold conjugate solution from section C-1.2 was used. Each conjugated pad in the size of 9 x 20 mm was immersed in the series volume of 4-ABE-Ab-Au solution, 0.5, 1.0 and 1.5 ml, for 30 min then picked up from the conjugated solution and dried in the air. The absorbing volume was determined. The gold conjugated pad was cut into 3 x 5 mm putting on the edge of reaction membrane at the same end of sample pad.

Test line was applied on the reaction pad that has been selected in section C-2.1. By using a fine capillary tube, a series volume, 10, 20, 30, and 40 µl, of 20 µg/ml BSA conjugating hapten solution was spotted on the surface of each reaction membrane at 10 mm from the edge of gold conjugated pad position.

For the control band, the series of volume in the range of 1 to 5 µl of 1:10 goat anti-rabbit-IgG in PBS pH 7.4 was applied on the reaction membrane in the upper position above the test line. The excess 4-ABE-Ab-Au that re-hydrated from conjugated pad would be reacted with the goat anti-rabbit-IgG.

To indicate the successful of testing, two pink or red color of test line and control line should be visible. The visible test line indicated that 3-APM-BSA was reacted with the 4-ABE-Ab-Au as well as the visible control line confirmed the validity of the test. The completely absent of gold-labeled antibody on conjugated pad was observed. The absorbing pad could absorb the excess 4-ABE-Ab-Au.

Table 3 The summarized of membrane preparation

No.	Sample volume (µl)	Test band (µl)	Conjugated pad (ml)	Control band (µl)
1	150	10	0.5	1.0
2	200	20	1.0	2.0
3	300	30	1.5	3.0
4	400	40	-	4.0
5	-	-	-	5.0

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

Procedure

Artificial urine, the component as shown in Table 4, was used as the diluent buffer for test sample. For test sample preparation, amphetamine or methamphetamine or ephedrine was spiked into artificial urine in the concentration range of 0 – 10,000 µg/L. The membrane immunoassay was used. The proper volume of sample was slowly dropped on the middle part of sample pad, the liquid sample was migrated by capillary diffusion through the conjugate pad, re-hydrating the gold conjugate and allowing the interaction of the sample analyte with the conjugate. The complex of gold conjugate and analyte then moved onto the membrane strip and migrated towards 3-APM-BSA, where it became immobilized and produced a distinct

signal in the form of a sharp red line. The zero concentration of standard solution was used as the negative control. The absent of red color of test line indicated the cut-off concentration of methamphetamine and ephedrine.

Table 4 Artificial urine component

Reagent	mmole/L	Reagent	mmole/L
NaCl	105.5	NaN_3	1.00
KCl	63.70	$MgSO_4$	3.85
Na_2SO_4	16.95	$CaCl_2$	4.00
$NaH_2PO_4 \cdot 4H_2O$	3.23	$Na_2C_2O_4$	0.30
$Na_3C_6H_5O_7$	3.21		

The accuracy of the cut-off was confirmed at 6 different concentrations. They are:

- low negative concentrations (zero drug)
- at 50% below the cutoff concentration
- at 25% below the cutoff concentration
- at 25% above the cutoff concentration
- at 50% above the cutoff concentration
- high positive concentrations

Those samples that could present the equal or less red color intensity of test line to the positive control was considered positive (+) in contrast to the negative (-) result should be presented the higher intensity of red test line.

C-2.3 Cross-reactivity study

Procedure

Measurement of cross-reaction were set up with cross-reacting compounds; amphetamine, pseudoephedrine, phenylpropanolamine, ranitidine, cimetidine, vitamin C, chlorpheniramine, chlorpromazine, caffeine, theophylline, diphenhydramine, paracetamol, aspirin, ampicillin, and ibuprofen. The tested concentration of 1.0, 10.0 and 100.0 mg/L in artificial urine, was tested in duplicate by using the lateral flow immunoassay. Methamphetamine 1.0 mg/L and the artificial urine with zero drug was tested as the positive and negative control, respectively.

Those cross reactants that created the equal or less red color intensity of test line comparing to the positive control was considered as positive (+) while the negative (-) result indicated by the higher intensity of red test line.