CHAPTER I

INTRODUCTION

Immunoassay is the analytical method that analyte as the hapten is indirectedly determined by relating to the label on the antigen or antibody. The immuno-analytical method is based on the competitive reaction between the analyte and the label in binding to the specific antibodies. It is the label that is analyzed. Therefore the method of label analysis is based on the types of label for example radioisotopes, enzymes, chemiluminescent molecules, colloidal gold and fluorescent molecules.

Antibody is crucial for immunoassays as it could direct the sensitivity and specificity of the methods. Three types of antibody involved in immunoassay method are polyclonal antibodies, monoclonal antibodies and recombinant antibodies (Wild, 1994).

Polyclonal antibodies (PAbs) are frequently obtained from the sera of immunized animals and comprised of a mixture of antibody populations. Meanwhile, monoclonal antibodies (MAbs) consist of a single mono-specific antibody population. MAbs are produced in cell culture by a single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells. The hybridoma cells can be propagated almost indefinitely in culture medium and generated the unlimited production of MAbs with constant characteristics. For recombinant antibodies (RAbs), the fragments of cloned antibodies are manipulated to be produced in a variety of organisms such as *E. coli*, baculovirus, yeast and human cells. The recombinant antibody technology is meaningful in therapeutic applications, i.e., ovarian, colorectal and testicular cancer. For general commercial immunoassays, only polyclonal or monoclonal antibodies are involved. However, the selection of a good MAbs is still expensive and time consuming; PAbs are therefore more widely used in immunoassays.

The main difference between monoclonal and polyclonal antibodies is the specificity of antibody in binding to specific site of analyte. Most polyclonal antibody

contains more than one specific binding sites thus binding to various antigens with similar chemical structure. The term cross-reaction is defined to indicate this specificity. The cross-reactivity could be observed in both monoclonal and polyclonal antibodies. Nevertheless, the presence of cross-reaction in monoclonal antibody could be designed in the cloning step that identical copies of a same antibody molecule with the same affinity and cross-reactivity are magnified (Crowther, 1995). For PAbs, it is hardly to control this cross-reactivity, since different lot of antibody induction in the same animal could exert different cross-reactivity. Therefore, cross-reactivity problem is the other concern in replacing MAbs with PAbs. Besides, the other proposal to overcome the non-specificity of PAbs in binding to other related substances, is to use heterology principle in the analysis.

Heterology, the difference in chemical structural between the hapten-protein conjugate used for immunization and enzyme conjugated derivative, is often incorporated into the design of competitive immunoassay (Van Weemen and Schuurs, 1975; Hosoda, Kawamura and Nambara, 1981; Yamamoto et al., 1985; Piran, Riordan and Silbert, 1990; Rose, Kamps-Holtzapple and Stanker, 1995; Kim et al., 2003; Holthues et al., 2005). Heterology is used as a means for reducing the enhanced binding affinity of the antibody-conjugate interaction.

Three types of heterology have been reported (Van Weemen and Schuurs, 1975; Piran et al., 1990; Khosravi, and Papanastasiou-Diamandi, 1993). They are bridge, site, and hapten heterology (Van Weemen and Schuurs, 1975; Piran et al., 1990).

Bridge, site and hapten heterologous combinations are named according to the difference in the chemical structure of the haptens in term of cross-linker (bridge) or position of the same linkage (site) or related hapten (hapten). These three heterology could also be combined such as bridge-hapten heterology (Van Weemen and Schuurs, 1975). It is named as multiple heterology. Although heterology have been incorporated into various immunoassays for either sensitivity or specificity improvement. No absolutely conclusion could be made in the pattern of heterology for any individual reaction. The

appropriate heterology for any immunoassay can only be obtained from the experiment for each compound (Hosada et al., 1980; Hosoda, Kobayashi and Nambara, 1983, Hosoda, Kobayashi, Kawamoto, 1983).

The principle of heterology has been applied to various immunoassays utilizing polyclonal or monoclonal antibodies; for example, radioimmunoassay (Khosravi and Papanastasiou, 1993; Piran, Silbert-Shostek and Barlow, 1993), nephelometric immunoassays (Nishikawa, Saito, and Kubo, 1984a, 1984b; chemiluminescent immunoassay (Sato et al., 1996), surface plasmon resonance-based immunoassay (Wu et al., 2002), and enzyme immunoassay (Van Weemen and Schuurs, 1975; Goodrow, Harrison and Hammock, 1990; Wortberg et al., 1995, 1996; Bruun et al., 2001; Kim et al., 2003).

With polyclonal antibody, it has been proven that the different type of heterology could effectively enhance the sensitivity of the assay (Van Weemen and Schuurs, 1975; Hosoda, Kawamura and Nambara, 1981; Yamamoto et al., 1985; Piran, Riordan and Silbert, 1990; Rose, Kamps-Holtzapple and Stanker, 1995; Kim et al., 2003; Holthues et al., 2005) with different degree of specificity (Van Weemen and Schuurs, 1975; Goodrow and Hammock, 1998; Loomans et al., 2003). The sensitivity of monoclonal antibody-based assays could be influenced by selecting haptens for which the affinity of the antibody is lower than for the analyte (Harison et al., 1991; Goodrow et al., 1995, 1998; Holthues, et al., 2005). For monoclonal antibodies, the specificity of heterogenous hapten group is dependent upon the structural design of hapten (Danilova, 1994; Abad, A., and Montoya, A. 1994, 1998; Manclus, Primo and Montoya, 1996; Goodrow et al., 1998; Bruun, et al., 2001).

Since 1995, P. Thongnopnua, et al. at Chulalongkorn University have applied the principle of heterology to solid-phase enzyme immunoassay of theophylline. It was indicated that the sensitivity and specificity of theophylline assay could be enhanced by bridge heterology (P. Thongnopnua, T. Hongratanaworakit and V. Lipipun, 1995; P. Thongnopnua, S. Pichayawasin and S. Vangveravong, 1998). With this heterologous

combinations, the cross-reaction of caffeine to polyclonal antibody of theophylline was reduced to less than 0.1% (P. Thongnopnua et al., 1998). The heterology principle for immuno-detection of amphetamine, methamphetamine and ephedrine have been explored since 1999 (P. Thongnopnua and W. Matapatara, 2000; P. Thongnopnua and A. Sirikatitham, 2004; W. Matapatara and P. Thongnopnua, 2003). By inducing the polyclonal antibody against amphetamine, methamphetamine and ephedrine, three patterns of heterology (bridge, hapten and bridge-hapten) were experimented. It was found that the patterns of immuno-detection of amphetamine, methamphetamine and/or ephedrine could be affected by heterologous immunoassay. It would be possible to manage the detection of these compounds by selecting the appropriate heterologous combinations of the assay.

Amphetamine and methamphetamine have been known as the major drug- abuse problem of Thailand for a long time. To confirm the illegal use of suspects, the result of specimen examination is one of the vital support. This requires the efficient and usable screening test of these compounds. Presently, the imported immuno-test kit is still needed for this process. It is therefore the strong intention of this study to investigate the manageable of heterologous immunoassay from polyclonal antibody to monoclonal antibody and then transferred to non-instrument membrane immunoassay. Ephedrine was also included in this study with two reasons. First, it shares the main phenethylamine structure as amphetamine and methamphetamine. Second, ephedrine is found contaminated in amphetamine or methamphetamine raw material either intentionally or un-intentionally in the synthesis process (D'Nicuola, 1992; Andrews, 1995; Lekskulchai and Kaewpongsri, 2004; Stout, Klette and Horn, 2004; Urine testing, 2006).

The objective of the study

1. To determine the effect of heterology on the sensitivity and selectivity for the detection of amphetamine, methamphetamine and ephedrine.
2. To determine the effect of heterology on polyclonal and monoclonal antibody in the detection of amphetamine, methamphetamine and ephedrine.
3. To determine the effect of heterology on membrane immuno-detection of amphetamine and/or methamphetamine and/or ephedrine.

The significance of the study

1. The sensitivity and selectivity of immuno-detection for amphetamine, methamphetamine and ephedrine could be enhanced via proper heterologous combinations.
2. The experience gained could possibly be applied to other drug analyse via immunoassays.

Theoretical background

Polyclonal antibody

Polyclonal antibodies (PAbs) are antibodies with different affinities and specificities from different group of cells, called polyclone cell. PAbs are mostly induced in animal such as rabbit, goat, sheep, etc. The sera containing antibodies obtained could usually recognize hapten containing similar chemical structure, limiting its specificity.

Monoclonal antibody

Monoclonal antibodies (MAbs) are the identical copies of antibody produced from the clone of a single cell, called monoclone cell. To obtain MAbs for any compound, the B lymphocyte cells from spleen of immunized mouse or rat have to be hybridized with the myeloma cells. Only hybrids that can produce the antibody would be selected and cultured for further mass production of antibody with constant affinity and cross-reactivities.

MAbs could be produced by the hybridoma technique as the following procedure. The initial step is the immunization of the desired immunogens to mouse or rat. The specific antibodies of mouse are screened and selected for the fusion procedure. Prior to fusion step, the spleen cell from the immunized mouse and myeloma cell are prepared. For hybridization, in general, one spleen is fused in each time. Spleen cells from the immunized mouse are fused with the previously prepared myeloma cells by using polyethylene glycol as the fusion reagent, a substance that causes cell membrane to fuse.

Then a number of antibodies for specific binding characteristics are screened, and when the desired antibody is found, the cells are cloned for producing more identical antibody. Expansion of antibodies from monoclone have to be grown by either in vivo, or mouse ascites method such that the selected antibody would be peritoneal injected, or

by in vitro tissue culture. Further processing of the mouse ascitic fluid and of the tissue culture supernatant might be required to obtain MAbs with the required purity and concentration. Each batch of the monoclone should contain the same affinity characteristics. The disadvantage of monoclonal antibodies is the high cost of isolating the right monoclone and cloning the corresponding cell line.

Various alternative chemical fusing agents for mammalian cells have been sought in the past. Simple chemical such as lysolecithin and polyethylene glycol (PEG) were found to be effective (Pontecorvo, 1975). PEG, presently widely used, is the standard fusing agent and used in this study because it is relatively nontoxic yet yields a high incidence of cell fusion (Steimer and Boettiger, 1977). Normally PEG of molecular weight 1500-4000 is used in the concentration of 40% or 50% (w/v). In addition for successful fusion, the temperature during fusion process should be maintained between 20 to 37°C.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is immunochemical methods using an antigen-antibody interaction for determination of substances such as drugs, peptides, proteins, antibodies and hormones. Either antibodies or antigen are passively adsorbed to the solid phase. The competitive reaction of non-label and labeling enzyme occur on the solid-phase. It is the enzyme-substrate reaction that is determined and calculated back to the amount of analyte. As shown in Figure 1, four methods of ELISA can be performed (Crowther, 1995).

1. Direct method: antibody or antigen attached to the surface is reacted directly with the enzyme labeled antigen or antibody.

2. Indirect method: the conjugated antigen is adsorbed to the surface and reacted to specific antibodies before adding the secondary enzyme labeled antibody.

3. Sandwich method: the antigen being assayed is held between two different antibodies, antibodies specific to analyte is attached to the solid phase. The detecting

antibodies are labeled with enzyme. Therefore, this antigen must have at least two different antigenic sites.

4. Competition: competition assays imply that two reactants are try to bind to antibody with the simultaneous addition of the two competitors.

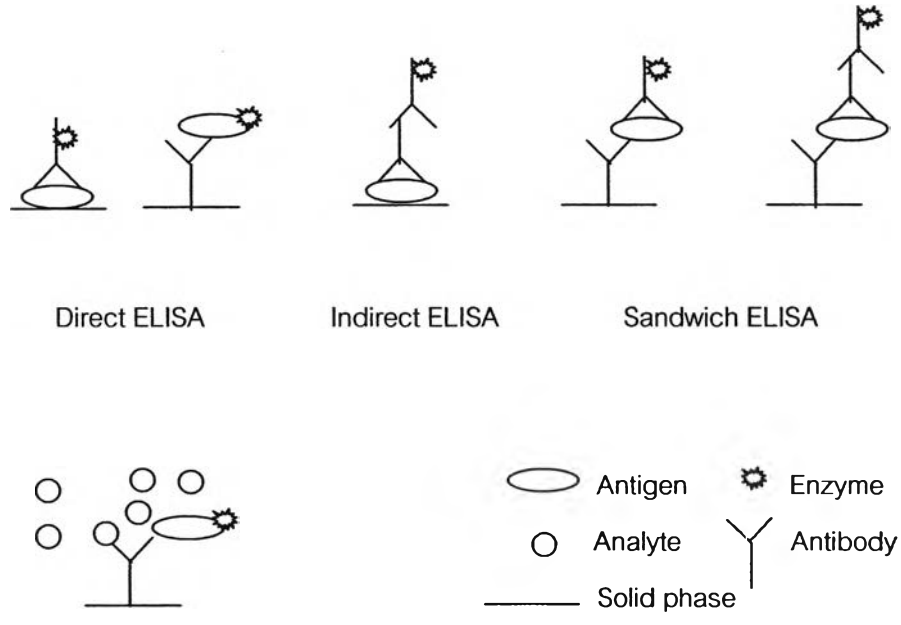

Figure 1 Enzyme-linked immunosorbent assay (ELISA)

Amphetamine and methamphetamine immunoassay

Instrumental immunoassay

Many commercial available kits for amphetamines immunoassays have been reported. One of the kits is manufactured by Syva Laboratories using Enzyme Multiplied Immunoassay Technique (EMIT) (Verstraete and Heyden, 2005). The EMIT Polyclonal kits used for detection of methamphetamine, could be cross-reacted with ephedrine (D'Nicuola et al., 1992). Other kits employ technique such as cloned enzyme donor immunoassay (Loor et al., 2000; Jeon, Yang and Andrade, 2004), the enzyme-linked immunosorbent assay (ELISA).

The other techniques have been invented such as Abuscreen Online® Radioimmunoassay (Ward et al., 1994), Coat-A-Count radioimmunoassay (Cody, 1990), ONTRAK® (Latex agglutination inhibition reaction tests) (Aoki and Kuroiwa, 1985), Roche Abuscreen online KIMS (Kinetic Interaction of Microparticles in Solution) (Verstraete and Heyden, 2005), and ADx and AxSYM (Fluorescence Polarization Immunoassay) (Poklis and Moore, 1995).

Non-instrumental immunoassay

There are a variety of commercials ready test-kits; One Step AMP Test Kit (Medimplex United Inc.,), QuickScreen One-Step Rapid Amphetamine Test (Craig Medical Distribution Inc.) and QuikPac II OneStep Amphetamine/Methamphetamine Test (Syntron Bioresearch, Inc.).

The Mandatory Guidelines for Federal Workplace Drug Testing Programs, proposed by NIDA-The National Institute on Drug Abuse and The Substance Abuse and Mental Health Services Administration (SAMHSA), require a laboratory to conduct two analytical tests before a urine specimen can be reported positive for a drug of abuse.

The first is the initial screening test using a single test kit and the second test is the confirmatory test utilizing gas chromatography/mass spectrometry (GC/MS), respectively (Jenkin, 1999).

Ephedrine immunoassay

Ephedrine is a sympathomimetic amine that mimics the stimulant action of amphetamine. Ephedrine has been found as the precursor material in process of amphetamines synthesis. Ephedrine is therefore classified in the Psychotropic Substances class 2 in Thailand since 1996 (สาธารณสุข, 2539).

The reported immunoassays for ephedrine are the radioimmunoassay and ELISA (Midha et al., 1983; Miagkova, Lushnikova and Polevaia, 1990). At Chulalongkorn University, the immunoassay for ephedrine either with ELISA or gold-labeled have also be developed (Thongnopnua and Sirikatitham, 2004). There is no available test kit for ephedrine detection.

Membrane immunoassay

Immunoassay on porous membranes can be generally divided into two groups, protein transfer and dot blots. Protein transfer refers to immunoblot assays which involved the transfer of electrophoretically separated protein from a gel to an immobilizing matrix. In dot blot assay the protein sample is applied directly to the membrane.

Membrane immunoassay or immunochromatographic tests are available in two formats; lateral flow, and flow through or transverse flow. The lateral flow format is used for the rapid detection of pregnancy, drug abuse, infectious disease or parasitology. It is commercially available as test device or dipstick kit. The flow through format is less common due to greater operator involvement.

Various membrane matrices could be used in membrane immunoassay. Nitrocellulose is one of the most commonly used. It is available as the pure cellulose nitrate ester and as cellulose nitrate-cellulose acetate mixed ester. Cationic nylon membrane, uncharged nylon 66 membrane, polyvinylidene fluoride (PVDF) membrane and modified polyethersulfone (PES) membrane are also applicable. One of the positive features of using membrane matrices in solid-phase immunoassay is their high protein-binding capacity compared to surface as polystyrene which is used in ELISA.

Currently, the lateral-flow immunoassay is available in rapid test and developed to allow for single-step assays that require only the addition of a sample. In these assays, the sample is added to one end of the device and flows by capillary action through the membrane in the device. Continuing along this flow path, the sample contacts gold-labeled antibody which then migrates with the analyte to a capture zone of hapten conjugate immobilized on the membrane. After the competitive reaction between analyte and hapten conjugate to bind with the gold-labeled antibody, un-reacted gold-labeled antibody continues to flow past this capture zone, normally to an end-of-assay indicator. Generally, absorbent material at the distal end of the membrane helps draw the sample through the test membrane. The result shows in the capture zone, no red line if the analyte is present in the sample, in contrast to the absence of analyte that red line can observe.

Factors influencing protein binding on the membrane including; ionic strength, acidity, co-precipitating agent, and ambient humidity. Nonetheless, the type of membrane selected could have significant effect on the protein binding.

Binding Mechanisms of gold labeled

Immunogold conjugation is a technique that protein, including antibodies and antigens, can be coupled to gold colloid to produce an immunogold complex. Although a variety of applications for such immunogold conjugates are still emerging, the current

primary applications are in rapid-test devices used for the diagnosis and monitoring of disease.

Mechanisms of immunogold conjugation relate to three particular amino acids of protein i.e. lysine, tryptophan, and cysteine. Lysine is highly positively charged and therefore naturally attracted to a negatively charged gold particle. Tryptophan works through hydrophobic interactions. Cysteine creates dative bonds through the formation of sulphur bridges with the gold surface, such that the antibody and gold particles could share electrons. With all the forces controlling the attachment of antibodies to gold particles, the conjugation is strong, permanent, and difficult to break.

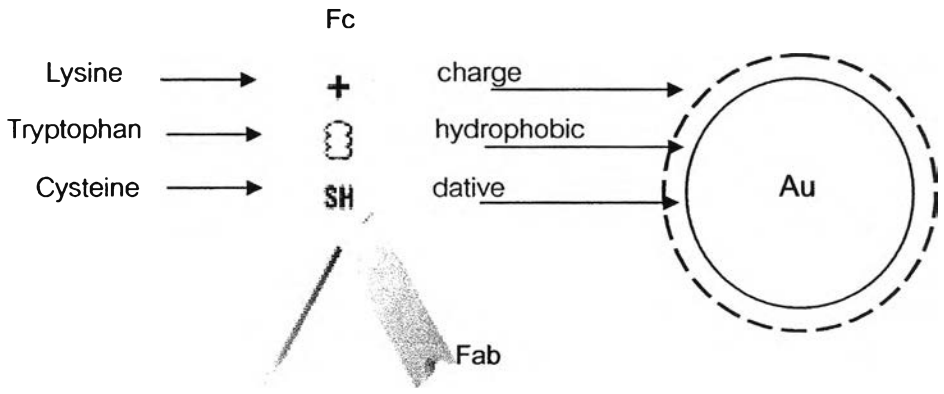

Figure 2 The charge distribution on surface of gold particles and the binding mechanism of gold colloid to antibody operates on three amino acids; lysine, tryptophan and cysteine.