IMMUNOAFFINITY SOLID PHASE MICROEXTRACTION

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my advisory committee.

I understand that my thesis may be made electronically available to the public.

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Abstract

Biological fluids are commonly analyzed in clinical and forensic studies for drug concentration measurements. Conventional quantification procedures are always associated with lengthy sample pretreatment steps to eliminate the interfering compounds that potentially exist in complex biological matrices. The objective of this study was to address these problems employing solid-phase microextraction (SPME) technique. Antibodies (Abs) were employed to serve as an extremely specific extraction phase for direct extraction of analytes from untreated biological matrices based on their exquisite selectivity for antigens (Ags).

Much of the research was focused on selecting the most appropriate antibody (Ab) for a particular application based on evaluation of characteristics of various types of Abs obtained from four suppliers. Abs' binding characteristics were evaluated before and after immobilization in terms of affinity, valence, homogeneity, capacity and cross-reactivity for three benzodiazepines. The performance of immunoaffinity probes of the same type provided by different suppliers was found to be comparable. Finally, the probes' utility for extraction of benzodiazepines from plasma samples was evaluated.

The limit of detection of the method developed in this work was 0.01 ng/mL with upper limits of quantification of 0.5 ng/mL in buffer and 2 ng/mL in plasma. The method's precision was 12% for extraction from buffer and less than 10% for extraction from plasma. With limits of detection similar to the current state-of-the-art methods available for quantification of drugs in biological matrices, the method presented in this thesis was found advantageous compared to other available methods due to its simplified sample preparation procedure.

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List of Abbreviations

Ab	Antibody
Abs	Antibodies
Ag	Antigen
APTES	Aminopropyltri(ethoxy)silane
Ags	Antigens
BGG	Bovine γ-globulin
BSA	Bovine serum albumin
CW/TPR	Carbowax/ templated resin
DADPA	Diamonidipropyl amine
ELISA	Enzyme-linked immunosorbent assay
GA	Glutaraldehyde
GC	Gas chromatography
GnHCl	Guanidine Hydrochloride
HPIAC	High performance immunoaffinity chromatography
HPLC	High performance liquid chromatography
Hs	Haptens
IAC	Immunoaffinity chromatography
IgG	Immunoglobulin G
Igs	Immunoglobulins
ISs	Immunosorbents
KLH	Keyhole limpet hemocyanin

LC	Liquid chromatography
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
MAb	Monoclonal antibody
MAbs	Monoclonal antibodies
MS	Mass spectrometry
MWCO	Molecular weight cut-off
PAb	Polyclonal antibody
PAbs	Polyclonal antibodies
PBS	Phosphate buffer saline
PPY	Polypyrrole
RSD	Relative standard deviation
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
ULOQ	Upper limit of quantification

Chapter 1 Introduction

Biological fluids are frequently analyzed in pharmaceutical studies for drug concentration measurements. These measurements are often used in clinical medicine to ensure appropriate therapeutic levels of drugs while minimizing the occurrence of toxicity.

Biological samples are considered complex matrices due to the presence of a variety of species such as proteins, salts and organic compounds. These compounds, which often exist at higher concentrations than analytes, can interfere with analyte concentration measurements. Therefore, determination of drug levels in biological samples, such as plasma or urine, cannot be performed directly.

Elimination of interfering compounds from the matrix is an inevitable step in the course of analyzing biological fluids and is commonly performed in a series of steps, referred to as sample preparation. Preparation of a pure, concentrated sample requires several steps during which the risks of operator error, sample loss and/or sample contamination increase with each additional step. As a result, in drug quantification procedures, a sample preparation method with a minimal number of steps is aimed for extraction and isolation of the analyte of interest.

Solid-phase microextraction (SPME), invented in the late 1980s by Belardi and Pawliszyn[1], has been exploited in a wide range of applications as a powerful sampling and sample preparation technique.[2] While sample preparation is generally laborious and time consuming, SPME integrates extraction, concentration and clean-up into a single, solvent-free

step with high sensitivity.[3,4] Therefore the present work focused on exploiting the SPME technique for accurate quantification of drugs in biological samples to avoid the time and labor intensive sample preparation steps frequently conducted in conventional pharmaceutical measurement studies.

1.1 Drug analysis by SPME

The SPME technique invovles exposing a small amount of extraction phase, coated onto a solid support, to the sample matrix for a defined period of time. Partitioning of analyte molecules onto the extraction phase occurs during the extraction time, following which the SPME device is removed from the sample. The extracted analytes are then desorbed from the extraction phase and introduced to an analytical instrument for separation, identification and quantification.[4] Quantification in SPME is based on the fact that the amount of extracted analyte is proportional to the initial concentration of analyte in the sample matrix.

SPME was primarily developed for extraction of volatile organic compounds from environmental samples. Consequently, commercially available SPME extraction phases are generally suitable for extraction of volatile compounds with relatively low polarity, which are typically analyzed using gas chromatography (GC).[5] Analysis of pharmaceuticals in biological matrices, on the other hand, is often performed using liquid chromatography (LC) due to the analytes' higher polarity, lower volatility and in some cases, thermal instability. The only commercialized extraction phase that is appropriate for LC applications is Carbowax/templated resin (CW/TPR), which is not always suitable for drug quantification in biological matrices.[5] Yet, commercial SPME fibers have been used in several studies mostly in conjunction with GC devices for drug analysis. [6-14]

Commercial SPME fibers have been employed extensively for drug analysis in toxicological cases of accidental or suicidal drug overdoses, rather than therapeutic drug monitoring studies.[10] This is due to the limit of quantification associated with commercial

SPME fibers which is often higher than therapeutic levels of drugs. In one approach, benzodiazepines were analyzed in blood with high sensitivity using a polypyrrole (PPY) extraction phase, which had lower detection limits than analyte concentration.[5] However, many drugs are commonly present at much lower concentrations than benzodiazepines. Thus, the search for extraction phases with higher sensitivity continues.

Furthermore, a minimum of one sample preparation step was shown to be essential in almost all drug analysis studies where currently available SPME fibers were used.[6-14] In the case of extraction from plasma samples in particular, deproteinization was found to be essential prior to extraction to avoid recoveries as low as 3% of the total drug concentration in plasma.[9]

Nevertheless, SPME is chosen over other conventional sample preparation methods due to its potential to eliminate all sample pretreatment steps by combining several steps into one step. Therefore, the aim of the present research was to design a SPME probe for direct extraction of drugs from biological matrices.

Adsorptive extraction phases have generally been employed in drug analysis. The amount of drug extracted is directly dependent on the affinity of the analytes to the adsorption sites as well as the amount of sorbent, and the analyte's concentration in the sample. Thus, selection of an appropriate extraction phase is the primary step in designing an SPME probe for any particular application.

As mentioned previously, none of the commercially available SPME probes are ideally suited for specific quantification of pharmaceuticals from complex matrices. The extraction phases developed for this purpose, such as PPY,[5] are general sorbents for which the affinity

coefficients of a broad range of compounds are generally understood to differ by less than three orders of magnitude.[15] Employing such sorbents, that extract a wide range of analytes with moderate affinities, is associated with co-extraction of competing compounds. Indeed, when analyzing real samples where trace levels of analytes exist along with interfering compounds at considerably higher concentrations, the issue of co-extraction becomes a major problem. Due to the fixed extraction capacity of the fibers, the compounds with lower affinities and higher concentrations compete with the analytes of interest for the sites on the extraction phase. In such cases, displacement of analytes by interfering compounds is likely to occur. This problem can be avoided by exploiting highly selective sorbents to extract a few structurally similar drugs with high affinity. Antibodies (Abs) have been chosen as sorbents in this work due to their exquisite selectivity and high affinity for the respective antigens (Ags).

1.2 Antibodies

The primary step in developing Ab-based sorbents is identification of Abs that can recognize one or a group of target analytes. For each application, the proper Ab is selected based on its characteristics, which determine the sensitivity and selectivity of the sorbent.

1.2.1 Antibody structure and classification

Abs, also referred to as immunoglobulins (Igs), are glycoproteins that belong to the "globulin" family of proteins in serum. The immune system of living organisms produces Abs in response to invasion by foreign molecules. Foreign substances capable of evoking such immune response are called immunogens. The presence of an immunogen activates a chain of reactions that ultimately triggers production of Abs by white blood cells (lymphocytes). The terms "antigen" and "immunogen" are often used interchangeably; however, it should be noted that, not all antigens can evoke an immune response.[16]

Although Abs carryout various functions in the immune response, all Abs exist as Y-shaped units consisting of four chains of polypeptides. The basic structure of each Y-shaped unit consists of two identical "heavy" chains (~ 50 kDa each) that are coupled with two identical "light" chains (~ 25kDa each) by a variable number of disulfide bonds. The resulting 150 kDa Ab has a central axis of symmetry that splits the Ab molecule into two identical halves.[16-18] The sequence of amino acids at the "tail" of the "Y" structure, the F_c fragment, determines the major class the Ab belongs to while the amino acid composition at the two tips of the "Y", the F_{ab} fragments, is responsible for its specific Ag-binding properties of Abs (Figure1).

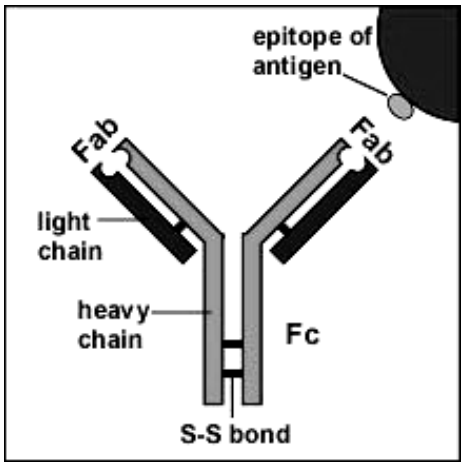


Figure 1: Structure of Immunoglobulin G showing F_{ab} and F_c fragments. Reference: Dr. G. E. Kaiser[19]

There are five major classes of Abs in higher mammals: IgA, IgD, IgE, IgG and IgM. These classes differ from each other in the number of Y units they have as well as the amino acid sequences in the heavy chain of their F_c fragments. In the development of immuno-based sample preparation techniques, Immunoglobulin G (IgG) is chosen to be used in almost all cases. This is primarily due to the fact that IgG is the most abundant class of Igs in serum following immunization; although other reasons include its stability during purification processes, its higher affinity for Ags as well as the availability of a wide range of commercial purification kits specifically for IgG. For all of these reasons, IgG was also chosen to be use in the present study.

Ags are also classified based on their binding properties. The sites of Ags and immunogens that interact with complementary sites on Abs are called epitopes (Figure1). Pharmaceuticals which are the target analyte in pharmaceutical studies each have a single epitope to bind to an Ab. Such Ags are also called haptens (Hs). Similar to other molecules with molecular weights smaller than 1000 Da, Hs are inherently unable to evoke an immune response. Thus, anti-hapten Abs are commonly produced by conjugating the desired hapten to

a carrier protein such as bovine serum albumin (BSA). To avoid confusion in the text, I have used Ag instead of H.

1.2.2 Antibody characteristics

In general, Abs are characterized based on three major qualities: specificity, cross-reactivity and affinity. Each one of these characteristics is explained in this section.

Specificity is defined as the ability of an Ab to bind only to the analyte of interest.[20] Since only a limited number of Abs can be immobilized on the sorbent support, specificity of the Ab batches that will be immobilized plays an important role in the development of immunoaffinity adsorbents. As mentioned previously, the chance of co-extraction of interfering compounds is significant when dealing with complex matrices. The possibility of co-extraction of other compounds increases if the Abs, which in this work, serve as the extraction phase, are not specific to the analyte.

Cross-reactivity is the tendency of Abs to bind to Ags other than target analyte(s). Although Abs have typically been employed in analytical techniques because of their unique specificity for their Ags, molecules with closely related structures (e.g. drug metabolites) are likely to cross-react. There is also a chance for cross-reaction with molecules that have dissimilar structures but are present in the sample at high concentrations. Cross-reactivity is often described as the amount of cross-reactant that displaces the target analyte by 50%. Some antibody-producing companies measure cross-reactivity in relation to relative affinity, meaning that 100% cross-reactivity is assigned to the Ag for which the Ab has the highest affinity and other Ags are assigned cross-reactivity percentages according to their relative activity.

Affinity of an Ab is defined as the association constant of the Ab complex with an Ag. Abs are known for identification of their specific Ags and subsequent formation of a stable complex with those Ags. The interaction of an Ab with its selective Ag is called the "primary interaction". This is a fairly rapid interaction that results in equilibrium between the free and the antibody-bound antigen.The general binding procedure is described by the following equations:

$$Ab + Ag \rightleftharpoons Ab - Ag \quad (1.1)$$

$$\text{Affinity} = K_a = [Ab - Ag] / [Ab][Ag] \quad (1.2)$$

where Ab-Ag is the antibody-antigen complex and K_a, generally referred to as "affinity" of an antibody for a specific antigen, is the association constant for binding of the Ab with the Ag. The square brackets correspond to molar concentration or surface concentration of each species at equilibrium.

The binding forces involved in the interaction of Ab and Ag include hydrogen bonding and hydrophobic forces along with coulombic and van der Waals interactions. Although these forces are relatively weak, complementarity of Ag and Ab binding sites takes place at a relatively large area, leading to rather large values of affinity (10^{8}- 10^{12} M^{-1}).[16, 21] Such large values of the association constant leads to the conclusion that the Ab-Ag complex is formed even when both Ab and Ag are present at very low concentrations. It is also expected that the dissociation of the Ab-Ag complex can only be achieved under extreme conditions.

1.2.3 Evaluation of affinity in solution

The evaluation of binding properties between proteins and small molecules was initially conducted by George Scatchard in 1949.[22] Consequently, current evaluation of an Ab's affinity for an Ag is commonly performed by Scatchard analysis. This method is a thermodynamic approach that is applied to the equilibrium between Ab and Ag in solution.

Experimental evaluation of Ab affinity is performed by incubating the Ab at constant concentration with an Ag at varying concentrations. To estimate Ab affinity, two assumptions are made: first, that both reactants, Ab and Ag, have to be pure and in solution and second, that the reactants should be homogenous with regard to the Ag's epitopes and the Ab's complementary binding sites.[23] Both assumptions can be satisfied for the Ag if it is a hapten. However, as discussed in the next section, Abs that are naturally produced in serum are heterogeneous both in structure and affinity. Purification of Abs helps in getting closer to the ideal situation.

Estimation of affinity is performed based on Equation 1.2. In this equation, K is to be identified. However, all other components in this equation are also unknown. To solve the equation for K, equation 1.3 is employed:

$$[Ab] = (Ab)_t - [Ab\text{-}Ag] \qquad (1.3)$$

where $[Ab]$ is the concentration of free Ab sites, $[Ab\text{-}Ag]$ represents the concentration of bound Ag or occupied Ab binding sites at equilibrium and $(Ab)_t$ represents the total concentration of Ab binding sites in the sample.

Equation 1.4 is used to determine $(Ab)_t$ as follows:

$$(Ab)_t = n\,(Ab) \tag{1.4}$$

where n represents Ab valence, defined as the number of binding sites for each Ab molecule, and (Ab) represents molar concentration of total Ab in sample. In experimental determination of antibody affinity, the total concentration of antibody shown as (Ab) is known.

Substituting Equation 1.3 and Equation 1.4 in Equation 1.2 results in the following equation:

$$K = \frac{[Ab-Ag]}{(n(Ab)-[Ab-Ag])[Ag]} \tag{1.5}$$

which is changed into Equation 1.6 following rearrangement:

$$\frac{K}{1+K[Ag]} = \frac{[Ab-Ag]}{n(Ab)[Ag]} \tag{1.6}$$

Solving Equation 1.5 for [Ag] and substituting it in the left side of Equation 1.6 leads to Equation 1.7 after rearrangements:

$$K - \frac{K[Ab-Ag]}{n(Ab)} = \frac{[Ab-Ag]}{n(Ab)[Ag]} \tag{1.7}$$

The term $\frac{[Ab-Ag]}{(Ab)}$ is the number of moles of Ag bound per mole of Ab. To simplify Equation 1.7, this term is shown by "r" in Equation 1.8:

$$\frac{r}{[Ag]} = -rK + nK \tag{1.8}$$ [23]

Equation 1.8 is the general Scatchard equation for an *n*-valent Ab. As can be seen from the Scatchard equation, in an ideal situation where both assumptions are true, a plot of $\frac{r}{[Ag]}$ versus *r* results in a line with a negative slope (K), which is the affinity of the Ab, and an x-intercept (n) which is the Ab's valence (Figure 2).

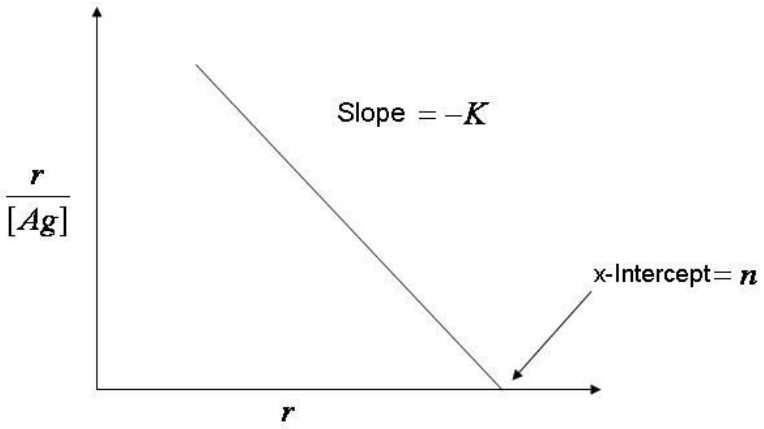


Figure 2: Scatchard plot of ideal antibody-antigen reaction

For an anti-hapten Ab with 2 binding sites per Ab molecule (such as IgG whose valence is 2), the Scatchard equation can be employed to calculate the affinity at the time when half of the Ab sites are bound ($r = 1$). Substituting the values for *n* and *r* in Scatchard equation ($n = 2$ and $r = 1$), the value of *K* is determined to be equal to $\frac{1}{[Ag]}$.

1.2.4 Evaluation of affinity on solid surfaces

The affinity of immobilized Abs is not estimated with Scatchard analysis. Since the number of immobilized Abs is limited by the space available on the immobilization surface, other techniques must be employed to accurately estimate Ab affinity.

As Ab-coated surfaces have defined sites to which Ags can be bound, it is expected that an Ab-coated surface acts in an adsorptive fashion. Therefore, higher concentrations of Ag lead to saturation of the Ab-coated surface. For this reason, an adsorption isotherm, which relates the concentration of Ab-Ag at equilibrium to the concentration of Ag in the sample, can be used to model the binding of an Ag to an Ab-coated surface.

The Langmuir Adsorption Isotherm has been commonly used to model such binding. Equation 1.6 is a form of Langmuir Adsorption Isotherm. This equation is a hyperbola, which is the typical saturation function (Figure 3). At low Ag concentrations, more Ag is bound when more is added to the sample. At high concentrations of Ag, saturation occurs as [Ab-Ag] approaches (Ab).

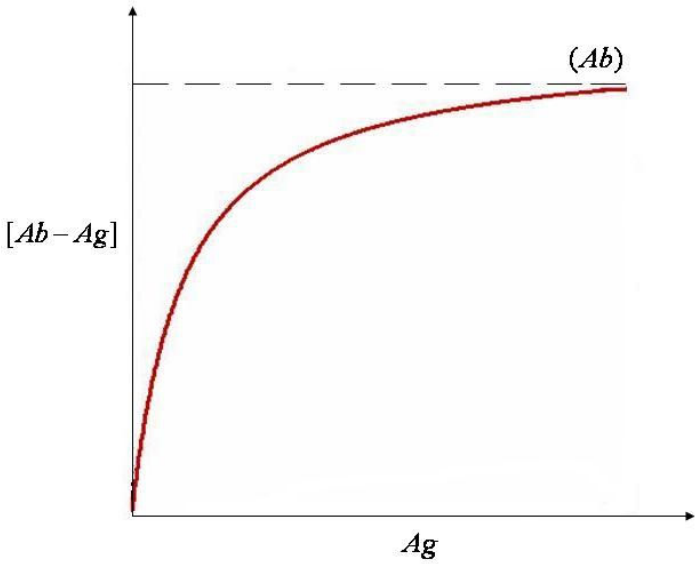


Figure 3: Graphical demonstration of general binding curve.

Since estimation of affinity from a non-linear function is complicated, the reciprocal of the Langmuir Adsorption Isotherm, which is a linear transform of the binding isotherm, has

typically been used to estimate Ab affinity. To achieve the linear transform, Equation 1.6 is rearranged to:

$$\frac{n(Ab)K[Ag]}{1+K[Ag]} = [Ab-Ag] \tag{1.9}$$

where $n(Ab)$ is equal to total binding sites of Ab immobilized on the surface. The maximum amount of Ab's active sites is the capacity of the adsorption surface and is shown with b in the text. Replacing $n(Ab)$ with b in Equation 1.9, the reciprocal of Equation 1.9 is as follows:

$$\frac{1}{[Ab-Ag]} = \frac{1}{bK}\cdot\frac{1}{[Ag]} + \frac{1}{b} \tag{1.10}$$ [23]

where K represents Ab affinity and n represents the capacity of the adsorption surface, defined as the maximum amount of Ab's active sites.

As can be seen from Equation 1.10, both affinity and capacity of the immobilized Ab can be obtained from the reciprocal Langmuir plot (Figure 4).

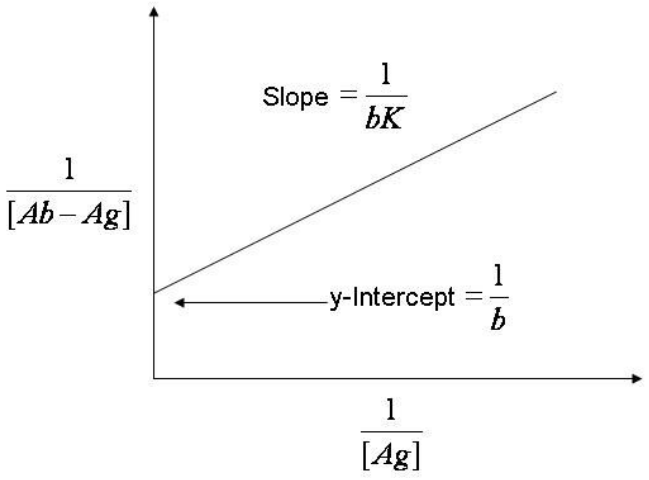


Figure 4: Reciprocal Langmuir plot of ideal antibody-antigen reaction

Similar to the Scatchard plot, the reciprocal Langmuir plot is obtained based on assumptions for homogeneity and purity of Abs. Due to the fact that Ab heterogeneity

increases upon immobilization, the reciprocal Langmuir plot often deviates from linearity. The deviation exists even when purified Abs are employed. This is due to the presence of range of affinities within the population of naturally produced Abs. The distribution of affinities in an Ab population has been described in terms of Sips equation (Equation 1.11).

$$[Ab-Ag]=\frac{b(K[Ag])^{a}}{1+(K[Ag])^{a}} \tag{1.11}$$

The Sips equation has been developed based on a model in which the Gaussian distribution function describes the distribution of Ab affinities. In this model, K is the average affinity and a, which is related to the width of the affinity distribution, is the heterogeneity index. Based on Sips equation, when a is equal to 1, a single value for K is obtained, demonstrating that Abs are homogeneous. The more heterogeneous the Ab population is, the smaller the value of a will be, which results in a wider distribution of affinities.

The Sips equation is frequently used in logarithmic form to produce a linear equation. The logarithmic form of the Sips equation is obtained by rearrangement of Equation 1.11 as follows:

$$[Ab-Ag]+[Ab-Ag](K[Ag])^{a}=b(K[Ag])^{a} \tag{1.12}$$

which can be rearranged further to:

$$\frac{[Ab-Ag]}{b-[Ab-Ag]}=(K[Ag])^{a} \tag{1.13}$$

The logarithmic form of the Sips equation is therefore obtained as:

$$\log\frac{[Ab-Ag]}{b-[Ab-Ag]}=a\log[Ag]+\log K^{a} \tag{1.15}$$ [23]

When $\log\frac{[Ab-Ag]}{b-[Ab-Ag]}$ is plotted versus $\log[Ag]$, a straight line with slope equal to the index of heterogeneity is obtained (Figure 5).

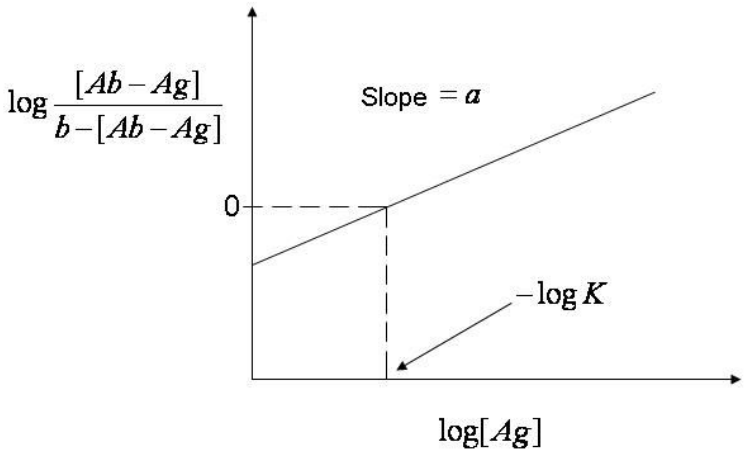


Figure 5: Logarithmic form of Sips plot

Similar to Scatchard analysis, for a divalent anti-hapten Ab, K can be determined from the logarithmic of the Sips equation at 50% Ab saturation. For anti-hapten IgG, when half of the Ab sites are bound, ($r = 1$), then $\log\frac{[Ab-Ag]}{b-[Ab-Ag]} = 0$. Thus, K is equal to $\frac{1}{[Ag]}$.

1.2.5 Antibody types

Two types of Ab have been produced to date: polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs). Although both types have been successfully employed in immunoaffinity sample preparation methods, there are advantages and disadvantages associated with each in terms of their binding characteristics, the procedures used for their preparation, and their applications. It has been found that the dissimilarity in their production processes is the root of the dissimilarity in their characteristics. However, both types have been successfully employed in immunoaffinity sample preparation methods. Since evaluation

of Ab characteristics is an essential part of developing any immunoaffinity method, the generation of both types of Ab is expressed in this section.

PAbs are the natural products of conventional immunization in living species. During the immunization process for commercial scale production of PAbs, a suspension of the specific immunogen is injected several times into a suitable animal, such as a sheep, horse, or goat.[18] Bleeding is performed at the peak of Ab production, which is determined experimentally. As mentioned before, drugs have to be bound to a carrier protein before being used as immunogens.

Upon immunization, a wide range of B-cell lymphocyte clones are activated which causes a series of reactions, ultimately resulting in the production of Abs by the B-cells.[24] Each immunogen has various epitopes against which Abs are produced. These Abs are secreted in the blood where they are mixed with the Abs produced against other Ags present in the organism.[16,24] The mixture of various Abs is called PAbs. As is apparent from the name, more than one clone of cells is responsible for the production of PAbs.

PAbs are a heterogeneous population of Abs with a range of affinities and specificities.[25] In fact, it has been reported that only 15% of polyclonal IgG is specific.[26] This is mainly due to the production of Abs against various epitopes and the presence of Abs against other Ags in the living system. However, Abs produced against a single epitope are still heterogeneous with regard to their affinity and specificity.[25]

Despite their heterogeneity, PAbs have been exploited in various research fields and clinical studies with great success.[25] Nevertheless, their usage is limited to assays for which

Abs with high specificity and consistent quality are not crucial. In cases where a homogeneous population of Abs with unique specificity is desired, MAbs are the better choice.

Since their introduction by Köhler and Milstein[27] in 1975, MAbs have been extensively employed in immunoaffinity assays. MAbs are produced *in vitro* from a single B-cell clone, which means they are a homogeneous population of Abs with desired specificity. The challenge of producing homogenous Abs from an isolated B-cell clone arises from the fact that B cells do not survive in cell culture for more than a few days of their isolation.[18, 28] Köhler and Milstein overcame the problem by fusing isolated B-cells with immortal cancerous cells.[27] Such B-cells, are able to generate identical Abs with equal affinity for a single epitope continuously.

The production procedure for MAbs begins with immunization of an animal, typically a mouse. This step follows the same principles governing PAb preparation.[18] The B-cells that are responsible for production of Abs are then isolated from the animal's spleen and fused to malignant plasma cells (also called myeloma cells). Hybrid cells, also known as "hybridomas", are produced upon culturing the fused cells.[16] The resulting hybridomas combine the properties of both B-cells and immortal malignant cells; such that they are capable of continuous production of consistent Abs for years while cultured in a tissue medium.[28] During screening, hybridomas that generate the specific Ab of interest are identified and isolated from the non-specific hybridomas. Various populations of fused B-cells that existed in the original clone are then separated and re-cloned. Following the re-cloning

step, the binding characteristics of the pre-selected hybridoma is evaluated. Once the desired hybridoma is identified, a MAb will be produced in large scale.[28]

1.2.6 Antibody selection

During the course of development of an assay based on Abs, the particular application of the assay determines the most suitable type of Ab. Several factors including the level of specificity and the degree of affinity desired for that application are to be considered when selecting a type of Ab. In this section, the two different types of Abs are compared with regard to different aspects that are generally considered in designing an assay.

As discussed previously, PAbs are heterogeneous, which means they consist of different classes of Igs. Therefore, lengthy purification procedures are required to isolate a specific class such as IgG which is the most favorable class in analytical assays.[29] Furthermore, due to its heterogeneity, a PAb mixture reacts with the epitopes on an Ag with a wide range of affinities.[29] To avoid production of a diverse population of irrelevant Abs, specific conditions need to be met with regard to their production. Indeed, aseptic areas must be available for housing immunized animals and, although they may not be accessible in some cases (e.g., for newly developed drugs), highly purified Ags must be used for immunization.[29]

The limited supply of PAbs is another disadvantage associated with them, making them irreproducible. Using different animals or bleeding the same animal at different times can result in significant batch to batch variation.[28] However, in spite of all their disadvantages, if the purified Ag is available, PAbs can be easily generated at relatively low cost.

The disadvantages of PAbs are eliminated for MAbs due to their production procedure. A batch of MAbs contains a specific Ab of a defined class (generally IgG) with high affinity towards a single epitope. Continuous culture of hybridomas allows unlimited production of consistent MAbs which are thus considered reproducible.[30] Furthermore, this procedure eliminates the need to use animals for bulk production. As a result, the number of animals used for MAb production is drastically reduced, in comparison with PAbs.[26] Finally, since a specific hybridoma is selected for production of a specific MAb based on its binding characteristics, highly purified Ags are not necessarily required for their production.[31]

There are also some disadvantages associated with MAbs. Chiefly, their production is laborious and time-consuming compared to PAbs, they are less available compared to the wide range of commercially available polyclonals and ultimately, their cost is considerably higher.

1.3 Immunoaffinity sample preparation techniques

Immuno-based sample preparation techniques are becoming increasingly popular in the analytical community. These techniques are based on molecular recognition and employ biological tools, such as Abs, for selective extraction of analytes. The interest in using Abs for sample preparation when dealing with complex matrices is due to their exquisite selectivity and high affinity for their respective Ags. Over the last decade, Abs have been utilized successfully in various forms including chromatography column packings,[32-35] solid phase extraction (SPE) sorbents [36, 37] and biosensors.[38, 39]

Immunoaffinity chromatography (IAC) is a term for any chromatographic method with Abs or Ab-related compounds bound to the stationary phase. The first reported use of IAC dates back to 1951 when Campbell et al. employed an immobilized Ag column for purification of an Ab.[40] Since its introduction, numerous IAC methods have been developed for separation of large molecules, such as antibodies, hormones, receptors and enzymes as well as smaller molecules including pesticides and drugs.[41-45]

In the IAC technique, Abs or Ab-related compounds that are suited for any particular application are immobilized to a support to serve as the stationary phase. Considerations for selection of Ab type and immobilization method is discussed in detail in sections 1.2.6 and 2.5, respectively. The two types of supports typically employed for IAC are low-performance supports, which can be used under gravity flow or slight vacuum, and higher efficiency supports, which can be exploited as high-performance liquid chromatography (HPLC) columns. Carbohydrate-related materials (e.g. agarose) or synthetic organic supports (e.g. acrylamide polymers) are generally low-performance supports. These materials are relatively

simple and inexpensive and have been used particularly in off-line immunoextraction methods. However, due to their slow rate of mass transfer and instability at high pressures and flow rates, more rigid materials such as derivatized silica are employed as supports for high-performance Immunoaffinity chromatography (HPIAC). These materials, which are mechanically stable, can be used with standard HPLC equipment, thus improving the speed and precision of analytical applications of IAC.

Once Abs are immobilized on the appropriate support, samples are applied to the IAC column. Ags are then allowed to bind to immobilized Abs over a certain period of time. During this time, equilibrium is established between the unbound molecules (Abs and Ags) and antibody antigen complex. Interfering compounds present in complex matrices are removed by performing a washing step after equilibration time which eliminates unbound compounds from the column.

As mentioned in the previous section, under physiological conditions, K_a is typically in the range of 10^8 to 10^{12} L/mol, which indicates very strong binding between analytes and immunoaffinity supports. Therefore, elution of analytes is performed by employing eluents that can change the IAC column conditions by decreasing the effective K_a value. An appropriate elution buffer is chosen based on its ability to elute the analyte as fast as possible without imposing any irreversible damage to the immobilized Ab. Buffers with extreme pH values, chaotropic agents (e.g. sodium iodide), denaturing agents (such as guanidinium) and organic modifiers, have been used as elution buffers. However, the proper elution buffer is determined for each case experimentally.

Following elution of analytes from the immunoaffinity column, the column is typically regenerated and stored in proper buffer for further use.

Immunoaffinity SPE is a form of IAC. The so-called immunosorbents (ISs) are prepared by linking Abs to a solid support, which is then packed into an SPE cartridge. Both low-performance and high-performance supports have been used in preparation of ISs. Following preparation of an immunoaffainity SPE cartridge, the same steps are followed as in the IAC.

In recent years, immunochemical methods have gained the interest of biosensor technology, which has resulted in the development of immunosensors. The additional advantage of immunosensors over other immuno-based sample preparation methods is that immunosensors combine specific extraction of analytes with direct detection. Since both isolation and detection of analytes happen at the surface of the sensor, the support for immobilization of Abs is different from the supports regularly used in immunoaffinity sample preparation methods. An immunosensor's support is chosen based on the method of detection and can be varied from synthetic polymers (such as polypyrrole) to inert metallic electrodes (such as gold electrodes).[38] While immunosensors have been successfully employed for separation and detection of analytes of interest in complex matrices, research continues, focusing on improving immobilization techniques for the sensors. [39]

The first attempt at the development of immunoaffinity solid phase microextraction was reported in 2001.[46] Theophylline, a drug used in treatment of respiratory diseases, was determined in serum samples using anti- theophylline Abs immobilized to glass rods and radioisotope detection. The technique has been further improved by purification of Abs into

drug-specific fractions and optimization of the immobilization procedure in recent work conducted by H. Lord et al.[47] The performance of the Immunoaffinity SPME glass rods have been demonstrated for determination of sub ng/mL concentrations of 7-aminoflunitrazepam, a metabolite of the club drug , flunitrazepam, in human urine samples.[48] This work is a continuation of the development of the immunoaffinty SPME probes that was initiated by H. Lord et al.

1.4 Thesis objective

The primary goal of the research discussed in this thesis was to overcome the limitations of SPME in monitoring trace levels of pharmaceuticals in biological fluids by exploiting the selective recognition and binding properties of Abs for quantification of drugs. This goal was achieved by extending the development of immunoaffinity SPME probes that had been initiated by H. Lord et al.[47, 48]

The research was focused on two main areas; the development of a broadly applicable technique for extraction of drugs from biological fluids based on immunoaffinity SPME, and the demonstration of the technique's application to plasma. The former objective was achieved by employing both types of Abs (PAb and MAb) from four different suppliers. Characteristics of all Abs were carefully evaluated in solution and when immobilized on silica-based support. Especial attention was given to the study of cross-reactivity of Abs for drugs within the same class, which led to interesting results. In the second phase of the research, the results for extraction of diazepam and its two major metabolites from plasma using all types of the immunoaffinity SPME probes were demonstrated.

It should be noted that while H. Lord et al. have successfully demonstrated the utility of immunoaffinity SPME probes for analysis of trace levels of benzodiazepines, their work was mainly focused on the investigation of the new device. The aim of the current research was to demonstrate the device's utility as a broadly applicable tool that can be employed with Abs produced in different species and by various companies. The final purpose of this research was to demonstrate the probes performance in plasma, the most analyzed biological fluid in pharmaceutical and toxicological studies.

Chapter 2 Materials and methods

In this chapter, specifications of materials used for the development of immunoaffinity SPME probes are outlined. In addition, the methods employed for purification of Abs, immobilization of Abs on to solid supports and the design of experiments performed for the current research are expressed in the following sections. The methods for purification and immobilization of Abs as well as the experimental design for affinity test of Abs prior to immobilization are very similar to those employed by H. Lord et al. These methods are briefly described in this chapter; further details can be found in publications by H. Lord et al.[47,48]

2.1 Materials

Anti-benzodiazepine Abs were obtained from four suppliers: Biotrend Chemikalien GmbH (Köln, Germany), Cortex Biochem, Inc., (San Leandro, CA, USA), Fitzgerald Industries International, Inc. (Concord, MA, USA) and United States Biological (Swampscott, MA, USA). For the sake of simplicity, short forms of the suppliers' names are used throughout the text.

Two types of PAbs were obtained from Cortex with different cross-reactivities for various benzodiazepines. One batch was indicated by the supplier to be cross-reactive towards all benzodiazepines and the other was specified as oxazepam-specific. All Abs supplied from other companies were described by the suppliers as cross-reactive towards benzodiazepines. PAbs and MAbs were raised in sheep and mice, respectively. Oxazepam was coupled to a carrier protein (bovine γ-globulin (BGG), keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) and used as the immunogen for preparation of Abs obtained from all companies except for Biotrend Chemikalien GmbH. All batches of MAbs were purified for IgG and supplied in Phosphate Buffered Saline (PBS) with pH ranging from 7.0 to 7.2. Sodium azide was also added to the purified Abs as a preservative. The MAb obtained from US Biological contained glycerol (in the amount of 40%) in addition to PBS. With exception of the Fitzgerald PAb, which was supplied in the form of purified IGg, PAbs were supplied as neat serum without any preservatives. All batches of PAbs, not purified for IgG by the supplier or containing reagents other than PBS and sodium azide were purified according to the procedure described in the next section.

Generic IgG was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) in the form of purified IgG in phosphate buffer. According to the supplier, generic IgG was separated from non-immunized sheep serum.

Human plasma was obtained from US Biological. As indicated by the supplier, the plasma had been obtained from healthy male donors and was mixed with the appropriate amount of potassium ethylenediaminetetraaceticacid to prevent coagulation of plasma proteins.

Benzodiazepines (diazepam, nordiazepam, oxazepam and lorazepam) were obtained from Cerilliant (Round Rock, TX, USA) in the form of 1 mg/mL certified standards in methanol or acetonitrile. Intermediate standards were prepared by dilution of the primary standards with methanol. Final standards to be used for extraction were prepared on the day of experimentation by dilution of intermediate standards with PBS.

PBS was prepared in-house with sodium chloride (0.1 M), sodium phosphate dibasic (11.4 mM), potasium chloride (2.7 mM) and potassium phosphate monobasic (1.8 mM). The pH was adjusted to 7.4 with 5M NaOH. PBS was prepared fresh every month and stored at 4 ^{o}C. A Corning pH meter (model 220) was employed along with Corning combination electrode with calomel reference electrode from Fischer Scientific (Ottawa, ON, Canada).

Purification of IgG from other proteins in serum was performed using a Protein G affinity column and reagents supplied in a "ImmunoPure® (G) IgG Purification Kit" by Pierce Biotechnology (Rockford, IL, USA). Another affinity kit from Pierce Biotechnology (Pharmalink™ Immobilization Kit) was employed for affinity purification of polyclonal IgG. The purification procedures are described in the following sections.

The immobilization reagents including aminopropyltriethoxysilane (APTES) at 99% purity and glutaraldehyde grade II (25% aqueous solution) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Abs were immobilized on borosilicate glass rods (diameter: 4 mm, length: 10 cm) that were obtained from the University of Waterloo glass blower.

All other chemicals were ACS grade or higher. HPLC-grade solvents and nano-pure water were used for preparation of buffers and mobile phases. The nano-pure water was collected from a water deionization system from Barnstead (Dubuque, IA, USA) at 18MΩ.

Centrifugal devices were obtained from Millipore Corporation (Bedford, MA, USA). Amicon Ultra-4 centrifugal filter units (volume: 4 mL) with molecular weight cut off (MWCO) of 30,000 and 10,000 Da were employed for concentration and buffer exchange of Abs. Ultrafree-MC centrifugal filter units (volume: 0.4 mL) with MWCO of 30,000 Da were used in affinity testing of Abs in solution.

A magnetic stirrer, 96-well plates and vials used for extraction were obtained from VWR Scientific (Mississauga, ON, Canada). The 96-well plate dryer was constructed at the University of Waterloo Science Shops from stainless steel and standard "yellow" pipette tips (1-200 μL) from VWR. The pipette tips could be replaced if contaminated.

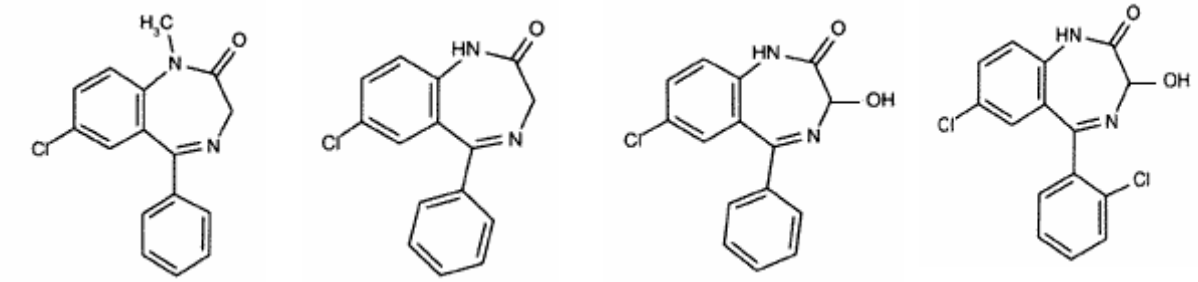
Samples were agitated during extraction and desorption processes using a Jeiotech SK-300 rotary shaker from Rose Scientific (Edmonton, AB, Canada). The ultraviolet spectrophotometer used for measuring protein concentration at 280 nm is a Spectronic Biomate 3 purchased from Thermo Scientific (Nepean, ON, Canada). An Eppendorf Repeater Plus obtained from Brinkman (Mississauga, ON) was used for reproducible dispensing of solutions.

Linear regression analysis was performed using Microsoft Excel 2000. Data for calibration of immunoaffinity SPME probes, which required non-linear regression, were fit to the Sips equation (Equation 1.11) in the form: $y = \frac{b(Kx)^a}{1+(Kx)^a}$ using SigmaPlot software (version 9.0). In this equation, y is the amount of Ag extracted (pg), x is Ag concentration at equilibrium, a is the heterogeneity index, b is the probe's capacity and K is the affinity of immobilized Abs.

2.1.1 Model drug selection

Four drugs, all classified as benzodiazepines, were selected for this research. Measurement of benzodiazepine concentrations in biological fluids has been the topic of numerous studies as drugs from this family are frequently prescribed as tranquilizers, anticonvulsants and sleep inducers. They have also been studied extensively in toxicological cases since they are often abused in large doses by illicit drug users.[49] As a result, their physiochemical properties and pharmacokinetics in a variety of animals are well defined. Having a liquid chromatography-tandem mass spectrometry (LC/MS/MS) system available for this work, the use of benzodiazepines in this research was additionally advantageous as they show good sensitivity in mass spectrometry (MS) analysis, and have been widely analyzed by chromatographic techniques coupled with MS.[49] Finally, benzodiazepines and their corresponding Abs are commercially available, which made benzodiazepines suitable as model drugs for the current work.

Diazepam, its two major metabolites (nordiazepam and oxazepam), and lorazepam were the drugs from the benzodiazepines' class chosen for these studies. Their chemical structures are shown in Figure 6.



Diazepam (mw: 284.7) Nordiazepam (mw: 270.7) Oxazepam (mw: 286.7) Lorazepam (mw: 321.2)

Figure 6: Chemical structures and molecular weights of the target drugs used in the studies

Diazepam, nordiazepam and oxazepam are closely related in structure and can co-exist in plasma. Therefore, studying specificity and cross-reactivity of the SPME probes for the parent drug and its significant primary metabolites was seen as important for the purpose of this research.

Lorazepam, which is a frequently prescribed benzodiazepine, was added to samples as the internal standard. Although it is from the same class of drugs, its structure is different from the other three in a way that unless added to the samples, there is no way for it to spontaneously arise in biological samples from diazepam metabolism. Conveniently, it also has good chromatographic properties for the liquid chromatography/tandem mass spectrometry (LC/MS/MS) method employed in the current work.

2.2 IgG purification

Serum contains various proteins (such as albumin) as well as all classes of Abs. All batches of Abs that were supplied either in the form of serum or in solutions other than PBS buffer were treated for isolating IgG and transferred to a solution of PBS containing 0.05% sodium azide using an IgG purification kit.[50]

IgG molecules were bound to an affinity column in which protein G had been covalently immobilized to a polymeric support. The binding was facilitated by mixing the Ab with a buffer provided in the IgG purification kit. Molecules other than IgG were not bound to the column and were washed out. IgG was then eluted from the column using an elution buffer that dissociated the bound IgG.

In the second step, IgG was transferred into PBS, with pH 7.4, by a "gel filtration" procedure using "D-Salt™ Excellulose™ Plastic Desalting Columns" provided in the IgG purification kit. During this procedure, molecules with molecular weights larger than 5000 Da which include IgG were eluted from the column faster, and were therefore separated from the buffer salts that passed through the column at a later time.[50]

2.3 Preparation of homogeneous IgG fractions

As described earlier, polyclonal IgG consists of Abs with a wide range of specificities and affinities. In the course of the current work, polyclonal IgG was fractionated into homogenous populations using an immobilized-ligand affinity column.

The column was made in-house according to the instructions provided by the manufacturer of the "PharmaLink™ Immobilization Kit".[51] Since oxazepam had been used as an Ag for preparation of anti-benzodiazepine Abs provided by most suppliers, oxazepam was coupled to the affinity column. The column consisted of diaminodipropyl amine (DADPA) that had been immobilized to 6% cross-linked agarose gel support. Oxazepam was covalently bound to the gel support by exploiting the "Mannich" reaction through condensation of oxazepam active hydrogen with formaldehyde and an amine.

The oxazepam-affinity column was stored in PBS containing 0.05% sodium azide and was used within a year of preparation. The concentration of the protein eluted from the column with each eluent was monitored by measuring absorbance of the 0.5-mL collected fractions at 280 nm, allowing baseline absorbance to be reached before applying the next eluting solution.

In the first step, polyclonal IgG was loaded on the oxazepam affinity column in fractions of 1 mL volume or less. Due to the volume capacity of the column, there is a limit of 1 mL sample application at a time, as indicated by the manufacturer.[51] Each 1 mL fraction was incubated for one hour to allow oxazepam-specific IgG to bind to the column before eluting unbound IgGs with PBS. IgGs with no affinity for oxazepam were eluted from the column.

After applying all of the polyclonal IgG to the column and removing unbound IgGs, the IgG molecules that were non-specifically bound to oxazepam were eluted from the column.

Elution of non-specific IgGs was performed using a series of solutions prepared in PBS (pH=7.4) in the following order: 0.1% Tween 20,[52] 10% ethylene glycol [53] and 0.3 M NaCl [54]. Employing these solutions, hydrophobic and electrostatic interactions associated with non-specific IgG were eliminated.

Subsequently, oxazepam-specific IgG molecules were eluted from the column sequentially with 0.5 M NaCl / PBS followed by 1.0 M NaCl / PBS, acidic phosphate buffer (pH=2), 7M Urea (pH=8) and finally 6M Guanidine Hydrochloride (GnHCl) (pH=4).

For over three decades, solutions with high salt concentrations along with acidic solutions have been used to recover specific Abs by disrupting the antibody-antigen affinity interactions.[54] Nevertheless, low recoveries indicate that many Abs are still bound to the ligand. It has been suggested that the acidic buffer is not chaotropic enough to disrupt all Ab-Ag interactions.[55] Denaturing agents such as urea and GnHCl were employed to disrupt the interactions of the remaining bound Abs on the column. These compounds are known to disrupt intramolecular interactions essential for stabilization of folded protein structures.[56] The tertiary structure of the Ab was unfolded by the urea solution, which resulted in partial denaturing of Ab.[56] The extremely chaotropic solution of GnHCl completely denatured the protein by unfolding the secondary structure so that any IgG remaining on the affinity column after urea elution was eluted in this solvent.[55, 56]

In the course of developing the method for preparation of specific IgG fractions from polyclonal IgG, one of the main challenges was refolding of the denatured Abs. Refolding was performed based on transferring the denatured fractions of Abs into PBS by ultrafiltration. GnHCl fractions were re-natured in two steps: first they were transferred into urea solution,

which partially restored IgG structure, and then they were transferred into PBS, resulting in completely refolded IgG.[55]

Imposing small changes to the procedure employed by H. Lord et al.[47, 48], re-naturing specific IgG was improved. IgG fractions collected from acidic phosphate and urea eluents were diluted three times with PBS containing 0.05% sodium azide immediately after collection. GnHCl fractions were diluted with urea. All fractions were then kept at 4^{o}C for 24 hours after which they were completely transferred to the diluting solutions by centrifugation. GnHCl fractions went through the same cycle once more before being transferred to PBS buffer.

The IgG fractions collected from various eluents were characterized in terms of specific binding, affinity and valence to evaluate the efficiency of various eluents in affinity purification of polyclonal IgG.

2.4 Evaluation of antibody characteristics in solution

Various fractions of Abs, including fractions collected from the oxazepam affinity column, generic IgG fractions, and polyclonal and monoclonal IgG fractions, which were raised against oxazepam, were evaluated in terms of their binding characteristics. The evaluations were performed by measuring the concentration of unbound oxazepam after incubation of a known amount of IgG with oxazepam standards at varying concentration. The drug concentration measurements were performed by an LC/MS/MS system. The results were used to select the IgG populations that were best suited for immobilization of glass rods.

Abs were allowed to bind to oxazepam during a 90 minute incubation period. The drug that remained unbound in the samples (also termed "free drug" throughout this text) was separated from the drug bound to IgG by ultrafiltration. Membranes with molecular weight cut-offs of 30,000 Da, which is lower than the molecular weight of IgG (~150,000) and higher than that of the free drug (~ 300), were used for this purpose. The free drug concentration was measured using a LC/MS/MS system. Oxazepam concentrations were chosen in such a way as to be able to differentiate between initial and equilibrium concentrations of oxazepam in the upper range of concentrations and to have sufficient amounts of unbound drug for accurate analysis in the lower range of concentrations.

The results of this test indicated the specific binding of each fraction of Ab to oxazepam at different Ab: drug molar ratios. The results were then employed to determine the affinity and valence of each fraction of Ab by Scatchard analysis.

2.5 Antibody immobilization

Ab immobilization has been reported for enzyme-linked immunosorbent assays (ELISA), biosensors and immunoaffinity chromatography separations using different methods. Since Ab characteristics can be changed upon immobilization onto a surface, attempts have been made for developing methods that impose minimal change to Ab binding characteristics.

Covalent immobilization of Abs has been performed using a linker compound such as glutaraldehyde (GA), while non-colvalent immobilization has commonly been performed by either adsorption to a surface or entrapment in a cross-linked matrix.[57, 58] The main advantage of non-covalent immobilization is that these methods are more likely to retain the native composition of the Ab molecules. On the other hand, covalent immobilizations improve antibody binding capacity and stability by eliminating partial denaturation, leaching and displacement of the immobilized Abs which have been reported for non-covalent immobilization methods.[59, 60] Covalent immobilization was therefore found to be more suitable for the purpose of this work. The method was initially employed by H. Yuan et al.[61] for preparation of immunoaffinity SPME probes, but was modified by H. Lord et al.[47, 48] and used for the current work.

The immobilization method that was used in this work is based on covalent binding of Ab amino groups to activated silica surfaces of 4 mm diameter glass rods through GA (a cross-linking reagent). The silica surface was silanized with a fresh solution of APTES in ethanol. GA was then reacted with an activated-silica surface followed by exposing the GA-activated surface to Ab solutions for Ab immobilization. Abs with high affinity and specificity were selected for the immobilization process based on the results of characteristic evaluation

experiments. SPME probes with generic IgG were also prepared for determination of non-specific binding. Concentrations of 0.2 mg/mL or greater were used for Ab solutions. In the final step, a solution of ethanolamine was used to deactivate the remaining aldehyde groups on the probes surface. Readers are referred to publications of H. Lord et al.[47, 48] for more details on the immobilization procedure.

2.6 Solid phase microextraction procedure

In this section, the general procedure that was followed for microextraction of target drugs by immunoaffinity probes is described.

The immunoaffinity probes, which were stored at 4^{o}C in PBS containing 0.05% sodium azide, were allowed to equilibrate to room temperature before performing extraction. The extraction solutions were prepared on the day of the experiment by spiking appropriate amounts of drug standards into PBS or plasma. Probes were immersed in 20 mL disposable glass vials, each containing 15 mL of sample in the case of extraction from PBS. Extraction from plasma was performed in 96-well plates using 1.5 mL of sample in each well. Extraction was performed for 45 minutes during which the samples were agitated at 100 rpm on a Jeiotech shaker. The probes were then removed from the samples and rinsed thoroughly with nano-pure water to remove PBS salts or plasma proteins that might have stuck to the probes. The rinse also reduces carryover of non-extracted drugs to the desorption solution.

Following the rinse, the probes were immersed in 500 µL of desorption solution which consisted of 75% methanol, 25% nano-pure water and 7.5 ng/mL internal standard (lorazepam). The composition of the desorption solution was reported to desorb >95% of extracted drugs while maintaining Abs affinity for the next experiments.[48] A positive displacement repeater pipettor (Eppendorf Repeater Plus) was used for reproducible and accurate dispensing of desorption solution. Desorption was also performed on the rotary shaker (100 rpm) for 15 minutes. Subsequently, the probes were rinsed briefly with water and stored in PBS containing 0.05% sodium azide at 4^{o}C for at least 24 hours before the next

extraction took place. This time was determined to be necessary for Abs to re-nature and regain activity.[48]

Desorption solution was then dried by flushing nitrogen through a 96-well plate dryer. The extracted drugs were reconstituted in smaller volumes of 75% methanol-25% water and injected to the LC/MS/MS system for separation and detection. The volume of reconstitution solution was dependant on the sensitivity required. A smaller volume (25 μL) was used when high sensitivity was required. Larger volumes (50-75 μL) however, allowed for multiple injections.

2.7 Separation and detection of analytes

Separation of analytes was performed using a Shimazu gradient LC system (model SLC 10 AVP) purchased from Mandel Scientific (Guelph, ON, Canada), an autosampler from CTC Analytics (model HTS PAL) purchased from Leap Scientific (Carrboro, NC, USA) with a 20 µL injection loop and a Symmetry Shield RP18 column (2.1 x 50 mm, 5 µm particle size) purchased from Waters Corporation (Milford, MA, USA). A gradient of two mobile phases (A containing 90% water, 10% acetonitrile and 0.1 % acetic acid and B containing 90% acetonitrile, 10% water and 0.1 % acetic acid) was used for separation of benzodiazepines. The run was started with 100% A which, after the first 30 seconds, was decreased to 10% A over 2 minutes. The mobile phase concentration was kept constant at 10% A for the next 1.5 minutes, after which it was brought back to 100% A till the end of run. Total run time for each sample was 5.0 minutes and a flow rate of 0.5 mL/min was used throughout the run.

Separated analytes were detected in a triple quadrupole tandem mass spectrometer (model API 3000) purchased from MDS Sciex (Concord, ON, Canada). Following electrospray ionization in positive ion mode, the analytes were analyzed with selected reaction monitoring. The transitions monitored for each target drug were as follows: 285.2/154.1 for diazepam, 271.1/140.0 for nordiazepam, 287.1/241.1 for oxazepam and 321.1/275.1 for lorazepam. The following conditions were used for MS: Nebulizer gas flow: 8 N_2, nebulizer voltage: 4500 V, drying gas: 7 L/min N_2, curtain gas: 12 N_2, CAD gas: 12 Ar and ion source temperature: 250 ^oC. Figure 7 shows chromatograms of the target analytes.

An electronic switching valve (model EV 750) and a six port valve (Rheodyne PEEK) from Waters (Milford, MA, USA) were employed to bypass the LC effluent for the first

minute of run time to the waste container. This procedure was performed for samples tested for IgG affinity in solution (contained 25% PBS) as well as samples from plasma extractions. In this way, the MS system was protected from salts or any other contaminants that could have been introduced to it from the samples.

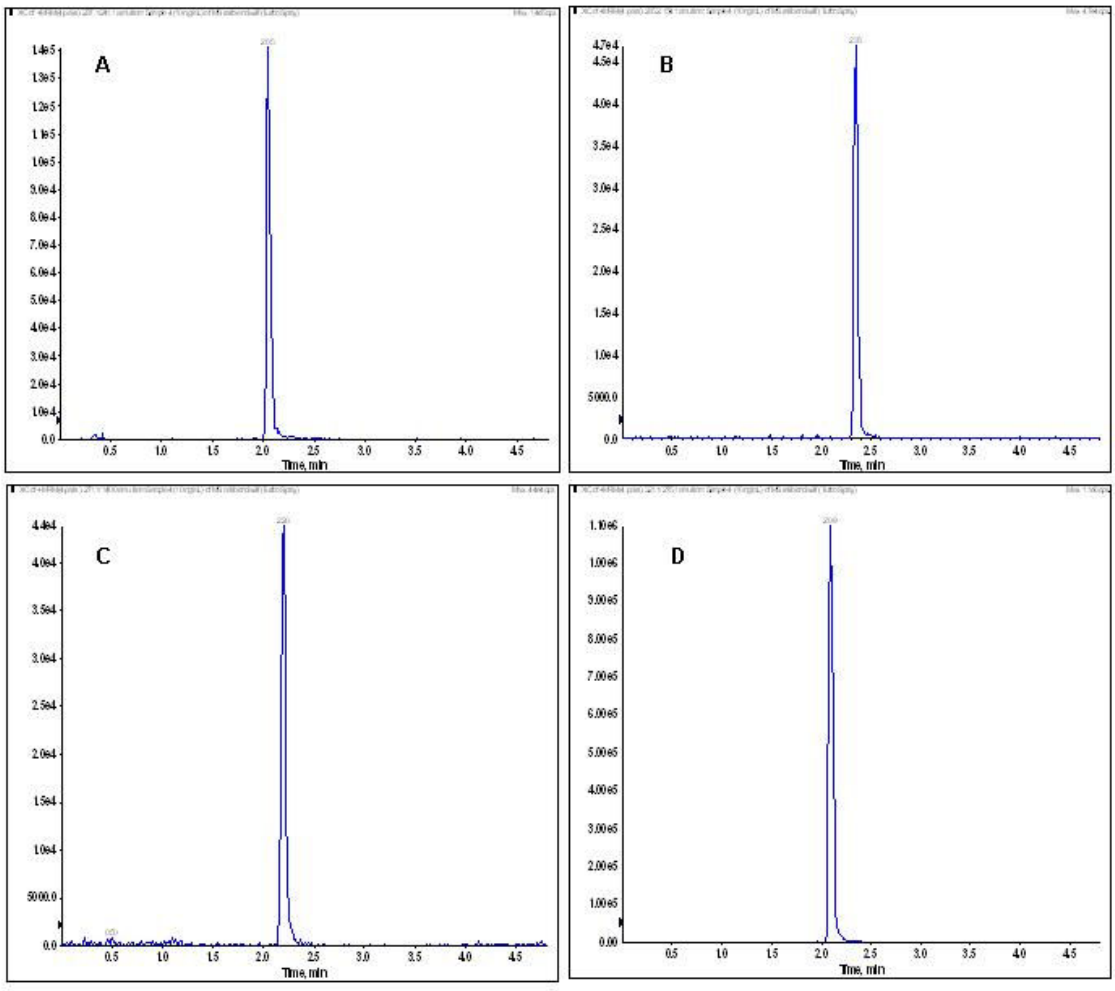


Figure 7: Sample chromatogram for diazepam (A), oxazepam (B), nordiazepam (C) and lorazepam (D).

Chapter 3 Results and Discussion

Polyclonal and monoclonal Abs supplied by four different companies were evaluated for the percentage of specific IgG in polyclonal batches as well as binding characteristics in solution and were immobilized on glass rods. The immunoaffinity probes were also evaluated for extraction from plasma. The results are presented in this chapter.

3.1 Affinity purification of polyclonal IgG

Purification of a fraction of polyclonal IgG with high affinity for benzodiazepines from the heterogeneous IgG pool was performed using an oxazepam-affinity column according to the process outlined in the Materials and Methods section. The result of a sample purification of Fitzgerald Polyclonal IgG is shown in Figure 8. A similar trend was also observed after affinity purification of polyclonal antibodies supplied by the other three suppliers.

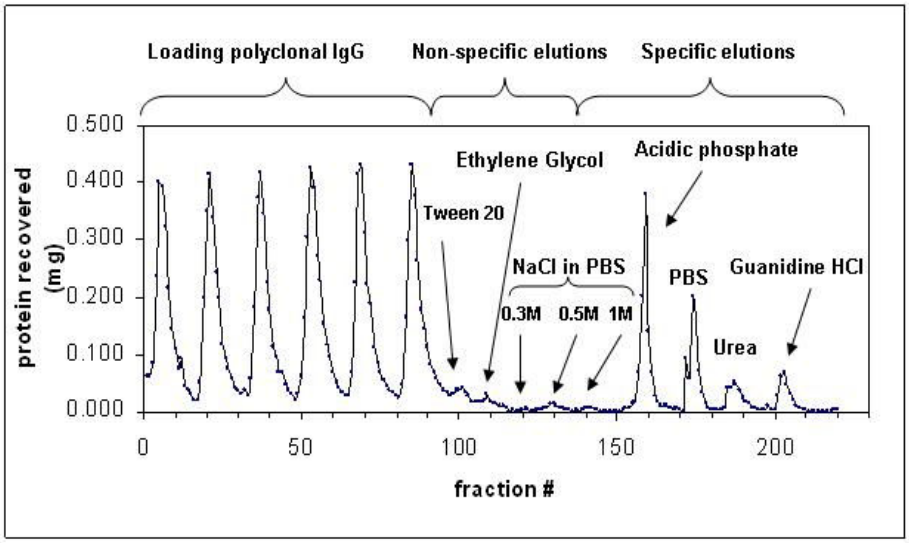


Figure 8: Sample of homogeneous IgG preparation from polyclonal IgG

In Figure 8, the x-axis shows the number of fractions collected while the y-axis represents the amount of protein euted from the column. The y-axis values were determined for each fraction by measuring its absorbance at 280 nm and using 1.35 mg^{-1} mL cm^{-1} as the absorptivity coefficient of IgG as indicated in the literature.

As shown in Figure 8, acidic phosphate eluent has the highest yield of specific IgG. For this reason up to now, acidic phosphate had been the only eluent that provided enough active IgG for immobilization on glass rods. [47, 48] However, having improved the process of re-naturing Abs eluted by urea and GnHCl as described in section 2.3, I was able to collect relatively large amounts of active IgG from GnHCl elution as well. Thus, along with acidic phosphate IgG, specific IgG from GnHCl elution was utilized in this work for preparation of immunoaffinity probes. The results are shown in the next sections.

Also notable in Figure 8 is the high amount of protein eluted with PBS buffer (pH=7.4) after acidic phosphate elution. This step was performed to neutralize the affinity column in preparation for further elutions. Although no report of such behavior was found in the literature, the same trend was observed during affinity purification of polyclonal antibodies from all the suppliers used in this study. Elution of IgG by PBS might be due to the charge induced on IgG molecules by acidic buffer. These charged molecules may be bound non-specifically to the column and released following column neutralization by PBS.

The results of affinity purification of PAb supplied from all four suppliers are compared in Table 1. The values are reported as the approximate percentage of total amount of PAb applied to the column. As apparent from Table 1, PAb of different suppliers show analogous results with respect to affinity purification. The amounts of IgG recovered from acidic phosphate and GnHCl elutions from all PAb batches were enough for preparation of immunoaffinity rods.

Supplier	Unbound IgG	Non-specific IgG	Acidic Phosphate	Urea	GnHCl
Biotrend	76%	6%	9%	3%	6%
Cortex	76%	7%	9%	3%	5%
Fitzgerald	75%	7%	8%	4%	6%
US Biological	74%	7%	9%	4%	6%

Table 1: Approximate amounts of IgG recovered from affinity purification of PAb from various suppliers

3.2 Affinity assessment of antibodies in solution

The fractions collected from specific elutions during affinity purification of polyclonal Abs were characterized along with polyclonal and monoclonal Abs in terms of specific binding as mentioned previously in the text. A fraction of "unbound IgG" which was expected to have relatively small specific binding towards oxazepam, was also included in each test to assure the effectiveness of the affinity column. The specific binding data was used to determine the affinity and valance of the original and fractionated Abs by Scatchard analysis as explained in section 1.2.3. The Scatchard plot of US Biological Abs is shown in Figure 9.

As shown in Figure 9, specific fractions of PAb show higher affinity towards oxazepam than the original PAb, confirming the effectiveness of the affinity purification method employed in the current research.

Furthermore, Figure 9 demonstrates a higher degree of linearity for more homogeneous fractions of IgG. This is expected due to the fact that in a homogeneous population of Abs all molecules have identical affinities, and therefore produce a linear Scatchard plot while heterogeneous populations of Abs, that have a range of affinities, are expected to produce plots with curved shapes. Correlation coefficient (R^2) values which indicate the degree of linearity of Scatchard plot for each fraction of Ab, are reported in Table 2.

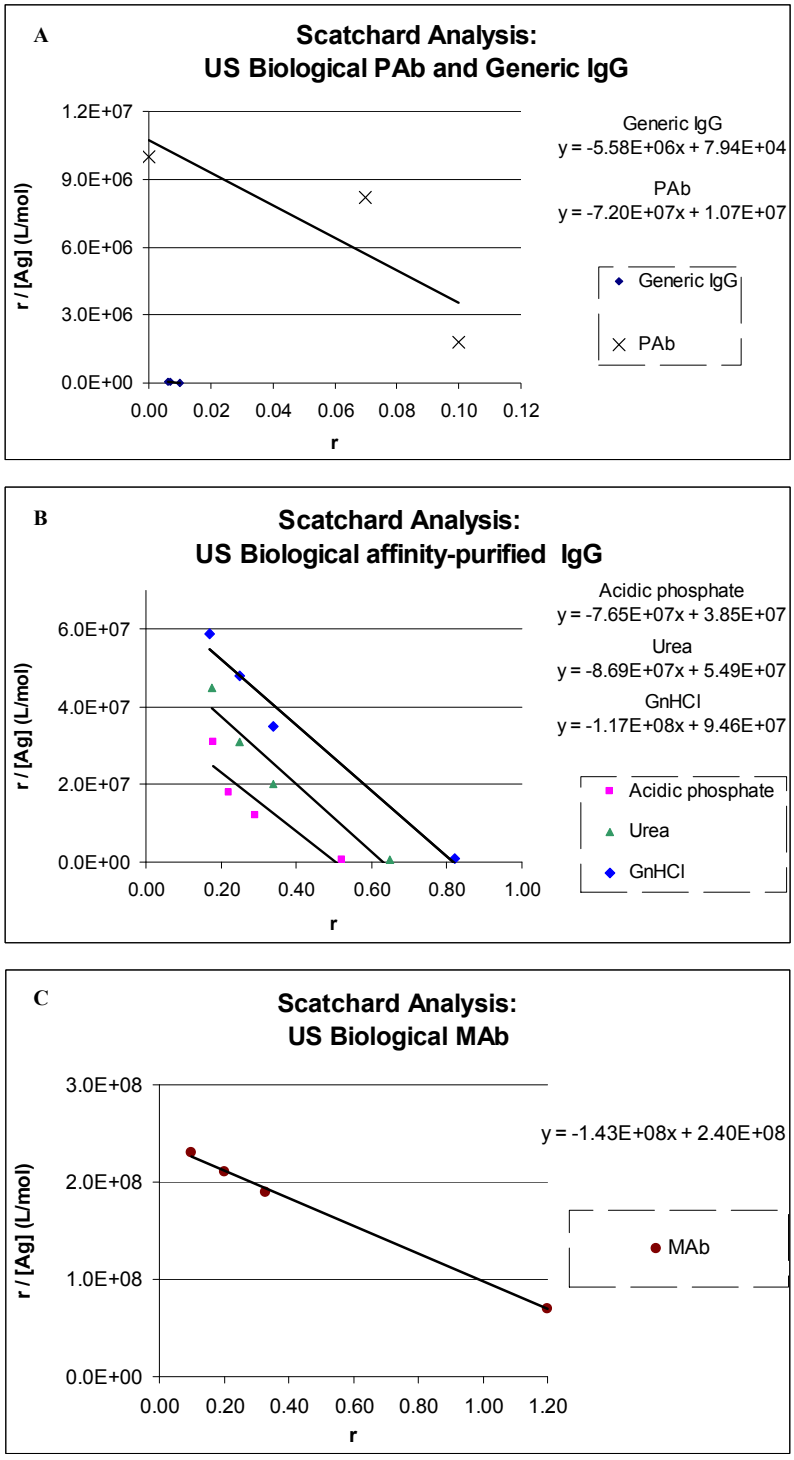


Figure 9: Scatchard plots of US Biological Abs including linear regression data

	PAb	Acidic phosphate	Urea	GnHCl	MAb
Correlation coefficient (R^2)	0.734	0.844	0.936	0.974	0.998

Table 2: Correlation coefficients associated with Scatchard plots of US Biological Abs demonstrated in Figure 9

Table 2 clearly demonstrates improved linearity with increased homogeneity of affinity purified PAb fractions, which verifies that affinity purification of PAb has resulted in more homogeneous populations of Abs. Interestingly, the linearity of GnHCl fraction data is found to be relatively close to MAb's which is expected to have a linear Scatchard plot ($R^2 = 1$). Similarly, as indicated in Table 3, which provides affinity and valence values calculated from Scatchard analysis for all batches of Abs, linear regression analysis reveals comparable affinities for GnHCl and MAb fractions. The Ab valence, however, is found to be quite different for these two fractions. The "generic IgG" was also included in the test and was found to have an affinity of 6E06 (L/mol) and valence of 0.01 which are in line with affinity and valence values for different "unbound IgG" fractions.

Antibody Supplier		PAb	Unbound IgG	Acidic Phosphate	Urea	GnHCl	MAb
Biotrend	K	8.29E07	8.01E06	9.17E07	1.28E08	3.31E08	3.59E08
	n	0.122	0.024	0.421	0.453	0.593	1.35
Cortex	K	6.46E07	6.51E06	7.49E07	9.55E07	1.27E08	1.48E08
	n	0.152	0.051	0.290	0.383	0.425	1.49
Fitzgerald	K	9.02E07	6.25E06	1.02E08	1.54E08	3.49E08	4.16E08
	n	0.104	0.043	0.372	0.491	0.552	1.08
US Biological	K	7.20E07	5.58E06	8.65E07	8.69E07	1.17E08	1.43E08
	n	0.117	0.014	0.472	0.614	0.805	1.68

Table 3: Affinity, K (L/mol), and valance, n, of various IgG fractions in solution, calculated from Scatchard analysis data

In comparing various Ab fractions provided by the same supplier, it is observed that the affinity and valence of Abs increase as homogeneity increases (Table 3). This trend, which demonstrates the efficiency of affinity purification of PAbs, is seen with Ab fractions from all four suppliers. The fraction of "unbound IgG" has the lowest affinity and valence compared to all other fractions of Ab from the same supplier, verifying that during the time that PAb was loaded on the oxazepam affinity column, no breakthrough of oxazepam-specific IgG was observed. The highest values for affinity and valance, on the other hand, are associated with

MAb fraction. It is also noted that GnHCl fraction, has similar affinity towards oxazepam compared to MAb. However, it has smaller valance than MAb fraction.

Although the same trend is observed for various fractions of the Abs from the same supplier in terms of affinity and valance values, different values are estimated for Abs of the same type obtained from various companies. This is expected as Abs from different suppliers had been produced in different conditions. Abs provided from a single company could also have different binding properties even if the same animal and the same conditions are used for immunization. This is due to the fact that the response of a living species to a foreign agent is different at different times. Therefore, unless Abs produced during one immunization procedure, holding the same lot number, are obtained from a company, the binding characteristics are expected to be different.

From Table 3, it is also noted that the valence values estimated for the Abs are comparable for Abs of the same type obtained from various suppliers. As stated previously, the valence of a homogeneous population of IgG is ideally equal to 2, since an IgG molecule has two equal binding sites. The valence values for MAb fractions obtained from all four suppliers were greater than 1.00 while PAb valance values were smaller than 0.200.

The affinity assessment of the Abs was initially used for evaluation of the affinity purification method and for comparing Abs supplied from various companies in terms of their binding properties. However, the results were also used for selecting appropriate fractions of Abs for immobilization onto the glass rods. Since the immobilization process took a minimum of one week per batch of Ab, the batches with low affinities (in the range of E06) were excluded from the immobilization process. Finally, the affinity values calculated from Scatchard data were used in the current work to determine the concentration of free oxazepam

that would saturate 50% of Abs' binding sites as described in section 1.2.3. Calculating these values (Table 4) allowed for estimation of the range of sample concentrations for which the Abs can be used to extract free oxazepam. Based on the values reported in Table 4, the Abs were expected to be appropriate for quantification of benzodiazepines in the range of low to sub ng/mL concentrations.

Oxazepam concentration (ng/mL) at 50% saturation	PAb	Acidic Phosphate	Urea	GnHCl	MAb
Biotrend	3.59	3.19	2.87	0.96	0.96
Cortex	4.78	4.10	3.19	2.87	2.87
Fitzgerald	3.19	2.87	2.87	0.96	0.72
US Biological	4.10	3.59	3.19	2.87	2.87

Table 4: Estimates of oxazepam concentrations that saturate 50% of Abs' binding sites for different Ab fractions

3.3 Evaluation of immobilized antibodies

The binding characteristics of Abs employed in preparation of immunoaffinity SPME probes had been previously evaluated in terms of affinity and probe capacity using two different methods of regression.[47] Since non-linear regression to Sips equation is the best known method to date for describing the binding characteristics of Abs, it was used for these evaluations. Cross-reactivity of the immunoaffinity probes was also evaluated for different combinations of two of the three drugs at a time.

3.3.1 Calibration of immunoaffinity probes in buffer

Immunoaffinity SPME probes were calibrated for oxazepam, diazepam and nordiazepam in PBS buffer individually. For each test, five different concentrations of each drug were prepared in the range of 0.01 – 2 ng/mL. Three probes from each type of Ab were used for extraction from each concentration. Calibration profiles of Fitzgerald Ab probes for each drug are demonstrated in Figure 10. Probes prepared with generic IgG, which has no specific affinity for benzodiazepines, were used in calibration tests to determine the amount of non-specific binding. As can been seen from the graphs in Figure 10, the amount of drug extracted by generic IgG probes were found to be insignificant in the range of concentrations used for calibration of probes. Thus, elimination of drugs that were non-specifically bound to Ab probes was not considered in the course of this work.

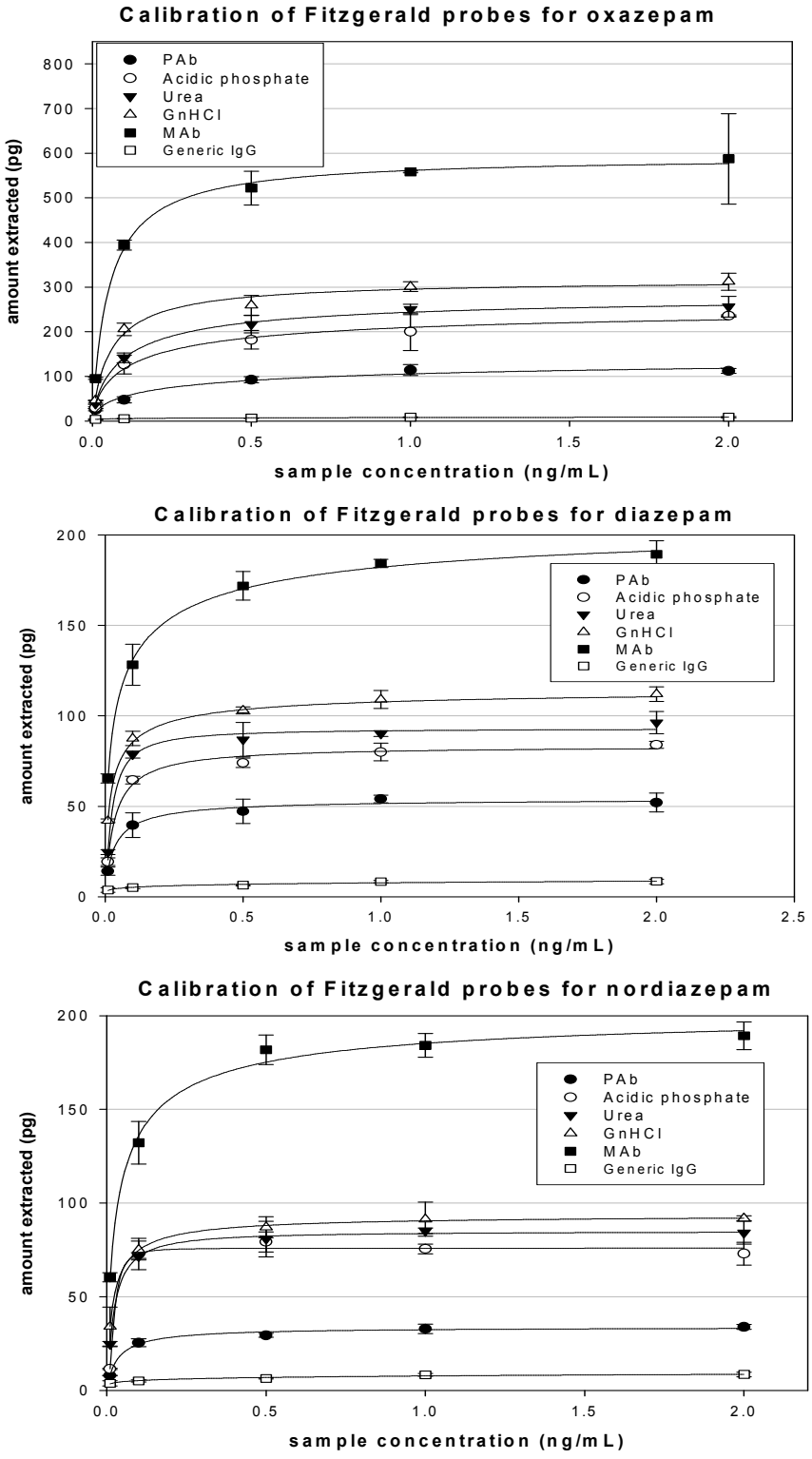


Figure 10: Calibration profiles of Fitzgerald and generic IgG probes for drugs of interest

The calibration data were fitted to the Sips equation as discussed in section 2.1 and the values of K (probe affinity), b (probe capacity) and a (heterogeneity index) were calculated. Linear regression analysis of immunoaffinity probe calibration can be performed based on the reciprocal of the Langmuir equation. However, as mentioned previously, the Langmuir equation does not consider Ab heterogeneity and therefore, in this work, non-linear regression to the Sips equation was chosen. It should be noted that based on previous research performed on Cortex Abs, for Abs with $a \geq 0.78$, affinity and capacity values estimated by either of the equations are expected to be similar.[46]

Table 5 summarizes the values calculated from regression to the Sips equation for Fitzgerald immunoaffinity probes. From the values reported in Table 5, it is apparent that the homogeneity, capacity and affinity values of Fitzgerald immunoaffinity probes (represented by a, b and K respectively) are increased for fractions purified from PAb, as more oxazepam-specific fractions of Abs are exploited for extraction of each drug (Table 5). The trend observed for these values confirms the success of the affinity purification procedure for preparation of more homogeneous fractions of Abs with higher affinities towards benzodiazepines. Comparing the binding values of each probe type for the three different target drugs in Table 5, equal performance is observed for diazepam and nordiazepam. In the case of oxazepam extraction, lower affinities and larger capacities are calculated with respect to the other two drugs. The heterogeneity indices are slightly higher for diazepam and nordiazepam although the range is comparable for all three drugs.

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.586	0.611	0.590
	b (pg)	156	54.4	33.8
	K (mL/ng)	3.56	29.9	29.4
	K (L/mol)	1.02E09	8.53E09	7.96E09
Acidic phosphate	*a*	0.702	0.852	0.836
	b (pg)	261	83.4	75.9
	K (mL/ng)	7.50	30.5	44.8
	K (L/mol)	2.15E09	8.69E09	1.21E10
Urea	*a*	0.793	0.907	0.902
	b (pg)	283	93.0	85.0
	K (mL/ng)	9.85	41.4	44.7
	K (L/mol)	2.83E09	1.18E10	1.21E10
GnHCl	*a*	0.922	0.956	0.956
	b (pg)	317	115	93.9
	K (mL/ng)	16.9	45.7	51.3
	K (L/mol)	4.85E09	1.30E10	1.39E10
MAb	*a*	0.971	0.994	0.989
	b (pg)	593	213	204
	K (mL/ng)	19.8	22.7	27.1
	K (L/mol)	5.51E09	6.47E09	7.34E09
Generic IgG	*a*	0.199	0.200	0.193
	b (pg)	18.5	20.1	18.0
	K (mL/ng)	0.011	0.010	0.011
	K (L/mol)	2.87E06	2.79E06	3.03E06

Table 5: Heterogeneity index (a), capacity (b) and affinity (K) values of Fitzgerald and generic probes

MAb probes exhibit lower affinities for diazepam and nordiazepam than PAb and affinity purified PAb probes, which is not unusual.[25] The affinity values of MAb for oxazepam are comparable to affinity values of GnHCl probes and are in line with the values calculated from Scatchard analysis of Abs prior to immobilization (Table 3). As expected, MAb probes are the most homogeneous and have the largest capacities (Table 5).

Generic IgG probes show incredibly low homogeneity for drugs of interest which is expected since generic IgG is a mixture of IgG molecules that exist in the serum of non-immunized animal and therefore, have been produced against a wide variety of compounds. For the same reason, the capacity and affinity values of these probes are also found to be low. The affinity value of immobilized generic IgG probes are in line with the values obtained from Scatchard analysis for free Ab.

From a more general point of view, the data in Table 5 demonstrate that the biospecific activity of Abs toward oxazepam has been retained during immobilization, confirming the suitability of the immobilization procedure for this work. Comparing affinity values for Fitzgerald Abs in Tables 3 and 5, similar trends are observed for both immobilized and free Abs. The absolute values of affinity, on the other, have increased after immobilization (Table 5). The difference in affinity values of the same fractions of Abs before and after immobilization could be due to the orientation of immobilized Abs on a solid support, which increases the number of sterically available antigenic sites of Abs, as opposed to random distribution of free Abs in solution, which can cause more steric hindrance for Ags to bind to Abs. Another reason for this discrepancy could be the difference in the accuracies of methods used for regression analysis. As mentioned previously in the text, all Abs, including MAbs,

exhibit a range of affinities. This range, which is larger for more heterogeneous fractions of Abs, is not taken into account in Scatchard analysis. In the Sips equation, on the other hand, distribution of affinities is considered and thus, more accurate estimates can be calculated from regression analysis of the Sips equation.

Table 5 also demonstrates the difference between capacity and affinity of immunoaffinity probes. These two terms, which are often confused, are not directly dependent on each other. As shown in Table 5, the capacity values of MAb probes are higher than all other types of probes for all three benzodiazepines, while their affinities for diazepam and nordiazepam are significantly lower than other Fitzgerald Ab probes. The capacity of an immunosorbent is defined as the number of specific immobilized Abs that Ag molecules can access.[62] It is different from the total number of immobilized Abs, termed "bonding density", because random orientation and steric hindrance prevent Ag molecules from accessing all Ab molecules immobilized on the sorbent.[62] Based on the definition, the capacity of immunoaffinity probes depend on the concentration of specific Abs in the fraction of Abs used for preparation of the probes. Therefore, MAbs are expected to have higher capacities than PAbs, even for drugs to which they bind with lower affinities.

The values of heterogeneity index, affinity and capacity calculated from the Sips equation for Abs obtained from other suppliers are reported in Tables 6 to 8.Although the estimated values are different for each type of probe, the trends observed for Fitzgerald immunoaffinity probes, as explained in this section, are also noted for Biotrend and US Biological Ab probes. Cortex probes also follow the trends observed for other immunoaffinity probes except for diazepam extraction. As Table 7 shows, Cortex probes extract higher

amounts of diazepam than the other two drugs. In case of all other probes, oxazepam is extracted more than diazepam and nordiazepam.

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.548	0.694	0.679
	b (pg)	159	80.1	54.0
	K (mL/ng)	3.32	28.3	31.1
	K (L/mol)	9.52E08	8.07E09	8.41E09
Acidic phosphate	*a*	0.699	0.881	0.859
	b (pg)	258	122	107
	K (mL/ng)	6.16	34.1	39.3
	K (L/mol)	1.77E09	9.70E09	1.06E10
Urea	*a*	0.786	0.922	0.907
	b (pg)	273	189	162
	K (mL/ng)	9.80	43.9	44.5
	K (L/mol)	2.81E09	1.25E10	1.20E10
GnHCl	*a*	0.943	0.970	0.965
	b (pg)	349	247	191
	K (mL/ng)	14.0	48.2	47.2
	K (L/mol)	4.03E09	1.37E10	1.28E10
MAb	*a*	0.985	0.996	0.994
	b (pg)	921	433	409
	K (mL/ng)	17.9	18.6	18.7
	K (L/mol)	5.15E09	5.30E09	5.07E09

Table 6: Heterogeneity index (a), capacity (b) and affinity (K) values of Biotrend probes

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.509	0.667	0.609
	b (pg)	150	125	90.5
	K (mL/ng)	2.28	20.6	21.0
	K (L/mol)	6.55E08	5.86E09	5.69E09
Acidic phosphate	*a*	0.612	0.875	0.805
	b (pg)	189	191	129
	K (mL/ng)	3.00	21.8	22.9
	K (L/mol)	8.62E08	6.22E09	6.19E09
Urea	*a*	0.753	0.938	0.892
	b (pg)	229	271	134
	K (mL/ng)	4.25	23.7	24.0
	K (L/mol)	1.22E09	6.74E09	6.51E09
GnHCl	*a*	0.896	0.977	0.951
	b (pg)	255	338	172
	K (mL/ng)	7.27	25.0	26.2
	K (L/mol)	2.09E09	7.11E10	7.10E09
MAb	*a*	0.972	0.999	0.987
	b (pg)	980	426	311
	K (mL/ng)	8.81	16.4	16.5
	K (L/mol)	2.53E09	4.68E09	4.46E09

Table 7: Heterogeneity index (*a*), capacity (*b*) and affinity (*K*) values of Cortex probes

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.527	0.620	0.568
	b (pg)	144	54.8	40.0
	K (mL/ng)	2.86	12.0	14.7
	K (L/mol)	8.21E08	3.42E09	3.98E09
Acidic phosphate	*a*	0.724	0.862	0.798
	b (pg)	440	150	65.8
	K (mL/ng)	3.14	13.2	18.5
	K (L/mol)	9.01E08	3.76E09	5.02E09
Urea	*a*	0.891	0.968	0.927
	b (pg)	451	171	97.6
	K (mL/ng)	3.86	14.8	19.4
	K (L/mol)	1.11E09	4.22E09	5.26E09
GnHCl	*a*	0.920	0.971	0.959
	b (pg)	486	180	125
	K (mL/ng)	5.09	16.1	20.0
	K (L/mol)	1.46E09	4.59E09	5.42E09
MAb	*a*	0.972	0.989	0.978
	b (pg)	1265	320	296
	K (mL/ng)	5.63	3.31	8.89
	K (L/mol)	1.62E09	9.42E08	2.41E09

Table 8: Heterogeneity index (a), capacity (b) and affinity (K) values of US Biological probes

3.3.2 Cross-reactivity assessment

In clinical samples, drugs other than the analyte of interest are commonly present. With respect to the chemical structure, these drugs can be similar, such as analyte metabolites, or totally different. Thus, it is of importance to examine performance of the probes while more than one analyte is present in the sample.

A series of experiments were performed to compare cross-reactivity of immunoaffinity probes. All Abs were indicated by the suppliers to be 100% cross-reactive for benzodiazepines. In addition to the probes described previously in the text, a new batch was included in the cross-reactivity experiments. This batch was prepared with "acidic phosphate" fraction of oxazepam-specific PAb obtained from Cortex. The Ab was indicated by the supplier to be specific for oxazepam with low cross-reactivity for other benzodiazepines, including diazepam and nordiazepam. Thus, its performance was of interest in terms of cross-reactivity compared to other Abs that were recognized as cross-reactive for all benzodiazepines. In this text, the latter Abs are termed "highly cross-reactive" while "oxazepam-specific" refers to probes prepared with less cross-reactive Abs.

The performance of immunoaffinity probes in the presence of diazepam and its two major metabolites, oxazepam and nordiazepam, has been evaluated in earlier studies.[47] In the current work, cross-reactivity of probes for each one of the target analytes was evaluated in the presence of another benzodiazepine. In each experiment, the probes were exposed to 15mL samples containing the target drug at a constant concentration of 0.2 ng/mL and the cross-reactant at 7 different concentrations ranging from 0 to 10 ng/mL. Cross-reactivity was measured as the concentration of the cross-reactant that inhibits extraction of the analyte by

50%. Since all probes, except for "oxazepam-specific" ones were prepared with highly cross reactive Abs, the cross-reactivity of probes with the same type of Ab obtained from various suppliers were expected to be in the same range. The results are summarized in Table 9. Consistent results were obtained from highly cross-reactive Abs from various companies. Hence, the average cross-reactivity values are reported for various types of Abs obtained from all four suppliers (Table 9).

Cross-reactivity (ng/mL)							
Drugs used in experiments		oxazepam-specific	highly cross-reactive				
Analyte	Cross-reactant	Acidic phosphate	PAb	Acidic phosphate	Urea	GnHCl	MAb
Ox	Dz	1	< 0.2	< 0.2	0.2	< 1	< 1
	Nd	< 1	< 0.2	< 0.2	< 0.2	< 0.2	0.2
Dz	Ox	< 0.2	0.2	0.2	< 0.2	< 0.2	< 1
	Nd	< 1	0.2	<0.2	<0.2	< 0.2	< 0.2
Nd.	Ox	< 0.2	0.2	0.2	< 0.2	< 0.2	< 7.5
	Dz	0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2

Table 9: Results of cross-reactivity experiments for immunoaffinity probes of different types. (Ox: oxazepam, Dz: diazepam, Nd: nordiazepam)

To better demonstrate the displacement of an analyte by a cross-reactant, graphical results of two types of Cortex probes for oxazepam extraction in the presence of diazepam at different concentrations are shown in Figure 11.

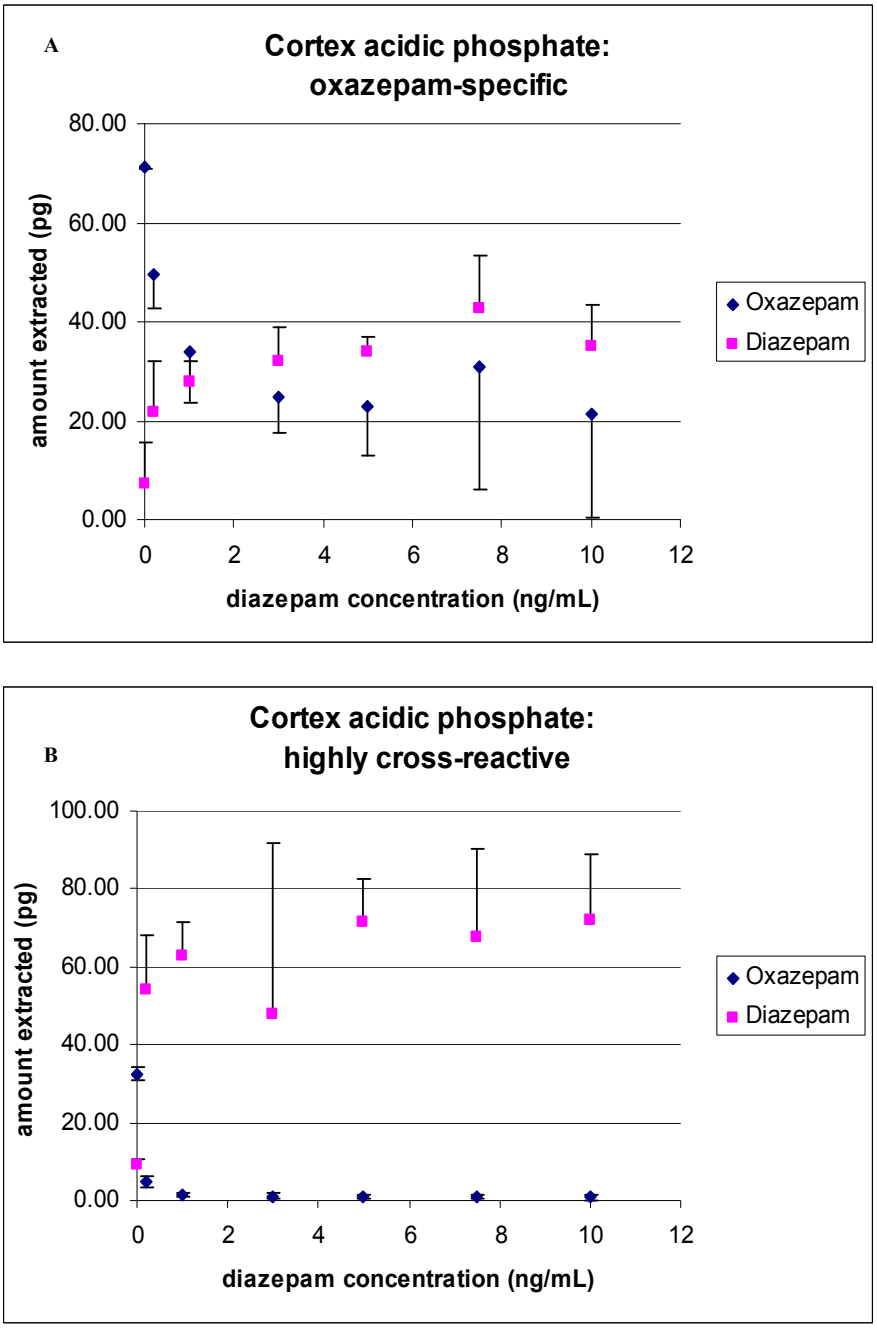


Figure 11: Displacement of oxazepam by diazepam for acidic phosphate probes

As the results clearly demonstrate, for oxazepam-specific probes, the 50% displacement of oxazepam by either of the other drugs occurred at much higher concentrations (nearly five times higher) than for the highly cross-reactive probes. Furthermore, 50% displacement of diazepam and nordiazepam was observed at oxazepam concentrations as low as 0.2 ng/mL. This suggests that the "oxazepam-specific" Ab, as described by the supplier, is in fact oxazepam-specific. When diazepam and nordiazepam were competing for Ab sites, oxazepam-specific probes were found to be more cross-reactive for nordiazepam rather than diazepam, while highly cross-reactive probes treated both analytes equally (Table 9). This result is in line with cross-reactivity values reported in Ab specification sheets provided by their suppliers.

As for highly cross-reactive probes, the PAb and affinity purified PAb probes were found to be equally cross-reactive for diazepam and nordiazepam. For oxazepam, on the other hand, the affinity-purified probes (acidic phosphate, urea and GnHCl) were found to be more cross-reactive towards oxazepam than the other two analytes. Once again, the results confirm that Abs purified on the oxazepam affinity column contained larger fractions of oxazepam-specific Ab than PAb fractions. Cross-reactivity of PAb probes was found to be comparable for all three drugs.

Similar to PAbs, MAbs were specified by the suppliers to be cross-reactive for all benzodiazepines. However, the cross-reactivity values of MAbs were not indicated in the Ab specification sheets. Based on the results of the cross-reactivity experiments (Table 9), MAbs have similar cross-reactivity for diazepam and nordiazepam. MAb probes were found to interact equally with diazepam and oxazepam when these two drugs were present in the same

samples. Nevertheless, it was observed that for MAb probes, nordiazepam was displaced by oxazepam at high concentrations (approximately 7.5 ng/mL) while oxazepam displacement (50%) by nordiazepam occurred at 0.2 ng/mL.

Apart from calculations for cross-reactivity, it was noted that oxazepam-specific probes extracted more than twice the amount of oxazepam as was extracted by highly cross-reactive probes in the absence of the competing drug (diazepam or nordiazepam). Thus, it is concluded that oxazepam-specific probes are more suitable for oxazepam extraction when diazepam and nordiazepam are at lower concentrations. It should be noted that even at the highest concentration used for this experiment (10 ng/mL), neither diazepam nor nordiazepam could completely displace oxazepam bound to "oxazepam-specific" probes.

3.4 Evaluation of extraction and desorption conditions

3.4.1 Evaluation of extraction conditions

As described in Chapter 2, the probes were used for extraction from 15 mL spiked PBS buffer samples. Based on sample concentrations, this volume had been chosen in previous studies of immunoaffinity probes because depletion of analytes was found to be negligible.[47] In order to have negligible depletion of analytes from extraction samples, the amount of drugs extracted by SPME probes should be comparable to the experimental error.[63] Such conditions are desired to generate consistent calibration profiles that are independent of sample volume. Since the conditions that allow negligible depletion have been studied for immunoaffinity probes in an earlier study, they will not be discussed further.[47]

Extraction from plasma samples, on the other hand, was performed from 1.5 mL samples mainly due to the limited volume of plasma that was available. The conditions for negligible depletion of analytes from plasma samples were carefully investigated. Assuming 95% protein-binding for the drugs in plasma, the amount of free drugs extracted by the probes were less than the experimental error which was calculated to be below 10% (see section 3.6).

The amount of time required for the probes to reach equilibrium during the extraction process was determined for various probes. Extraction was performed from 15 mL sample of 0.1 ng/mL oxazepam on a shaker with 100 rpm. Vigorous agitation is expected to speed up the equilibration by facilitating mass transport conditions. However, previous studies on immunoaffinity probes have shown no improvement in extraction rates beyond 100 rpm. Thus,

it can be concluded that the rate of extraction for agitated samples is controlled by the kinetics of antigen-antibody reaction rather than mass transport.

The extraction time was varied from 1 minute to 45 minutes at six time points. The equilibrium time profile of Biotrend probes is shown in Figure 12 as an example. Eliminating MAb probes results, the y-axis is extended on Figure 12-B to better show the equilibration time profiles of other probe types.

The equilibrium times for different types of probes were estimated based on the times that a plateau was reached. Based on Figure 12, the equilibration times are as follows: 10 minutes (PAb), 20 minutes (Acidic phosphate and Urea), 30 minutes (GnHCl) and 45 minutes or longer (MAb). As expected, probes with higher capacities are associated with longer equilibrium times. The equilibration time profiles for Abs obtained from different suppliers were comparable.

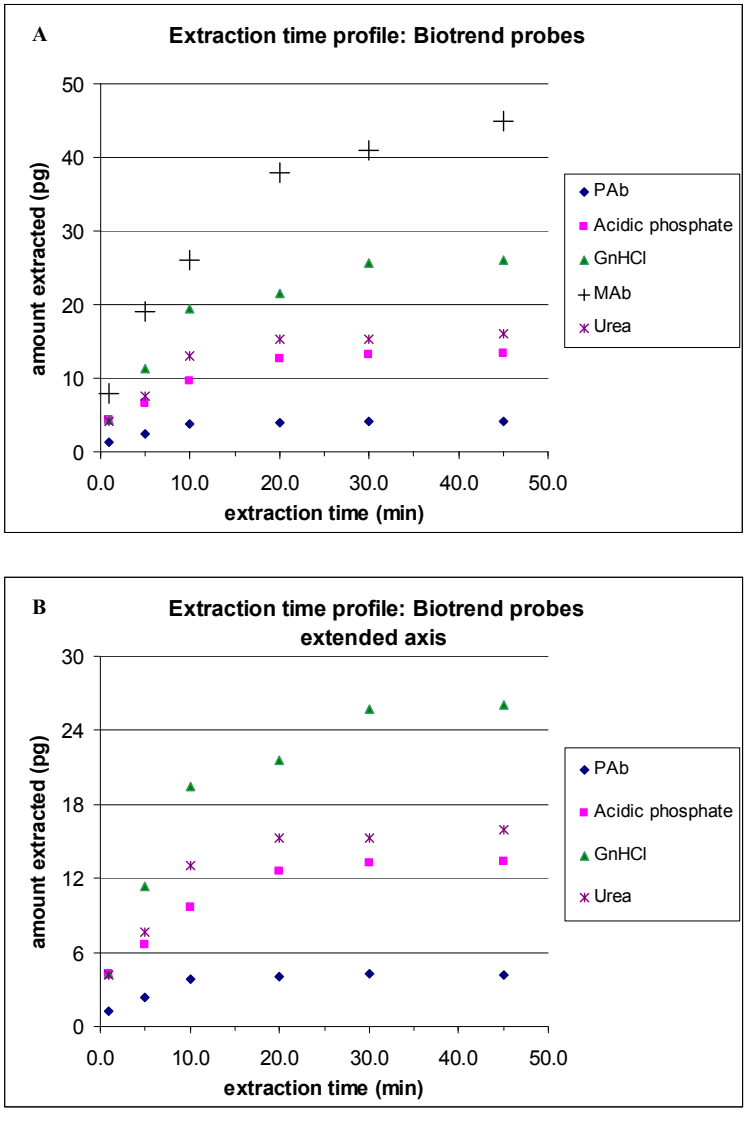


Figure 12: Equilibrium time profiles of Biotrend probes

Comparing the amounts extracted by the probes at the plateaus with the maximum capacity of the probes for oxazepam (Table 6), it is confirmed that the plateaus were not caused by probe saturation. It has been known for over three decades that the rate of dissociation of Ab-Ag complex determines the Ab affinity towards a hapten. The association rate, on the other hand, is fairly constant for Abs with different affinities.[64] Thus, different equilibrium times observed for Abs with different affinities are mainly due to different capacities of the probes. Shorter equilibrium times are also expected for extraction of diazepam and nordiazepam due to their smaller probe capacities (Table 6).

Based on the results, extraction time of 45 minutes was used in this study to allow equilibrium extraction for all probes including MAb. The extraction time can be shortened to 30 minutes when MAb probes are not employed in an experiment.

3.4.2 Evaluation of desorption conditions

Extracted analytes were desorbed in 500 μL of 75% methanol containing internal standard (lorazepam). The volume and composition of the desorption solution were determined in earlier studies as a good compromise allowing 95% of extracted drugs to be desorbed.[47] The desorption time profiles for the immunoaffinity probes were evaluated at six time points ranging from 1 minute to 45 minutes at 100 rpm. Prior to desorption, all probes were immersed in 15 mL samples of 0.1 ng/mL oxazepam for 45 minutes on a shaker at 100 rpm. The desorption profiles of Biotrend probes are shown in Figure 13. As shown in this figure, the time required for desorption of extracted drugs increases as probe affinity increases: 15 minutes (MAb probes), between 10 to 15 minutes (GnHCl probes), 10 minutes (Acidic phosphate and Urea probes) and 5 minutes (PAb probes).

As mentioned earlier, Ab affinity, which is inversely proportional to the dissociation rate constant, is mainly dependent on the rate of dissociation of the Ab-Ag complex. Therefore, Abs with higher affinities are expected to have slower dissociations which is in line with the results shown in Figure 13. The probes from other suppliers performed similarly. The longest desorption time observed for the probes was 15 minutes and was used throughout this study.

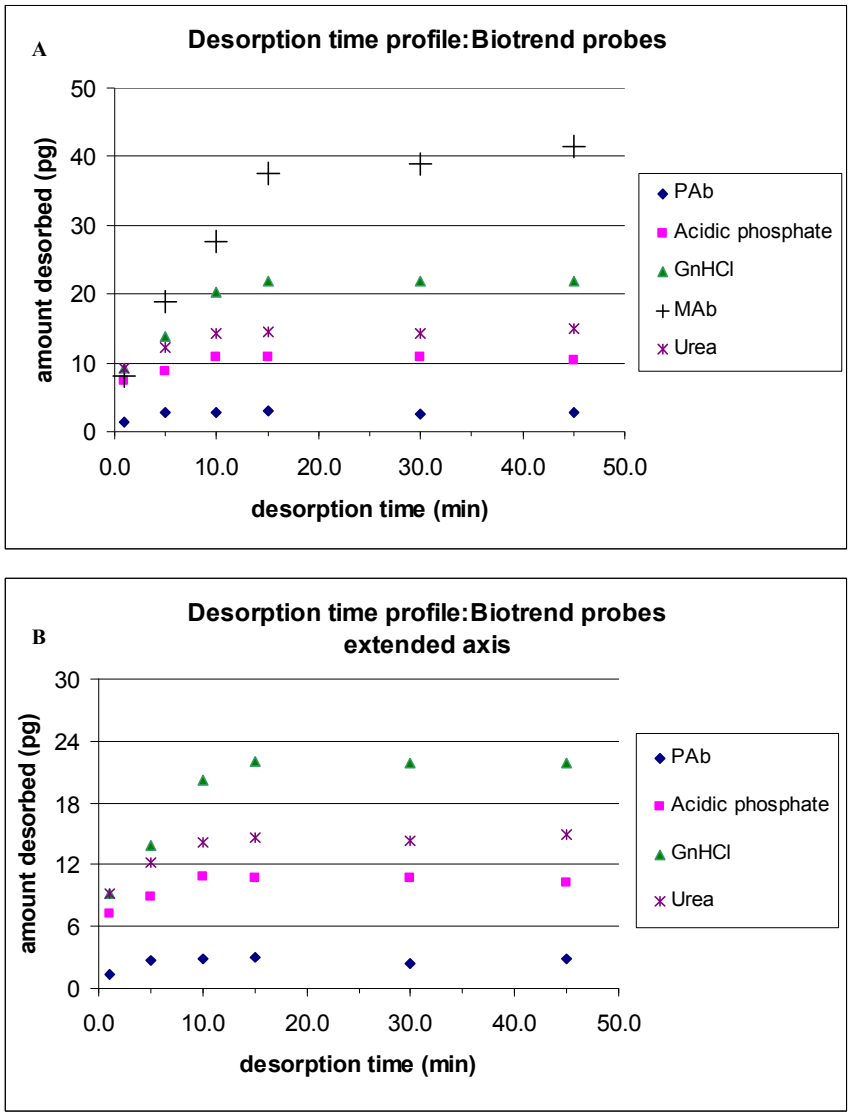


Figure 13: Desorption time profiles of Biotrend probes

3.5 Evaluation of probe stability

Abs are fairly expensive and preparation of immunoaffinity probes is laborious and time consuming. Therefore, it was of importance to evaluate probe stability upon storage and reusability following a number of extractions. The extraction efficiency of probes was studied in earlier works for up to three months storage time.[47] In the current study, this experiment was extended for up to six months from the day of preparation of the first set of rods. Each month, a set of the so called "fresh" probes were prepared from Cortex PAb which was stored in PBS buffer and 0.05% sodium azide at 4^{o}C. Their performance on extraction of oxazepam was compared to the so called "old" probes that had been prepared at the beginning of the six-month period. The latter probes were prepared from the same batch of Abs and were stored in PBS buffer containing 0.05% sodium azide at 4 ^{o}C. The experiment was performed once every month employing a set of four "old" probes that had never been employed for extraction before and a set of four "fresh" rods. The results obtained over a six month period are shown in Figure 14.

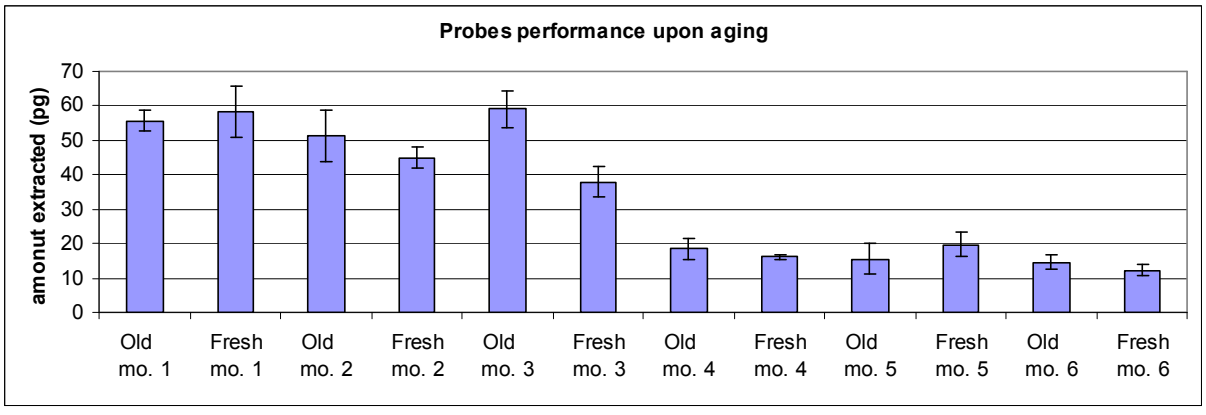


Figure 14: Evaluation of probe stability in six months

No significant decrease in extraction efficiencies was observed for old probes over the first three months (Figure 14). However, the extraction efficiency was considerably lowered after the third month. By the end of the six-month period, the extraction efficiency of "old" probes was reduced to 75% of the first month's extraction efficiency. It can be concluded that the probes can be used for extraction within three months of their preparation without a major decrease in extraction efficiency.

A gradual decrease in extraction efficiency is observed for "fresh" probes after the first month (Figure 14). The results demonstrate that during the three-month storage, immobilized Abs retain extraction efficiency while free Abs, stored in the same conditions, gradually lose their extraction efficiency by 30%. After the three-month period, the free and immobilized Abs' performance is comparable.

The reusability of immunoaffinity SPME probes for multiple extractions has been evaluated in previous studies in which the probes were found to have lost 30% of their capacity after a dozen extractions while the affinity values were not changed significantly.[47]

3.6 Method validation

The method developed for extraction and quantification of benzodiazepines in this research was validated by estimating the precision, limit of detection and dynamic range of the method. Precision of the method using various types of Abs was estimated at 12 % based on relative standard deviation (RSD).

The limit of detection (LOD) was estimated at approximately 0.01ng/mL as determined by the sensitivity of the tandem mass spectrometry system. LOD was calculated based on three times the standard deviation of samples containing 0.01 ng/mL of each drug individually in PBS buffer.

Fitting the calibration data to the non-linear Sips equation allowed for larger dynamic range. Thus, the dynamic range of the method was extended to concentrations that result in 80-90% saturation of the probes. The upper limit of quantification (ULOQ) of the probes is summarized in Table 10 along with the method's precision and LOD. The values reported for each type of Ab are the average of values for Abs of the same type provided by different suppliers.

Antibody type	Validation parameters	Oxazepam	Diazepam	Nordiazepam
PAb	% RSD	8%	9%	7%
	LOD (ng/mL)	0.001	0.001	0.007
	ULOQ (ng/mL)	0.5	0.2	0.2
Acidic phosphate	% RSD	8%	6%	7%
	LOD (ng/mL)	0.002	0.005	0.011
	ULOQ (ng/mL)	0.5	0.2	0.1
Urea	% RSD	5%	7%	5%
	LOD (ng/mL)	0.002	0.005	0.002
	ULOQ (ng/mL)	0.5	0.2	0.1
GnHCl	% RSD	5%	5%	8%
	LOD (ng/mL)	0.001	0.007	0.006
	ULOQ (ng/mL)	0.5	0.2	0.1
MAb	% RSD	12%	10%	10%
	LOD (ng/mL)	0.004	0.006	0.003
	ULOQ (ng/mL)	0.5	0.2	0.2

Table 10: Summary of validation data for immunoaffinity probes employed in this research

3.7 Application of immunoaffinity probes for drug analysis in plasma

Following the evaluation of immunoaffinity probes in buffer, the probes' applicability for drug analysis in biological fluids was examined by direct extraction of benzodiazepines from plasma. Therapeutic and toxic effects of drugs are usually found to have a good correlation with the drug concentrations in plasma. As a result, plasma is the most commonly sampled body fluid in drug analysis and was therefore chosen for this research. Plasma consists of large amounts of proteins to which the drugs can bind to various degrees. In the SPME technique, a small portion of the free drug is extracted. This is advantageous in drug analysis since pharmacological effects of drugs in plasma are exhibited by the unbound fraction of the drugs.

Immunoaffinity probes were used for extraction from 1.5 mL plasma samples which had been spiked with one of the target benzodiazepines. Three probes of each probe type were used for extraction from plasma samples with seven concentrations ranging from 0.01 ng/mL to 5.0 ng/mL. The concentration range of extraction solutions was extended to 5.0 ng/mL as it was observed that, unlike the calibration experiment in buffer, the saturation plateau was not reached at 2.0 ng/mL. Calibration profiles of Fitzgerald probes for each of the target drugs are shown in Figure 15. The data are fitted to the Sips equation as was done for the calibration experiment in buffer. The concentrations of free Ag (in the x-axis) were calculated assuming 95% plasma protein binding for all drugs.

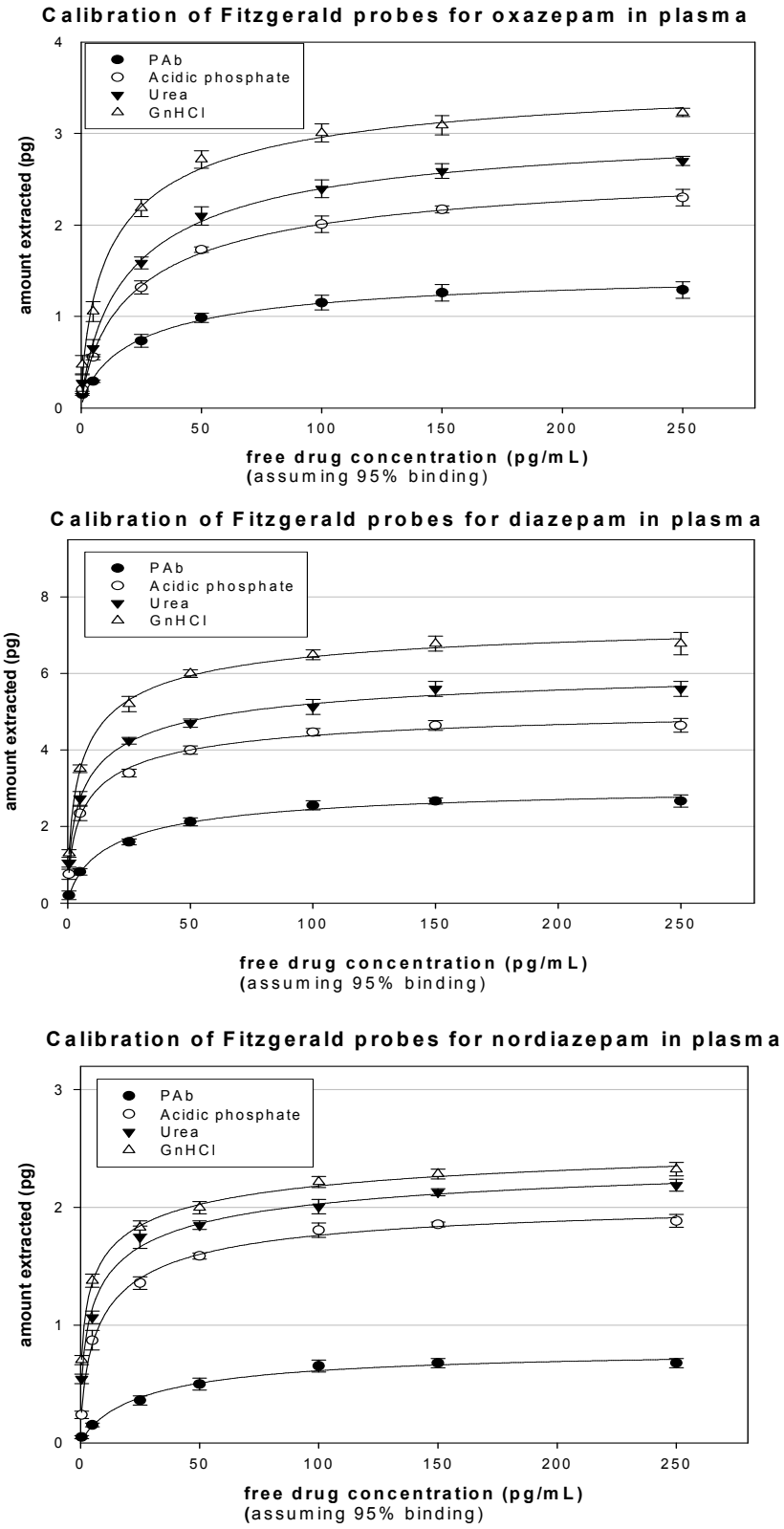


Figure 15: Calibration profiles of Fitzgerald probes for benzodiazepines in plasma

In spite of the high homogeneity and large capacity values of MAb probes for benzodiazepines in PBS buffer (Table 5), MAb probes were found to be inappropriate for extraction of benzodiazepines from plasma and were therefore excluded from the calibration curves represented in Figure 15. The same situation was observed when MAb immunoaffinity probes prepared with US Biological MAbs were employed for extraction from urine.[48]

Following the calibration experiment in plasma, the performance of MAb immunoaffinity probes were examined for extraction from buffer. The results were similar to the extraction from plasma samples which indicates that the plasma matrix has imposed an irreversible impact on the MAb probes. The cause of such behavior is unknown, however, it is suspected that one or a group of compounds present in plasma and urine interfere with the binding of drugs to antigenic sites of MAbs.[48] PAbs and their purified fractions suffer from such interference as well. However, due to their higher heterogeneity, they may exhibit alternative binding sites for which the matrix constituents do not compete with the analyte.

Comparing the results of calibration of Fitzgerald immunoaffinity probes in plasma (Figure 15) with the results in buffer (Figure 10), it is apparent that the amounts of drugs extracted from plasma samples are significantly lower than the amounts extracted from buffer using the same probes. This is due to high plasma protein binding of benzodiazepines which is in the range of 95-99%.[65,66] Thus, the amounts of drugs extracted from plasma samples are expected to be 95-99% less than the amounts extracted from PBS buffer with equal concentration of drugs.

As reported in section 3.3.1, amounts of oxazepam extracted by the different types of immunoaffinity probes are higher than diazepam and nordiazepam. The same trend would

have been expected for extraction from plasma if all three drugs were bound to plasma proteins to the same extent. As Figure 15 demonstrates, the amounts of diazepam extracted by Fitzgerald probes are nearly twice the amount of extracted oxazepam and three times the amount of extracted nordiazepam. Biotrend, Cortex and US Biological immunoaffinity probes have shown the same trend as well. Based on these results, it can be concluded that diazepam protein binding was less than the other two drugs.

Comparing Figure 10 and Figure 15 for each drug, it is also evident that the shapes of the antibody-antigen binding curves are different. For most types of immunoaffinity probes, saturation was reached at 0.5 ng/mL in buffer. In the case of extraction from plasma, on the other hand, the probes were saturated at 2.0 ng/mL or higher concentrations, generating smoother binding curves. Based on the shape of the calibration curves presented in Figure 15, lower values are expected for affinity of Fitzgerald probes in plasma. The affinity values along with capacities and heterogeneity indices of the immunoaffinity probes are estimated for extraction from plasma samples by non-linear regression of calibration data to the Sips equation and are presented in Tables 11 to 14.

The affinity values of various types of probes for benzodiazepines are found to be less in plasma than in buffer (Tables 5 to 8 and 9 to 12). Competition of plasma constituents for some antigenic sites of the Abs was suggested as a reason for failure of the MAb probes to perform adequately in plasma. Lower affinity values of other probes in plasma can also be explained by the possibility of such interference exerted by matrix components.

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.572	0.617	0.601
	b (pg)	1.51	3.21	0.805
	K (mL/ng)	1.88	2.28	1.84
	K (L/mol)	5.41E08	6.49E08	4.98E08
Acidic phosphate	*a*	0.705	0.849	0.812
	b (pg)	2.50	5.46	2.13
	K (mL/ng)	2.52	5.38	5.28
	K (L/mol)	7.22E08	1.53E09	1.43E09
Urea	*a*	0.766	0.911	0.899
	b (pg)	2.97	6.54	2.51
	K (mL/ng)	2.59	5.55	7.39
	K (L/mol)	7.42E08	1.58E09	2.00E09
GnHCl	*a*	0.919	0.958	0.950
	b (pg)	3.55	7.81	2.73
	K (mL/ng)	4.00	6.98	10.2
	K (L/mol)	1.15E09	1.99E09	2.75E09

Table 11: Heterogeneity index (a), capacity (b) and affinity (K) values of Fitzgerald immunoaffinity probes in plasma estimated from non-linear regression of data to Sips equation

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.512	0.688	0.682
	b (pg)	1.44	3.92	1.08
	K (mL/ng)	1.79	2.32	2.07
	K (L/mol)	5.14E08	6.60E08	5.60E08
Acidic phosphate	*a*	0.709	0.885	0.860
	b (pg)	2.97	6.13	1.63
	K (mL/ng)	2.08	6.05	4.36
	K (L/mol)	5.97E08	1.72E09	1.18E09
Urea	*a*	0.763	0.902	0.898
	b (pg)	2.59	9.05	3.55
	K (mL/ng)	2.41	7.18	5.78
	K (L/mol)	6.92E08	2.04E09	1.56E09
GnHCl	*a*	0.929	0.968	0.959
	b (pg)	3.16	11.72	3.78
	K (mL/ng)	3.58	8.95	8.53
	K (L/mol)	1.03E09	2.55E09	2.31E09

Table 12: Heterogeneity index (a), capacity (b) and affinity (K) values of Biotrend immunoaffinity probes in plasma estimated from non-linear regression of data to Sips equation

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.516	0.674	0.599
	b (pg)	1.43	5.87	1.59
	K (mL/ng)	0.972	1.92	2.42
	K (L/mol)	2.78E08	5.47E08	6.94E08
Acidic phosphate	*a*	0.609	0.890	0.824
	b	1.90	9.41	1.89
	K (mL/ng)	1.47	2.67	2.82
	K (L/mol)	4.22E08	7.60E08	8.10E08
Urea	*a*	0.745	0.933	0.897
	b (pg)	2.16	12.9	2.57
	K (mL/ng)	2.51	3.38	3.94
	K (L/mol)	7.21E08	9.62E08	1.13E09
GnHCl	*a*	0.902	0.980	0.964
	b (pg)	2.38	18.8	3.12
	K (mL/ng)	3.08	4.96	5.30
	K (L/mol)	8.84E08	1.41E09	1.52E09

Table 13: Heterogeneity index (a), capacity (b) and affinity (K) values of Cortex immunoaffinity probes in plasma estimated from non-linear regression of data to Sips equation

Antibody type	Sips equation values	Oxazepam	Diazepam	Nordiazepam
PAb	*a*	0.510	0.628	0.573
	b (pg)	1.39	2.36	0.640
	K (mL/ng)	1.47	3.61	5.82
	K (L/mol)	4.22E08	1.03E09	1.58E09
Acidic phosphate	*a*	0.716	0.867	0.800
	b (pg)	4.12	6.96	0.924
	K (mL/ng)	1.82	3.87	7.09
	K (L/mol)	5.22E08	1.10E09	1.92E09
Urea	*a*	0.886	0.974	0.922
	b (pg)	4.47	8.05	1.38
	K (mL/ng)	2.02	4.49	8.95
	K (L/mol)	5.80E08	1.28E09	2.42E09
GnHCl	*a*	0.924	0.980	0.962
	b (pg)	4.60	8.97	2.06
	K (mL/ng)	3.28	5.16	9.87
	K (L/mol)	9.41E08	1.47E09	2.67E09

Table 14: Heterogeneity index (a), capacity (b) and affinity (K) values of US Biological immunoaffinity probes in plasma estimated from non-linear regression of data to Sips equation

While the decrease in probe affinities in plasma were found to be as large as an order of magnitude, relative to the values in buffer, the amounts of extracted drugs were reduced to below 1% for oxazepam, 5-7% for diazepam and 1-2% for nordiazepam (Tables **9-12**). The low amounts extracted from plasma are expected to be based on the high plasma protein binding of benzodiazepines as stated earlier in the text. The heterogeneity indices are in the same range for plasma and buffer samples and are increased for probes with the purity of Abs used for preparation of the probes. Despite the differences in the values calculated from the Sips equation for buffer and plasma, the patterns are analogous in terms of changes in affinity and capacity values for different types of probes and different drugs.

The sensitivity of the detector used for the analysis of extracted drugs determined the LOD which was calculated based on 3 times the standard deviation for extractions from 0.01 ng/mL samples. The LOD for extraction from plasma samples ranged from 0.001 ng/mL to 0.008 ng/mL. Since calibration data are fitted to a non-linear equation, the dynamic range was extended to 2 ng/mL at which 80-90% of antigenic sites of Abs were bound to drugs (80-90% saturation). It should be noted that this upper limit of quantification (ULOQ) depends on the affinity of the Abs. Lower affinity values (as observed for plasma samples) result in saturation at higher concentrations which subsequently result in higher limits of quantification. The precision of probes in plasma (estimated by measuring %RSD) was calculated to be less than 10% for all three drugs.

The results demonstrate that the immunoaffinity probes prepared with PAb and affinity-purified fractions of PAb can be successfully employed for measuring benzodiazepines at low ng/mL concentrations in plasma samples.

Chapter 4 Conclusions

Development of a SPME device that would allow for direct extraction of trace levels of pharmaceuticals from biological matrices is an important step towards improvement of sample preparation in drug analysis. Immunoaffinity SPME probes were evaluated for such analyses in this study.

A purification method had been developed based on affinity chromatography for fractionation of polyclonal antibodies into more homogeneous fractions.[47] During the course of this work, this method was improved and resulted in production of three more homogeneous fractions of antibodies with higher affinities and capacities towards benzodiazepines.

As antibodies serve as the extraction phase, evaluation of their binding characteristics was important for the purpose of this research. It was also of great interest to evaluate MAbs along with PAbs from different suppliers for binding characteristics and performance upon immobilization on silica probes. Five different types of immunoaffinity probes were prepared with Abs provided by each supplier. The probe types were as follows: PAb, MAb and three affinity purified fractions of PAb referred to as acidic phosphate, urea and GnHCl. The values estimated for valance and affinity of the Abs towards oxazepam prior to immobilization were found to be comparable for Abs of the same type obtained from different suppliers. The affinity and valance values increased in the following order: PAb < acidic phosphate < urea < GnHCl < MAb.

The binding characteristics of Abs were evaluated following immobilization to glass rods. For each batch of Ab, the affinities were increased after immobilization. Although different values were estimated for affinity and capacity of immunoaffinity probes prepared with Abs from various suppliers, the probes with the same type of Abs performed similarly in terms of extraction of benzodiazepines from PBS buffer. All probes were found to exhibit higher capacities and lower affinities for oxazepam compared to the other two drugs.

The probes prepared with highly cross-reactive Abs, as indicated by the suppliers, were found to have similar cross-reactivity for all three drugs while affinity purified fractions of Abs were slightly less cross-reactive towards diazepam and nordiazepam. A batch of probes that were prepared with oxazepam-specific Abs was found to have lower cross-reactivities for diazepam and nordiazepam. Thus, the oxazepam-specific probes can be successfully employed for extraction of oxazepam from samples that contain multiple drugs, without significant interference from diazepam and nordiazepam. In case of real samples, a variety of other molecules may be present that will compete for extraction with the analyte(s) of interest. Since such conditions are based on each individual sample, the cross-reactivity of probes for the potentially interfering compounds should be determined for each specific application.

The extraction and desorption conditions were optimized for the probes. Extraction was performed for 45 minutes at 100 rpm while desorption was conducted in 15 minutes with agitation at 100 rpm. The probes were stored in PBS buffer containing 0.05% sodium azide at $4^{o}C$, and were found to be stable with regard to extraction efficiency for three months following preparation.

The applicability of various immunoaffinity probes for extraction of trace amounts of benzodiazepines from spiked plasma samples was demonstrated. In spite of their high

capacities and homogeneities, MAb probes were found to be ineffective for such applications. While performance of PAb probes in plasma samples were acceptable, affinity purified fractions of PAbs outperformed the other probes in extraction of benzodiazepines from plasma. Since MAb probes failed to adequately extract the benzodiazepines from plasma samples, affinity purification of PAbs was confirmed as a useful method for preparation of immunoaffinity probes to be used directly in biological matrices. The applicability of immunoaffinity SPME technique is extended by the use of PAbs for immunoaffinity probes because a wider variety of PAbs are commercially available and they are more cost-effective than MAbs.

Non-specific binding was found to be insignificant in the range of concentrations used in this study. Excluding MAb probes, the method precision is below 10% for buffer and plasma extractions. The method's LOD is 0.01 ng/mL and ULOQ is estimated to be 0.5 ng/mL and 2.0 ng/mL respectively.

The pattern observed for Abs supplied by different companies was found to be similar in terms of affinity, homogeneity, capacity and cross-reactivity for the three benzodiazepines. This clearly demonstrates the usefulness of immunoaffinity SPME as a broadly applicable technique for extraction of small molecules from complex matrices.

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