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A study of the performance characteristics of immunoaffinity solid phase microextraction probes for extraction of a range of benzodiazepines

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Abstract

Immunoaffinity solid phase microextraction (SPME) probes have been developed with antibodies specific for the benzodiazepine class of drugs, covalently immobilized to glass rods. This involved both purification of the polyclonal antibodies to isolate the drug-specific fraction, and optimization of the immobilization procedure. Such probes have been used previously for the extraction of 7-amnoflunitrazepam. This article presents a comprehensive study of their performance and characteristics beyond that described previously, and an evaluation of their application to additional benzodiazepines. The influence of non-specific drug binding (nsb) was determined, with the result that nsb was found to be insignificant for the probes when used in their dynamic range. Immobilized antibodies had specific affinities in the range of 10^9–10^{10} M^{-1}. Cross-reactivity was evaluated both for a range of benzodiazepines as well as a structurally unrelated molecule (erythromycin). For analysis of benzodiazepines individually or in the presence of erythromycin, limits of detection were 0.001–0.015 ng/mL depending on the antibody, and the dynamic range (based on 80–90% antigenic site occupancy) extended to 0.2–2 ng/mL.

Keywords: Immunoaffinity sample preparation; Solid phase microextraction; LC-MS/MS; Polyclonal antibodies; Antibody affinity

1. Introduction

The general adsorbents for solid phase microextraction (SPME) drug analysis for LC applications, such as divinylbenzene and polypyrrole have been shown to be good extraction phases for a wide range of small molecule drugs and many of their phase I metabolites [1–3]. While broad applicability to a range of target compounds (small molecules with intermediate polarity) is beneficial for some applications, allowing many of these compounds to be extracted simultaneously, those with stronger affinities may displace those with weaker affinities. This complicates quantification of one compound in the presence of another with higher affinity [4]. Several reviews of the application of SPME technologies for the analysis of drugs are available [5–9]. The development of immunoaffinity extraction probes by covalent immobilization of drug-specific antibodies may provide an alternative to circumvent the limitations of the general sorbents. A specific antibody is expected to have a high degree of selectivity for the target class of drugs compared to non-structurally related compounds [10,11]. While it would still extract different compounds with similar structures similarly, many of the potential competing compounds would be eliminated. Unlike general sorbents, antibody affinity can also be selectively inactivated through the use of non-physiological conditions, typically by temporary denaturation or unfolding of the protein [12]. Thus, desorption of extracted analytes should be much simpler than for the general adsorbents.

To date, drug analysis applications with SPME probes employing antibodies covalently immobilized to glass rods have been developed for theophylline [13] and 7-aminoflunitrazepam [14] but a comprehensive study of their performance and characteristics has not been described. The techniques involved in their preparation are similar to those employed in immunoaffinity sorbent preparation [15,16], although significantly miniaturized with sample volume independent quantification. In the present article we describe considerations in the design, development, evaluation, and implementation of immunoaffinity SPME

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probes as well as considerations for the rational selection of antibodies and application to additional benzodiazepines.

1.1. Antibody immobilization

Numerous antibody immobilization techniques are reported in the literature. Many of these have been developed for biosensor, immunoaffinity chromatography, and ELISA techniques. The strategies can be broadly divided into three categories: (1) non-covalent immobilization to a substrate, (2) immobilization via entrapment in a cross-linked matrix such as sol–gel or acrylamide, and (3) covalent immobilization to a substrate utilizing a linker molecule [17]. It has been recognized that covalent immobilization is preferred when the coated substrate is subjected to flow or extended times in solution as leaching of the immobilized molecule from the substrate is prevented [18,19]. Covalent immobilizations are used extensively in immunoaffinity chromatography and immunoextractions [20,21].

For the work presented here, glutaraldehyde cross-linking was used. In the most straightforward form of the reaction, this bifunctional aldehyde reacts with both protein amines and an amine functionalized surface through formation of Schiff bases. These are unstable under acidic conditions and break down to regenerate the original aldehyde and amine. Reaction with a reductant such as cyanoborohydride, before the aldehyde regenerates, yields stable secondary amine linkages. In practice other cross-linking reactions are feasible, and may even predominate [22,23]. Some researchers have found that immobilized proteins treated with reductant exhibit higher activity and stability than untreated proteins. However, it has been established that GA treated proteins do not regenerate lysine, indicating that alternate reactions that do not produce Schiff bases are functioning [24]. It was of interest to determine the effect of reduction of Schiff bases on the stability of the new probes.

1.2. Research goals

In this manuscript, we describe the full development process for the SPME probes described previously for 7-aminoflunitrazepam analysis [14] and evaluate their performance relative the generally accepted standards for immunoaffinity analysis devices. Whereas the previous report focused on extraction of 7-aminoflunitrazepam, the current work extends the application by addressing considerations for analysis of other members of the benzodiazepine class of drugs. In particular, aspects of the probe development which were not significant for the previous application, but that are important for continued development or application to other drugs, are addressed here. Considerations for linear and non-linear calibration, and evaluation of parameters such as affinity, affinity heterogeneity, probe capacity, non-specific binding, and extraction kinetics are discussed. Also, strategies for the rational selection of antibodies, optimal preparation of the probes, conditions for elution of extracted analytes, an evaluation of surface binding densities of active antibodies, antibody cross-reactivity and its effect on calibration are presented.

2. Materials and methods

2.1. Materials

Polyclonal antibodies were obtained from Cortex Biochemical (San Leandro, CA) as immune serum. Generic IgG was isolated from non-immunized sheep serum obtained from Bioreclamation Inc. (Hicksville, NY). All antibodies were purified prior to use as described below. The affinity columns for antibody purification were obtained from Pierce Biotechnology (Rockford, IL). Benzodiazepines were obtained from Cerilliant (Round Rock, TX) as certified standards (1 mg/mL). Fig. 1 shows the structures of the benzodiazepines used here. Methyl ^{3}H-diazepam was obtained from Perkin-Elmer (Boston, MA, 76 Ci/mmol, 1 mCi/mL).

Phosphate buffered saline (PBS; pH 7.4) was prepared in-house. Borosilicate glass rods (4 mm × 10 cm) were obtained from the University of Waterloo glass-blowing shop. The conductivity meter (VWR brand) was from VWR Scientific (Mississagua, ON). The centrifugal filtering devices for antibody preparation and concentration were Amicon® Ultra-4 (4 mL volume) with 30,000 MWCO high flow-rate membranes from Millipore Corporation (Bedford, MA). The ultrafiltration microconcentrators (UFMC) for free antibody affinity tests (Scatchard analysis) were Amicon Ultrafree-MC (0.4 mL volume) with 30,000 MWCO low flow-rate membranes from Millipore Corporation. Tritium counting was performed on a Beckman LS 1701 liquid scintillation counter (Mississauga, ON). The scintillation cocktail (CytoScint-ES) was from MP Biomedicals (Irvine, CA). Other materials required were as described previously [14].

Non-linear regression analysis was performed using SigmaPlot ver. 9.0 software. Data were fit to the Sips equation in the form: $y = b(Kx)^a/(1 + (Kx)^a)$ where x is the equilibrium

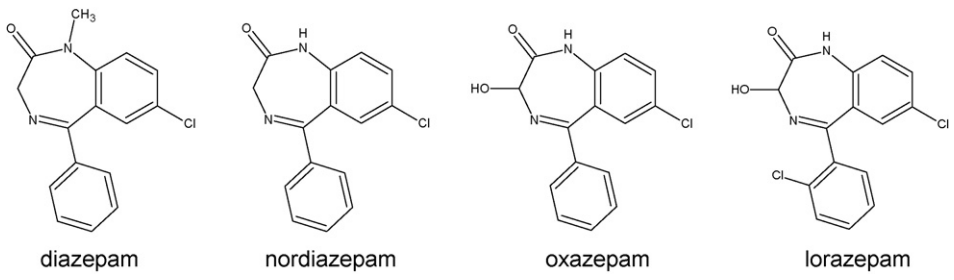

Fig. 1. Structures of the benzodiazepines employed in the current study.

sample concentration of free analyte (ng/mL), y is the amount extracted by the probes (pg), b is probe capacity (pg), K is antibody affinity (mL/ng), and a is the Sips index of homogeneity ($a = 1.0$ at maximum homogeneity) [25,26]. Constraints for the fittings were: $a > 0$, $a \leq 1.05$, $K > 0$. y was fitted to the function with a weighting $1/y^2$. Linear regression analysis was performed using Microsoft Excel 2000.

2.2. Antibody purification

Antibodies were initially purified to isolate IgG using a protein G affinity column as described previously [14]. A portion of the anti-oxazepam polyclonal antibody was further purified to enrich the benzodiazepine specific fraction using an oxazepam affinity column as described previously [14]. For fractionation, 15–20 mg of the protein G purified polyclonal antibody was loaded onto an oxazepam affinity column. Un-bound and non-specifically bound antibody was removed. The first useful specific antibody (PO4 fraction) was eluted with phosphate buffer (0.1 M, pH 2) in 0.4 mL fractions into tubes containing 100 μL of 1 M phosphate buffer pH 7.5. Fractions containing high concentrations of IgG were pooled and immediately transferred to PBS containing 0.05% sodium azide (NaN_3).

Fractions with higher specific binding were obtained from the oxazepam affinity column by elution with higher strength eluents (7 M urea in 50 mM sodium acetate buffer pH 4 (urea) or 6 M guanidine hydrochloride in 50 mM sodium acetate buffer pH 4 (GnHCl)) [27]. The pools from urea and GnHCl elutions were re-natured after elution [27]. In the case of the urea pool, where the denaturation reportedly involved loss of only tertiary structure in the protein, renaturation was accomplished simply by transferring the protein to PBS + NaN_3 through a minimum of four exchanges using the 4 mL centrifugal filtering devices. This was followed by a 10× dilution with the new buffer. Finally, protein was stored for a minimum of overnight at 4 °C. In the case of the GnHCl pool, where denaturation reportedly involved loss of both secondary and tertiary structure, renaturation was accomplished by first transferring the protein to urea and allowing overnight storage at 4 °C, and next by transferring to PBS + NaN_3 with a second overnight storage. In all cases, IgG concentrations were estimated by measuring absorbance at 280 nm, and converting to concentration (mg/mL) using a molar absorptivity of 1.35 mg^{-1} mL cm^{-1}. Purified antibody was stored in PBS + NaN_3 either at 4 °C for short-term storage or at −20 °C for long-term storage of up to 1 year.

2.3. Characterization of the antibody preparations

After purification, free antibody preparations were characterized for oxazepam valence, affinity, and specific binding by incubating a known amount of protein with different concentrations of oxazepam in PBS + NaN_3 (400 μL) as described previously [14]. Briefly, after equilibration, ca. 40 μL of the buffer containing un-bound drug was removed by ultrafiltration. Because just 10% of sample volume was removed in this process, the equilibrium between bound and unbound analyte in the sample was not expected to be significantly disrupted. Thirty microliter of filtrate was mixed with 90 μL of methanol containing IS (lorazepam, 75 ng/mL), and the concentration of un-bound drug was determined by LC-MS/MS ($n = 3$). The analysis was used to confirm activity in the IgG fractions eluted, to monitor the degree of purification, and to provide a rough estimate of affinity. Generic IgG and the polyclonal IgG prior to oxazepam affinity purification were also monitored for comparison.

2.4. Immobilization of IgG to glass rods

After the activity of the IgG of interest was verified, antibodies were covalently immobilized to glass rods by glutaraldehyde cross-linking as described previously [14]. Briefly, glutaraldehyde activated probes were immersed in antibody solution (0.2–0.6 mg/mL in PBS + NaN_3) to a depth of 2.5 cm with agitation for 10 h or overnight. Afterward, unreacted glutaraldehyde was deactivated by immersing in an aqueous ethanolamine solution (0.3 M, adjusted to pH 7.5 with HCl). Probes were stored in PBS + NaN_3 + 0.2 mg/mL sodium cyanoborohydride ($NaCNBH_3$) at 4 °C for 24–48 h to reduce the imide to amine and stabilize the covalent linkage. For long-term storage, the probes were stored in PBS + NaN_3 at 4 °C with the storage solution changed every 1–2 months. It should be noted that the actual probes used in the current and previous [14] studies were not necessarily the same.

2.5. Extraction of samples

The following general extraction procedure was used, with deviations noted in the specific sections of the discussion. Prior to extraction, probes were allowed to warm to room temperature. Samples (15 mL) were prepared just prior to an experiment by spiking intermediate standards into PBS (room temperature). Care was taken to ensure that final methanol concentration in the samples was well below 1%, a level that was determined to not impact antibody binding of drug. The extraction time was 30 min with gentle shaking on a rotary shaker. After extraction the rods were rinsed with a stream of nanopure water from a wash bottle for ca. 5 s each. This procedure had been previously determined to minimize carryover of sample to the desorption solution. The probes were immediately set into desorption solution (500 μL of 75% methanol–25% water containing 7.5 ng/mL lorazepam as internal standard) contained in a 96-well, deep-well plate with 1 mL well volume and round well bottoms. The desorption time was 30 min with shaking. After desorption, probes were rinsed briefly with nanopure water and returned to the storage bottles. The plates were dried under a stream of UHP grade nitrogen by means of a 96-well plate dryer. For analysis, wells were reconstituted with a solution of 75% methanol–25% water (25–75 μL). A smaller volume was used if optimal sensitivity was required. A larger volume was used if multiple injections from each well were required.

2.6. Chromatographic analysis

The chromatographic system used comprised a Shimadzu gradient LC system with a model SCL 10 AVP system controller,

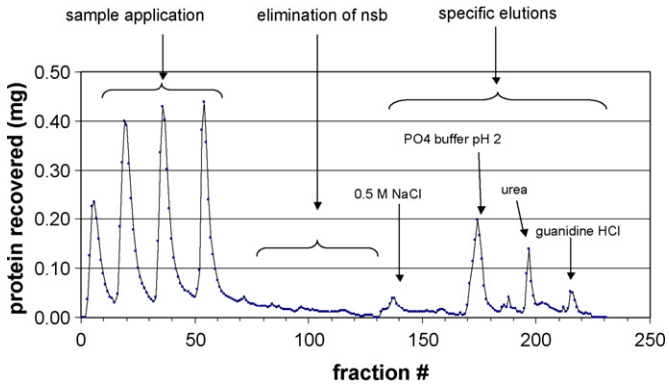

Fig. 2. Amounts of protein recovered at each step during fractionation of polyclonal antibody with oxazepam-specific affinity column.

two model LC 10 AVP dual piston pumps, and a model DGU 14A on-line mobile phase degasser (Mandel Scientific, Guelph, ON), a CTC analytics model HTS PAL autosampler from Leap Scientific (Carrboro, NC), and a Sciex model API 3000 turbo ionspray tandem mass spectrometer (Toronto, ON). The column was a Waters Symmetry Shield RP18, 2.1 mm × 50 mm, 5 μm particle size (Waters Corporation, Milford, MA). Gradient elution was used to minimize total analysis time (5 min total), while allowing for sufficient separation of analytes from elution of non-retained substances and a column cleaning after each run. Mobile phases were as follows: (A) acetonitrile–water (10:90) with 0.1% acetic acid; and (B) acetonitrile–water (90:10) with 0.1% acetic acid. Mobile phase flow was 0.5 mL/min and the gradient used was as follows: 0% B for the first 0.5 min, ramped to 90% B over 2 min, held for 1.5 min, and finally returned to 0% B for 1 min. A 20 μL injection volume was used. The HPLC effluent was analysed after ESI in positive ion mode with selected reaction monitoring. Transitions monitored were; diazepam, 285.2/154.1; nordiazepam, 271.1/140.0; oxazepam, 287.1/241.1; lorazepam, 321.1/275.1. Other details are provided elsewhere [14].

3. Results and discussion

3.1. Antibody fractionation

In a polyclonal preparation a significant proportion of the total antibody is non-specific for the target analyte (generic IgG). Where available surface area far exceeds that required to provide sufficient capacity, as would typically be the case for immunosorbent preparation, the amount of generic IgG present in a polyclonal pool is irrelevant. For SPME probes where surface area is limited, there is a significant advantage to using as high a purity of specific IgG as possible. In order to increase probe capacity polyclonal antibodies were purified to remove as much of the generic IgG as possible, and to isolate and concentrate fractions of high specificity antibody. Fig. 2 illustrates the results of an affinity chromatography fractionation of the PAb using an immobilized oxazepam affinity column. Fraction number is plotted on the *x*-axis and protein (mg) in each fraction is plotted on the *y*-axis.

Table 1
Summary of proportion of protein eluted with each fraction from the oxazepam affinity chromatography column

	mg	%
Applied	14.6	
Unbound	10.0	68.2
nsb elutions	1.1	7.6
0.5 M NaCl	0.5	3.7
PO4 pH 2	1.4	9.3
Urea	0.7	4.8
GnHCl	0.4	2.8
Unaccounted	0.5	3.7

Table 1 presents the amounts of protein recovered in each fraction shown in Fig. 2. In this case, the protein was applied in four aliquots due to a limited column reservoir volume. The first contained 2.6 mg; each of the next three contained 4 mg. The four large peaks eluting before fraction 70 are the unbound IgG that eluted after each sample was applied, and appears in Table 1 labelled *unbound*. The *unaccounted* protein may be either protein that did not elute from the column even after treatment with GnHCl, or it may simply be representative of cumulative error in the volume estimations for each fraction. The fractions from the affinity column were first characterized for affinity and specific binding and the high affinity fractions were immobilized to produce the SPME probes.

3.1.1. Characterisation of purified free antibodies

Scatchard analysis was used for characterization of the eluted fractions. The goal was to verify the effectiveness of the fractionation and the activity of the antibodies prior to immobilization. From the results, specific binding was calculated (ng drug bound per mg protein) and affinity and valence were estimated. The results of Scatchard analysis of the antibodies for five pools from the PAb fractionation and the original protein G purified PAb are presented in Table 2. The linear regression data were used to provide an estimate of affinity (K) and valence (n) of the samples. Because only three data points were plotted for each sample, due to the limited protein available, there is expected to be a high degree of error in the results. Even so, the trends observed in the data indicate a successful fractionation. A more homogeneous population of antibodies will produce a more linear correlation and it was observed that the degree of linearity in the data improved with successive fractions. Correlation coefficients (R^2) for the Scatchard plot data (not shown) in were as follows: PAb, 0.761; PO4, 0.834; urea, 0.944; GnHCl, 0.999. Increases

Table 2
Summary of Scatchard characterization of antibody preparations prior to immobilization: oxazepam affinity (K), valence (n), and specific binding

	K (M^{-1})	x-intercept valence (n)	Spec. binding (ng/mg)
Sample 4	1.0×10^7	0.023	38
0.5 M	2.1×10^7	0.022	111
PO4	4.3×10^9	0.314	576
Urea	7.6×10^9	0.272	590
GnHCl	6.0×10^9	0.414	773
PAb	6.5×10^7	0.092	139

in valence, affinity and specific binding with purification were also observed.

From Table 2 it is seen that the pool from the fourth sample application has low specificity and affinity, which indicates that there was no significant breakthrough of specific IgG during sample application. The affinity seen in this fraction is likely due to non-specific affinity, as benzodiazepines have a high degree of non-specific general protein binding (see below). It is also seen that the pool eluting with 0.5 M salt has a slightly higher affinity than the sample application protein, as well as a specific binding similar to the polyclonal antibody. This fraction was considered as the first specific fraction eluted, but because of its low affinity and specific binding, was not used for immobilization. The affinity appears to be lower for GnHCl than urea, although the difference may not be significant relative to the experimental error, or it may be due to incomplete renaturation of the antibody.

A high degree of selectivity is regarded as one of the primary reasons for employing immunoaffinity sample preparation. From these data we see that non-specific binding can have affinities on the order of just two orders of magnitude lower than for specific binding. This may, however, be a worst-case scenario as the benzodiazepines are recognized for a very high degree of protein binding to serum proteins (>90%). This being said, non-specific binding may be a significant consideration for immunoaffinity sample preparation from samples with non-target analytes having an inherent high level of protein-binding. This leads to the conclusion that non-specific binding of interferents could complicate extractions if such compounds are present in samples at concentrations of 100× higher than that of the target analyte. Fortunately, most interfering substances in biological samples have a relatively low degree of protein-binding.

From the calculated affinity values and with reference to Eq. (1), the approximate free drug concentrations [H] that would produce 50% oxazepam saturation of the antigenic sites ([AbH] = [Ab]) are determined to be in the range of 10^{-9} M (~0.3 ng/mL), which is a range appropriate for quantitative analysis of trace concentrations of drug.

$$K = \frac{[AbH]}{[Ab][H]} \quad (1)$$

3.2. Evaluation of effect of cyanoborohydride on probe stability

As discussed, it was of interest to determine the effectiveness of $NaCNBH_3$ treatment of the probes on their performance and stability. Rods with PAb immobilized were evaluated (n = 3) several times over 2 months to evaluate the effect of imide bond reduction by $NaCNBH_3$ on overall stability of the probes. The samples were ^{3}H-diazepam in PBS (4 ng/mL). One set of rods was treated with $NaCNBH_3$ after preparation and the other was not. After extraction bound drug was eluted as described above. While the rods initially had similar performance, the untreated rods lost about 50% of their extraction capability over the 2 months while the $NaCNBH_3$ treated probes actually showed a 50% increase in amount extracted over this time. It was not clear why an improvement in performance would result, but all subsequent probe preparations utilized $NaCNBH_3$ treatment.

3.3. Performance of probes with different immobilized antibodies

Different antibody preparations were immobilized to glass rods to evaluate the effect on probe performance. Preparations evaluated were: generic IgG, the original polyclonal (PAb) antibody, and the acidic phosphate and urea fractions of PAb. The probes were used to extract a solution of diazepam in buffer (1 ng/mL, 30 min extraction, static). Fig. 3 shows a comparison of the amounts extracted by the different probes. As expected, probes prepared with higher affinity and specific binding proteins extracted drug to a higher extent. The fraction recovered from acidic phosphate buffer elution was obtained in the highest quantity, and with the most consistent affinity characteristics. While the urea-eluted fraction was of interest for its higher amount of drug extracted, it was obtained in lower quantity, and its specific binding was not as consistent from batch to batch. This difficulty could be circumvented in future by isolating specific IgG from a larger batch of antibody. For these reasons, only the fraction eluted with acidic phosphate buffer was used in subsequent immobilizations. In the remainder of the text 'frac. PAb' refers to the fraction of PAb eluted from the oxazepam-affinity column with acidic phosphate buffer.

In order to fully evaluate the properties and characteristics of the immunoaffinity probes on glass rods, three sets of probes with 21 probes in each set were prepared. One set was prepared for each of generic IgG, PAb, and frac. PAb. The probes were then used for seven point calibrations (n = 3) individually for each of the drugs of interest (diazepam, nordiazepam, and oxazepam). The results are shown in the left panels of Fig. 4. The figures describe the adsorption isotherms for each drug and antibody. The plateau region of each curve is indicative of the region of antibody saturation. From the data for oxazepam, we see that the drug concentration resulting in half-saturation of the frac. PAb antibody occurs at about 0.1 ng/mL. This is in good agreement with that estimated from the Scatchard analysis of free

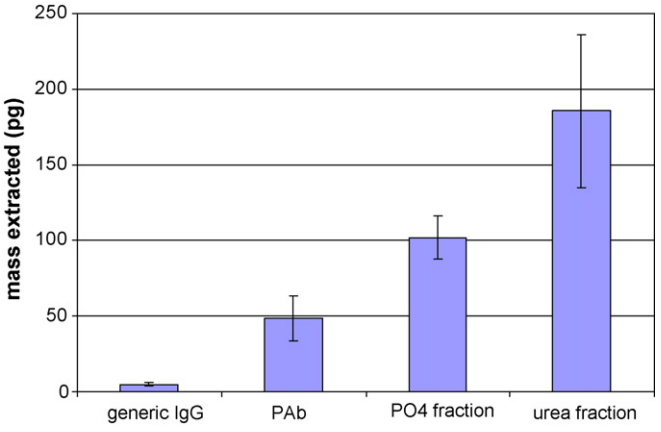

Fig. 3. Comparison of probe extraction performance with different antibodies immobilized. Sample, diazepam 1 ng/mL in PBS, n = 3. LOD: 0.6 pg extracted; PO4 fraction: acidic phosphate fraction.

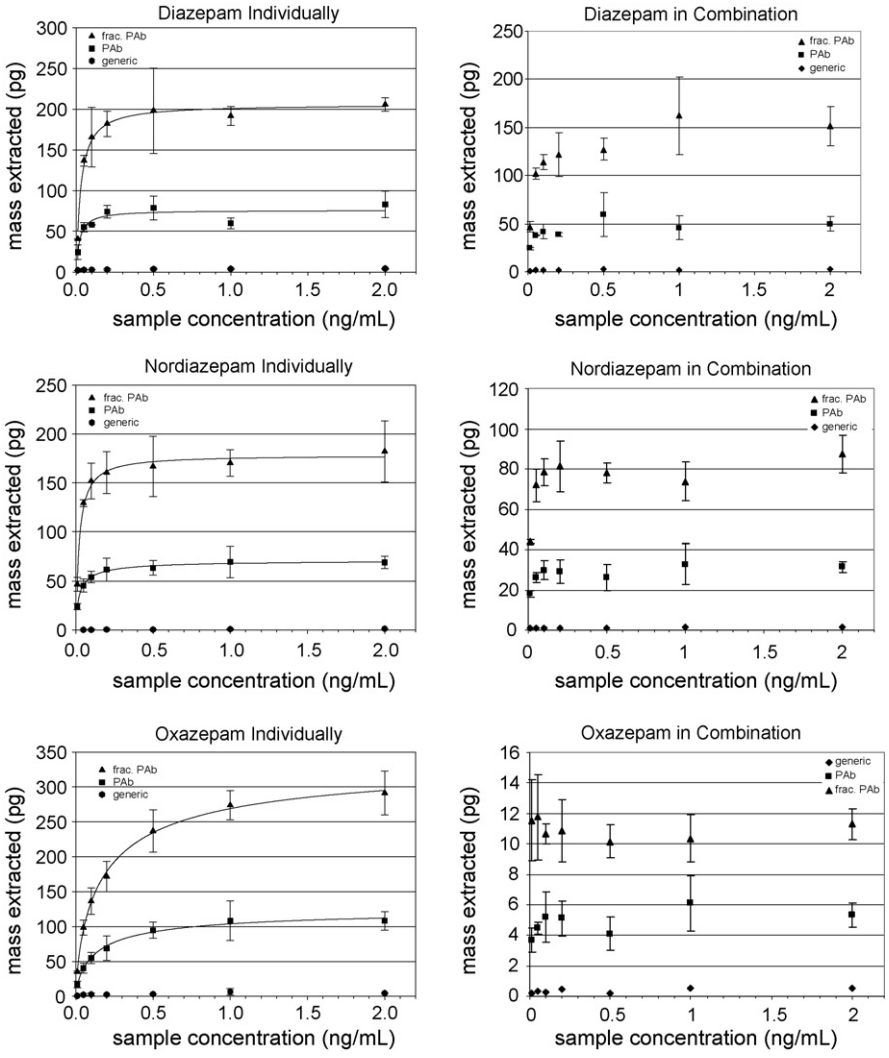

Fig. 4. Comparison of benzodiazepine calibrations—drugs individually and in combination, $n = 3$. Calibration data were fitted to the Sips equation using non-linear regression analysis. Left panels: only the stated drug was present in the samples. Right panels: diazepam, nordiazepam and oxazepam were each present at the concentration indicated.

antibody (0.3 ng/mL), which indicates that antibody affinity was not lost on immobilization, as is commonly encountered [28].

3.4. Contribution of non-specific binding

It is important to determine the contribution of non-specific binding (nsb) to total observed binding, particularly given the foregoing discussion of specific versus non-specific binding affinity for the benzodiazepines, which have a high degree of generalized protein-binding. Calibration can be compromised if nsb is significant. Generic IgG has no specific affinity for benzodiazepines, so all binding to these probes is non-specific. It can be assumed that this degree of non-specific binding is also a component of the binding of the specific antibody probes. As can be seen from Fig. 4, the contribution of nsb to total binding is not significant ($\leq$5%) in the concentration range tested. For some applications, a sorbent with sorbed analyte is exposed to a non-ionic surfactant solution to remove non-specifically bound material prior to elution of specifically bound analytes. When the immunoaffinity SPME probes with extracted drug were soaked in a Tween 20 solution (0.1%) for 30 min prior to solvent desorption, the amount of non-specifically bound drug was reduced by ca. 50%, but the amount of specifically bound drug was also reduced by ca. 30%. For these reasons, no effort was made to eliminate non-specifically bound drug from the probes prior to elution of the specifically bound drug.

3.5. Evaluation of conditions permitting negligible depletion

Smaller sample volumes are convenient for handling, but because of the high affinity of the immunoaffinity probes, significant depletion of analyte may occur at the sample concentrations appropriate for the probes (low to sub-ng/mL) when sample volume is small. During extraction, conditions of negligible depletion are preferred to allow for consistent calibration profiles regardless of sample volume, and to permit the fitting of the data for non-linear regression analysis (see Section 3.6). Under conditions of appreciable depletion, initial sample concentration may not be substituted for the equilibrium sample concentra-

tion required by the analysis and accurate regression analysis is significantly complicated.

Negligible depletion of the sample generally means that the proportion of total analyte in the sample extracted by the SPME probe is in line with experimental error, and so may safely be ignored [29]. In the case of the immunoaffinity probes, 10% depletion may be considered negligible as this typically corresponds to the degree of experimental error. For lower capacity probes prepared with the polyclonal antibody, sample volume variation from 1.5 to 15 mL does not impact the shape of the calibration profile, even though proportion extracted approached 15% for low concentration samples from the 1.5 mL volume extractions. For the higher capacity probes, however, a significant proportion of total drug is extracted from the low concentration samples when 1.5 mL sample volumes are used, and a noticeable difference in the shapes of the calibration curves emerges in the region of the calibration where depletion is not negligible (data not shown). From the data in Fig. 4 (left panels) where 15 mL samples were used, it was observed that depletion was negligible (≤10%) for all data points except PAb: 0.01 ng/mL and frac. PAb: 0.01 and 0.05 ng/mL, and there was no difference in the shape of the calibration curves if free versus initial sample concentrations were plotted on the *x*-axis. All subsequent extractions with the glass rods were conducted from 15 mL samples when concentrations below 0.5 ng/mL were used in an experiment.

3.6. Langmuir analysis of probe affinity and capacity

The nature of extraction with immobilized antibody probes is of sorption of the analyte to defined active sites. Therefore, it is expected that the probes will act in an adsorptive rather than an absorptive fashion. If this is valid, then it is possible to model the adsorption isotherm (the dependence between the equilibrium concentration of a compound associated with a sorbent and its concentration in a sample in contact with it) based on the Langmuir adsorption model [29]. Several requirements must be met for the Langmuir model to be valid: (1) the adsorbing molecule must adsorb into an immobile state, (2) all sites must be equivalent, (3) each site must hold only one of the adsorbing molecules, and (4) there can be no interactions between adsorbed molecules on adjacent sites so that the equilibrium constant is independent of the coverage of the adsorbed species. These assumptions are valid for an immobilized antibody surface, with the understanding that there is greater homogeneity in active sites for some antibody preparations than for others. In addition, several specific models have been developed based on Langmuir adsorption, to describe the extraction profile and affinity of free and immobilized antibodies, with an accounting for antibody heterogeneity. Here the performance of the Sips equation and non-linear regression analysis, which has been applied to both free and immobilized antibodies [25], is compared to that of the model developed for general solid sorbent SPME probes.

3.6.1. Langmuir analysis of solid sorbent SPME probes

As described above, the immobilized antibody SPME probes are expected to perform similarly to other solid sorbent SPME probes. If affinities are assumed to be homogeneous, the affinities of the immobilized antibodies on the SPME probes may be calculated from the calibration data, according to the calculations provided by Gorecki [30]. Briefly, the amount of analyte extracted by the probe is given by:

$$C_{fA}^{\infty} = \frac{C_{f\,\max}\, K C_{sA}^{\infty}}{1 + K C_{sA}^{\infty}} \tag{2}$$

where C_{fA}^{∞} is the analyte concentration on the probe at equilibrium, $C_{f\max}$ is maximum concentration of active sites on the probe, K is antibody affinity as defined in Eq. (1), and C_{sA}^{∞} is the free analyte concentration in solution at equilibrium. Eq. (2) is based on the assumption that extraction by the antibody immobilized probes follows a Langmuir model. Because C_{sA}^{∞} appears in both the numerator and the denominator of Eq. (2) the relationship cannot be linear. A linear relationship will, however, be possible when the product KC_{sA}^{∞} is much less than 1 (≤0.1) as it can be ignored in the denominator of the equation. This of course occurs at low sample concentrations ($C_{sA}^{\infty} \leq 0.1/K$). By taking the reciprocal of Eq. (2) and multiplying both sides by a term representing the volume of antibody active sites on the probe (V_f), Eq. (3) is obtained,

$$\frac{1}{n_{fA}^{\infty}} = \frac{1}{n_{f\,\max}} + \frac{1}{n_{f\,\max} K C_{sA}^{\infty}} \tag{3}$$

where n_{fA}^{∞} is the equilibrium amount of analyte adsorbed on the probe and $n_{f\max}$ is the maximum amount of analyte that can be adsorbed on the active sites on the probe, which corresponds to the maximum amount of active sites, assuming a 1:1 ratio of active sites to adsorbed analyte at saturation. Where sample depletion is not negligible, analyte concentration in the sample at equilibrium C_{sA}^{∞} is calculated by subtracting the amount of drug extracted from the total amount of drug originally added to the sample. A plot of $1/n_{fA}^{\infty}$ versus $1/C_{sA}^{\infty}$ yields a straight line with a slope of $1/n_{f\,\max}K$ and a *y*-intercept of $1/n_{f\,\max}$. Thus, $n_{f\max}$ and K may be calculated from the linear regression equation, and $n_{f\max}$ should correlate with the amount extracted at the plateau region of the non-linear calibration curve, providing internal verification that the estimate of K is accurate. This technique is referred to below as a *Reciprocal Langmuir analysis*. The '*f*' in the subscripts of several of the foregoing terms arises from the discussion of Gorecki [30] and refers to the fact that in that case the probe was a fibre. For simplicity the term $n_{f\max}$ is abbreviated to $n_{\max}$ in further discussion. This analysis has an advantage in that sophisticated modelling software is not required, but a limitation in dealing with heterogeneity in antibody affinities.

An example of Reciprocal Langmuir analysis for nordiazepam and oxazepam is presented in Fig. 5. The results of the analysis of the linear regression equations to calculate affinity (K) and capacity ($n_{\max}$) for all three compounds are presented in Table 3. The data for 'average' coefficients were calculated from the linear regression line drawn through all of the calibration data points. From Fig. 5 we see that in both cases linearity is better for the frac. PAb than for the original PAb. This indicates that the fractionated PAb is more homogeneous in affinity than the original PAb, as is expected. For diazepam by comparison,

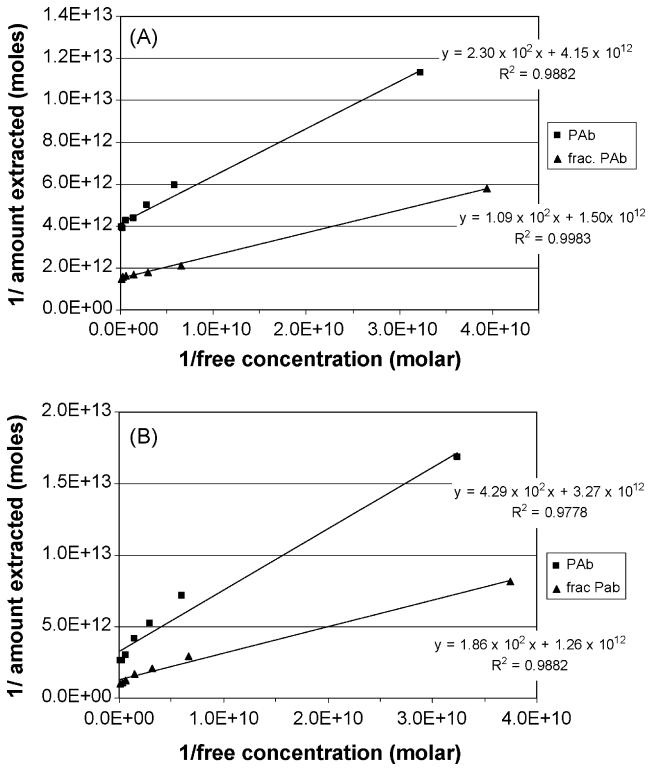

Fig. 5. Reciprocal Langmuir analysis of antibody affinity for benzodiazepines, graphical presentation, $n = 3$. Panel A: nordiazepam; Panel B: oxazepam. The diazepam data were highly linear and so are not shown.

the correlation coefficients were 0.991 for PAb and 0.997 for frac. PAb, indicating a high degree of homogeneity for both. In a heterogeneous protein, the high affinity antibodies dominate the overall affinity of the protein at low sample concentrations, whereas the low affinity antibodies are dominant at high sample concentrations. If affinities are calculated from the tangents to the curve at low and high sample concentrations, the range of affinities dominating extraction in each region may be estimated. In practice this was accomplished by estimating linear regressions from low and high concentration data sets, rather than calculating tangents *per se*. Table 3 also presents a summary of these affinity estimates.

Table 3
Summary of affinities and capacities determined by Reciprocal Langmuir analysis for probes prepared with PAb vs. frac. PAb

		Drug concentrations		
		Low	High	Average
Diazepam				
PAb	K (M^{-1})	1.8×10^{10}	7.7×10^{9}	1.5×10^{10}
	n_{max} (pg)	71.2	84.9	78.6
frac. PAb	K (M^{-1})	7.5×10^{9}	1.8×10^{10}	9.0×10^{9}
	n_{max} (pg)	252	204	219
Nordiazepam				
PAb	K (M^{-1})	2.2×10^{10}	4.1×10^{9}	1.8×10^{10}
	n_{max} (pg)	62.7	76.7	69.2
frac. PAb	K (M^{-1})	1.3×10^{10}	5.0×10^{9}	1.4×10^{10}
	n_{max} (pg)	204	195	191
Oxazepam				
PAb	K (M^{-1})	1.0×10^{10}	2.7×10^{9}	7.6×10^{9}
	n_{max} (pg)	70.3	114	87.8
frac. PAb	K (M^{-1})	8.9×10^{9}	2.6×10^{9}	6.8×10^{9}
	n_{max} (pg)	184	294	228

K is antibody affinity (1/M) and n_{max} is probe capacity for drug (pg).

It is interesting to note that the original polyclonal actually has a slightly higher affinity at low sample concentrations than does the frac. PAb. This result is not unexpected. As much as 10% of the protein applied to the column remained on the column after the acidic phosphate elution (Table 2). This would have been the very high affinity fractions that were either eluted with urea or GnHCl, or may have even remained uneluted on the column. These high affinity isotypes would however be present in the original PAb, providing the high affinity seen at low sample concentrations.

3.6.2. Comparison to Sips analysis of antibody affinity, capacity and heterogeneity

Affinities, capacities and degree of affinity heterogeneity were also calculated from a non-linear regression analysis of

Table 4
Summary of affinities, capacities and indices of heterogeneity determined by Sips equation analysis for probes prepared with PAb vs. frac. PAb

	Diazepam		Nordiazepam		Oxazepam	
	coeff.	S.E.	coeff.	S.E.	coeff.	S.E.
PAb						
a	1.05	0.25	0.78	0.14	0.71	0.09
b (pg)	72.61	5.91	69.47	4.78	127.33	18.53
K (mL/ng)	40.64	11.98	42.20	11.06	6.06	3.05
K (M^{-1})	1.16×10^{10}	3.42×10^{9}	1.14×10^{10}	3.00×10^{9}	1.74×10^{9}	8.76×10^{8}
frac. PAb						
a	1.05	0.12	1.05	0.15	0.74	0.05
b (pg)	205.14	10.49	176.64	8.62	340.10	26.89
K (mL/ng)	27.94	5.05	38.46	6.82	5.45	1.41
K (M^{-1})	7.96×10^{9}	1.44×10^{9}	1.04×10^{10}	1.85×10^{9}	1.56×10^{9}	4.06×10^{8}

Note: a and b are the coefficients of the Sips equation for heterogeneity and capacity, respectively; coeff. refers to the value of the coefficient being reported and S.E. refers to the standard error of the calculation of the coefficient. By definition heterogeneity is at a minimum at 1.0 (maximum homogeneity), but fitting was performed with 'a' constrained to a maximum of 1.05 to allow for 5% error. Capacity (b) is reported in picograms. Affinity (K) is reported both in mL/ng and the more familiar 1/M.

the Sips curve fitting to the calibration data. The graphical data are presented in Fig. 4 (left panels) while the calculated coefficients with their associated standard error (S.E.) are presented in Table 4. A comparison of these data with those generated from the Reciprocal Langmuir analysis allows a determination of the extent of the limitation of that method in dealing with heterogeneity in antibody affinities. We can see from a comparison of the data in Tables 3 and 4 that the diazepam and nordiazepam affinity values calculated from the Sips curve fitting fall within the range of those calculated from the Reciprocal Langmuir analysis. For oxazepam where heterogeneity is higher, the Sips curve fitting affinities compare better to the high drug concentration range data of the Reciprocal Langmuir analysis. The same is true for capacities calculated by the two methods. Both data sets indicate comparable affinites between the two protein preparations, with the PAb having slightly higher affinity, particularly at low sample concentraitons. Capacity is clearly higher with the frac. PAb, which is representative of the higher purity the protein and indicates the success of the fractionation. Oxazepam affinity is lower in nearly all cases relative to that of diazepam and nordiazepam. For the heterogeneous affinities, nordiazepam affinity to PAb ($a = 0.78$) has good agreement between the two methods, whereas for the oxazepam affinities ($a = 0.71$ and 0.74) agreement close but is not as good.

From the data it appears that the two techniques of estimation of affinities and capacities generate comparable data, particularly where $a \geq 0.78$. This indicates that the theory developed for solid sorbent SPME probes is applicable to immobilized antibody probes with a low degree of heterogeneity. Given that better extraction performance is seen with probes utilizing purified antibody with a low degree of heterogeneity, probes with more heterogeneous antibody would not likely be used in practice. Thus, the more sophisticated curve fitting to accommodate affinity heterogeneity would not have a significant benefit in routine use.

3.7. Evaluation of calibrations, precision and limits of detection

3.7.1. Analytes individually

Extraction calibration may be conducted from either non-linear or linear regression analysis, depending on the range of sample concentrations being analysed. As discussed previously, linear regression is feasible for a homogeneous antibody at up to $C_{sA}^{\infty} \leq 0.1/K$ or about 10% sorbent saturation. For the probes described here, regression is expected to be linear up to ca. 0.05 ng/mL. In practice, therefore, linear regression would only be possible if a higher sensitivity detector or higher capacity probe were used. This is feasible as higher sensitivity detectors than that used here are available and capacity may be increased by using multiple probes for extraction with desorption into the same solution, as has been described previously [31].

For external calibrations of individual compounds in the range of analyte concentrations tested here, non-linear curve fitting (Sips equation) was used. The dynamic range employed extended from the limit of detection (0.001–0.015 ng/mL), which was determined by the sensitivity of the detector and calculated based on 3 times the standard deviation of a 0.01–0.05 ng/mL extraction, to a point resulting in 80–90% sorbent saturation for single analyte analysis (0.2–2 ng/mL). This upper limit of quantification is determined by the antibody affinity. While it is a limitation where it is necessary to analyse high concentration samples, the only way to increase it would be to employ antibodies with lower affinity. This, however, would mean that non-specific binding would be a larger component of overall binding, thus eliminating the advantage of selectivity. A better strategy to deal with high concentration samples would be to dilute the samples. Method precision with the frac. PAb probes was less than 15% relative standard deviation (R.S.D.). Table 5 shows a summary of method performance for both the PAb and frac. PAb probes when analytes are extracted individually.

Table 5
Summary of quantitative data for calibration of samples containing individual analytes

	Diazepam	Nordiazepam	Oxazepam
frac. PAb			
ULOQ (ng/mL)	0.2	0.2	2
LOD (ng/mL)	0.007	0.004	0.001
%R.S.D.	10.4%	14.5%	10.4%
PAb			
ULOQ (ng/mL)	0.2	0.2	1
LOD (ng/mL)	0.015	0.005	0.008
%R.S.D.	11.8%	12.5%	19.3%

ULOQ: upper limit of quantification, LOD: limit of detection determined from 3× the standard deviation of a calibration point at approximately the limit of quantification; %R.S.D.: percent relative standard deviation.

3.7.2. Analytes in combination

While it is helpful to gauge the response of the probes to individual analytes, as is shown in the left panels of Fig. 4, in clinical samples the probes may be exposed to multiple analytes at once. This may be either structurally similar metabolites, or entirely different compounds. While antibodies are generally prized for their exquisite ability to discriminate between target and non-target analytes, antibodies raised to small molecules typically demonstrate a high degree of cross-reactivity to structurally similar analytes. In addition, the antibodies described thus far have been selected because they were characterized by the supplier as highly cross-reactive to the class of benzodiazepines, a fact which has been demonstrated experimentally.

Fig. 4 (right panels) illustrates the effect of having drugs combined in the sample (equivalent concentrations, w/v) relative to drugs introduced individually (left panels), on the shape of the calibration curve. In all cases the apparent capacity for individual drugs is reduced when drugs are present in combination. This is due to the fact that when saturation of the active sites is approached, competition for the active sites occurs among the analytes, and those with higher affinity are preferentially extracted. In all cases though, there are fewer total sites available for each drug. It can be seen that while there is a significant impact on the calibrations for diazepam and nordiazepam, which have higher affinity constants, the effect on the calibration of the relatively lower affinity oxazepam is drastic. It would be impos-

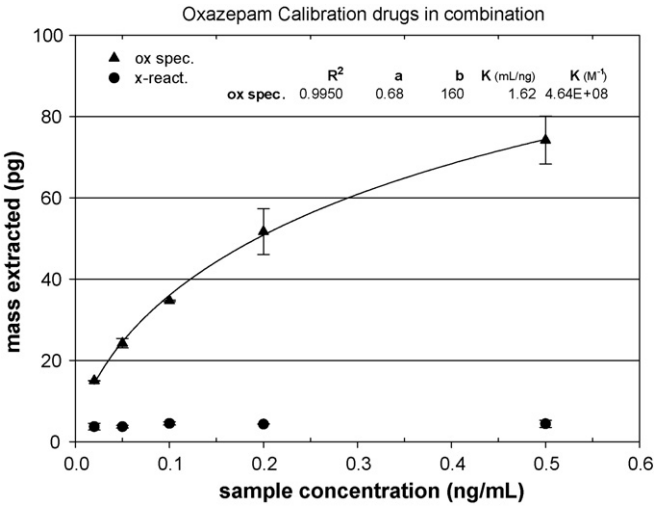

Fig. 6. Oxazepam calibration in the presence of nordiazepam and diazepam, use of oxazepam-specific antibodies, $n = 3$. Legend: ox spec., acidic phosphate fractionated PAb described by the supplier as highly specific for oxazepam; x-react., frac. PAb presented in Fig. 3.

sible to even semi-quantitatively estimate sample concentrations from this response curve.

As discussed, it is possible to accurately measure concentrations of one analyte in the presence of a competing analyte when the degree of total sorbent saturation is limited to about 10%. For the current analysis, however, even at the lowest concentration tested (0.01 ng/mL) the total amount of analyte on the sorbent at equilibrium was significantly higher than this. A higher capacity sorbent or higher detection sensitivity would be required for such an analysis.

It may also be possible to solve this difficulty by judicious selection of antibodies for immobilization. For instance, when an antibody reported to be highly specific for oxazepam, and having a low cross-reactivity for diazepam and nordiazepam, is fractionated and used in a similar experiment, an improved calibration may be achieved from a sample containing multiple drugs, as is presented in Fig. 6. In this experiment diazepam and oxazepam were present at equivalent concentrations (mass-to-volume), with nordiazepam present at twice the concentration. These concentrations were selected to mimic the ratios of the three drugs present under physiological conditions. While the observed affinity for oxazepam binding in the presence of diazepam and nordiazepam was not as high as hoped, it was also discovered that the antibody received was not as highly selective for oxazepam over other benzodiazepines (in terms of measured affinities) as had been indicated by the supplier. A truly drug specific antibody would be expected to perform better in this competitive experiment. Regardless, a significant performance improvement is seen here over that of the cross-reactive antibody.

3.7.3. Dissimilar analytes in solution

While calibration from a sample containing multiple structurally related analytes is problematic unless highly selective antibodies are selected, it is expected that the immunoaffinity probes will perform exceptionally well when structurally dissimilar analytes are present in combination with the analyte of

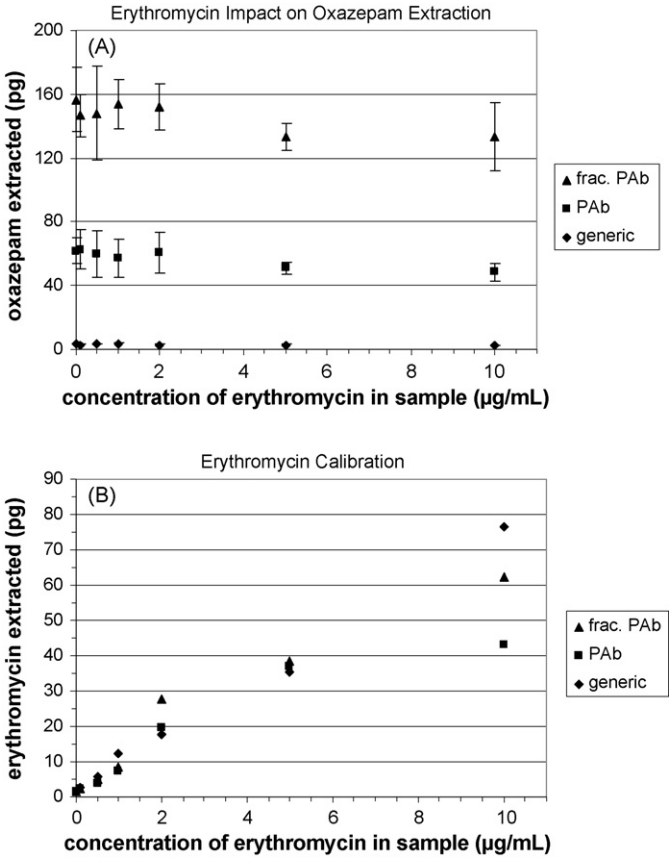

Fig. 7. Oxazepam extraction in the presence of erythromycin (A) and calibration of non-specifically bound erythromycin (B). Sample: PBS containing 0.5 ng/mL oxazepam and erythromycin up to 10 μg/mL, $n = 3$. Panel A: *y*-axis shows amounts of oxazepam extracted, panel B: *y*-axis shows amounts of erythromycin extracted. LOD: 1 pg oxazepam extracted, 0.5 pg erythromycin extracted.

interest. This is expected to be a primary advantage of these probes. To evaluate this, erythromycin at therapeutically relevant concentrations was added to buffer samples containing benzodiazepines in the determined calibration range. Samples contained 0.2 ng/mL oxazepam and erythromycin from 0.1 to 10 μg/mL in PBS. The results are presented in Fig. 7. From panel A where erythromycin is included at increasing amounts with oxazepam present at a constant level, it can be seen that erythromycin does not have a significant effect on the amount of oxazepam extracted. It is interesting to also note from panel B, which reports on erythromycin extraction from the same experiment, that there is a relatively linear and equivalent response of all the probes to erythromycin concentration, at least to 5 μg/mL. Literature data indicate percent free erythromycin in plasma in this range of concentrations is 55% [32], indicating that non-specific binding to antibodies should be expected. From this it can be concluded that specific extraction is unaffected even when nsb of unrelated analyte is high.

3.8. Evaluation of equilibration time profiles

The equilibration time profiles for the three types of probes developed were assessed from a solution of 0.05 ng/mL oxazepam in buffer. For extraction the plate containing the

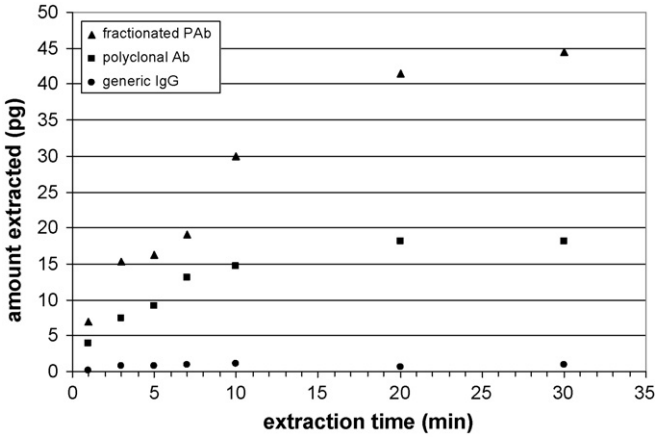

Fig. 8. Oxazepam equilibration time profiles at sample concentrations below the antibody saturation level. Sample: 0.05 ng/mL oxazepam in buffer, $n = 3$. LOD: 1.2 pg extracted. Legend: triangles, frac. PAb; squares, PAb; circles, generic IgG.

probes was shaken gently on a rotary shaker (100 rpm). It was previously determined that agitation conditions greater than this did not enhance extraction. Under static extraction conditions the extraction rate was controlled by diffusion through the boundary layer. Under any significant agitation conditions, extraction rate was controlled by the antibody–antigen reaction rate constant [33]. Therefore, in the current experiments it is expected that extraction rate was controlled by reaction kinetics. Reaction kinetics for the other benzodiazepines are expected to be about an order of magnitude higher, as it is known that the dissociation rate constant is similar for different antigen–antibody complexes, and the overall affinity constant is typically determined by the association rate constant [26]. Thus, equilibration times for the other benzodiazepines are expected to be faster than seen here for oxazepam.

From the data in Tables 3 and 4 it can be verified that the plateaus seen in Fig. 8 are not due to sorbent saturation as the amounts of drug extracted at the plateaus of the curves are well below the sorbent capacities. From the figure, equilibration times are estimated as: frac. PAb, 20 min; and PAb, 10 min based on the time to achieve 90% of the plateau. These data are consistent with the fact that higher capacity probes require a longer time period to reach equilibrium, due to the requirement of greater mass transfer.

In many methods, an equilibration time of 10–20 min would be acceptable. If shorter extraction times are required, analysts should consider pre-equilibrium extraction, as equilibration times cannot be improved further without drastically lowering the antibody affinity or reducing probe capacity, both options which would be undesirable in practice.

3.9. Performance of rods upon aging

It was of interest to determine how long the antibody-immobilized rods would retain their extraction efficiency during storage (time between preparation and use). This would indicate the underlying stability of the prepared rods, in order to have a degree of confidence in comparing variations in probe response seen from one experiment to the next, over both the short and long terms. To evaluate shelf-life, a set of rods was prepared, initially tested for drug extraction efficiency, and then tested just once each month for 3 months thereafter. The rods performed about 20% better on the first use than on subsequent uses. This may be due to the presence of a proportion of adsorbed rather than covalently immobilized antibodies on the freshly prepared rods. Adsorbed antibodies would likely be removed during the desorption steps and so not participate in extraction for subsequent uses. Afterward, performance was stable over the subsequent 3 months. Longer time periods were not tested.

While it was important to determine that the probes were stable during long-term storage, it was also important to determine the stability of the probes during repeated use. For this purpose, a probe extraction calibration experiment was exactly repeated after 6 weeks. In the intervening time, the probes were used approximately a dozen times, with storage at 4 °C in buffer containing 0.05% sodium azide. The performance of the probes was compared in terms of observed affinity and capacity. It was observed that the probes lost about 30% of their capacity in this time, but affinity was not affected. This is consistent with either a physical loss or a degradation of a portion of the immobilized antibodies, with the remainder being unaffected. In this case the probes had already been used twice prior to the first calibration, so a loss of weakly adsorbed antibodies was not a contributing factor here. The probes were evaluated again 6 months after preparation, with the result that their performance was further reduced but still acceptable. It can be concluded that the probes may be stored and used for extended time periods, but that comparative experiments should be conducted within 2–3 weeks of each other.

3.10. Effect of variation in pH and ionic strength

Variations in pH and ionic strength may be significant among in vitro samples and such variations are known in general to have a significant impact on protein structure. These may reasonably be expected to affect the ability of immobilized antibody probes to extract drugs of interest. The effect of pH and ionic strength variation on drug extraction was determined by preparing samples either in unbuffered solutions with different pH values (pH 5.4–9.4) but with isotonic salt concentrations, or in neutral pH buffered solutions with salt concentrations ranging from about 0.5 to 1.5× isotonic. The salt concentrations tested (0.08–0.12 M NaCl) corresponded to conductivities of 10.2–18.8 mS. For both PAb and frac. PAb there was not a significant impact of either pH or ionic strength variation in the range tested (data not shown). While it is possible to have in vitro samples with pH or ionic strength outside of these ranges, it is normally a simple matter to adjust both parameters in the samples prior to analysis by adding a standard amount of buffer, salt or water to each sample, with the result that narrow range variations are seen in practice. This is recommended for routine use.

3.11. Calibrations to high concentration

Although it was well established that the immobilized antibody probes saturated at sample concentrations <2 ng/mL, linear

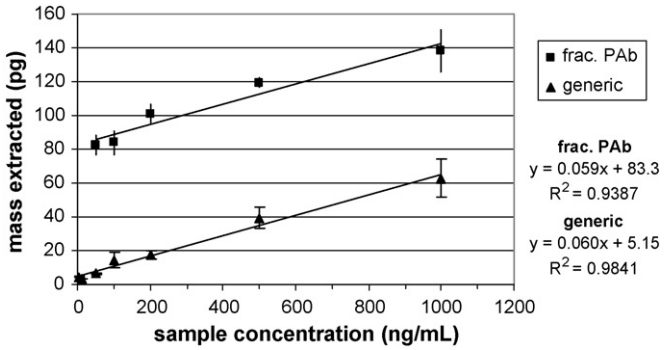

Fig. 9. Comparison of specific and generic IgG for calibration to high diazepam concentrations, $n = 3$.

calibrations had been observed to concentrations much higher than this. It was of interest to determine if this apparently linear calibration above antigen-binding site saturation, was due to non-specific binding, as it is also well understood that benzodiazepines have strong generalized protein binding affinity. This was tested by comparing protein-binding calibrations to 1000 ng/mL, between benzodiazepine specific probes (frac. PAb) and probes with generic antibody immobilized. Fig. 9 shows that the slopes of the calibration curves for the two IgGs are nearly identical, indicating that the same binding mechanism is at work in each case. The *y*-intercepts are significantly different and are indicative of the specific binding capacity, which in the case of generic IgG is essentially zero. The frac. PAb probes were older at this point so their specific capacity was reduced relative to that seen in Fig. 4.

The data further illustrate the importance of understanding which portion of the adsorption isotherm is responsible for extraction with these probes from different concentration range samples. When operating at sample concentrations above the saturation point of the antibodies, the benefits of antibody selectivity are largely lost as nsb dominates the extraction. Selective extraction is achieved from samples with analyte concentrations matched to the antibody affinity. In practice it would be appropriate to dilute high concentration samples to a concentration appropriate for the affinity of the probes being used, in order to avoid the complications of non-specifically bound analytes.

3.12. Evaluation of probe carryover

In the optimisation of the analyte elution, which was performed by extraction of ^{3}H-diazepam, it was observed that a small proportion of drug remained on the probes after the desorption with 75% methanol, and was only removed with harsher elution conditions. In routine application this would produce carryover associated with the fact that some drug is bound to very high affinity antibodies, which cannot be desorbed by 75% methanol. This carryover was estimated at 3.5%. It would be possible to routinely desorb drug with a higher strength eluent. For instance an elution with acidic acetonitrile is expected to remove all remaining traces of bound drug from the sorbent [15]. However, it was decided to not pursue higher efficiency desorption as the harsher treatment during desorption was expected to accelerate degradation or loss of antibody from the probes and it was estimated that the proportion remaining was too small to have a significant impact on the results.

It was also important to evaluate carryover associated the degree of incomplete desorption of drug that is elutable by the 75% methanol desorption solution routinely employed. From the high concentration extractions described in Section 3.11 it was observed that there was up to 5% carryover of this type. This was also determined to be insignificant for the current analyses. A larger volume or second desorption would be expected to reduce this carryover further if required.

3.13. Surface binding densities of antibodies

It was of interest to estimate the mass of antibody immobilized per area of the rod surface, in order to determine if antibody binding to the glass rods was as efficient as expected. Literature references indicate 2 ng/cm^2 is expected for PAb, in terms of active sites immobilized [18,34]. Insufficient binding density was identified in a previous report of immunoaffinity SPME as a limitation of the method [13]. From the probe capacities, it was possible to estimate binding density of active antigen-binding sites. The capacities of the probes for oxazepam when relatively new were: PAb, 90 pg (0.3 pmol); frac. PAb, 230 pg (0.8 pmol). These molar amounts should correspond to twice the molar amounts of antibody on the probes (two drug molecules bound per antibody). Given the IgG molecular weight of 150,000 g/mol and a rod surface area of 3.1 cm^2 (4 mm dia., 25 mm length coated), the maximum amounts extracted correspond to the surface binding densities (active antibody molecules) as follows: PAb, 7 ng/cm^2; fractionated PAb, 19 ng/cm^2. Thus, the current results are consistent with literature data.

4. Conclusions

SPME probes with specific affinity for benzodiazepines were prepared by immobilizing drug-specific antibodies onto glass rods, and were characterized for performance in a series of in vitro experiments. The characterization was extended significantly beyond that presented previously for the analysis of 7-aminoflunitrazepam. A comparison was made of probe performance for three different but related members of the benzodiazepines class of drugs (diazepam, nordiazepam and oxazepam).

The good performance of polyclonal antibodies observed is expected to widen the field of applicability of this technique, as polyclonal antibodies are much more available and less expensive than the monoclonals often used for immunoaffinity sample preparation. The polyclonal antibodies selected for this work were fractionated prior to immobilization, to isolate just the drug-specific IgG fraction. The purified antibody preparations were characterized for affinity and homogeneity prior to immobilization, in comparison to unfractionated and non-specific antibody. It was observed that the characteristics of the free antibodies were retained on immobilization, making this evaluation valuable as a predictive tool to screen antibody preparations for potential application to a particular analysis.

The evaluation of the SPME probes revealed that analyses to very low limits of detection were feasible, and that in fact the probes were most suited to these analyses. Regardless of probe capacity, the adsorption isotherms for the probes became saturated at analyte concentrations in inverse proportion to the antibody affinity, as expected. If analyte concentrations higher than this were used, the adsorption isotherm resembled that of non-specific affinity to IgG, rather than that of specific affinity to the antibody's antigen-binding site. Non-linear regression analysis was employed for both calibration and evaluation of bound antibody affinity, heterogeneity and probe capacity. It was observed that the relatively homogeneous antibody preparations obtained by fractionation of a polyclonal antibody pool performed optimally in terms of sensitivity, precision and capacity, relative to the unfractionated antibody. In addition, the adsorption isotherm of the probes with fractionated antibody was modelled to a high correlation coefficient with a standard Langmuir fitting. A more sophisticated Langmuir model that takes antibody heterogeneity into account (Sips) was only advantageous for probes prepared with the unfractionated antibody. Because the overall performance of these probes was less optimal than those prepared with fractionated antibody, the latter are expected to be more applicable for general use. Thus, sophisticated curve fitting for calibration or estimation of affinity and capacity would not be necessary for routine use.

The high affinities of the probes described had an advantage in eliminating co-extraction of structurally dissimilar analytes but a disadvantage in the narrow range of sample concentrations that could be quantified. Because of the limitation of the detector available for the current analysis, it was not possible to conduct extractions in the linear range of the probes. Not surprisingly, when antibodies that are cross-reactive to a class of drugs are employed, calibration of individual drugs in the presence of other members of the class is not possible unless degree of sorbent saturation restricted to a low level (<10%). With a state-of-the-art detector, an improvement in limit of detection of up to two orders of magnitude is anticipated, which would permit analyses in the linear range of the probes, and allow quantification of one drug in the presence of other co-extracted drugs. This may also be achieved if probe capacity could be further increased. This would be best achieved by increasing the surface area or surface binding density of active sites. If capacity were increased by adding depth to the immobilization substrate, an increase in extraction time would be anticipated. It may be more reasonable to increase capacity through the use of many small fibres or the binding of smaller-sized immunoreactive particles such as Fab particles or engineered peptides containing just the antigenic binding site.

The technique provides effective elimination of unwanted co-extractants from samples containing trace levels of analyte. Non-specific binding and carryover were both shown to be insignificant and equilibration times were relatively short (~20 min). Probe performance was unaffected in samples with varying pH and ionic strength, antibody binding density was shown to be comparable to literature values, and the probes were seen to be stable for either storage or repeated use over several months. Extraction conditions permitting negligible depletion were verified, further facilitating simplified calibration using standard adsorption models.

A significant strength of the technique is simplified sample preparation, even for complex samples. Clean-up between extraction and injection is minimized and the number of steps and total time required for a sample analysis is significantly shorter than with other immunoaffinity sample preparations. This reduction in complexity of analysis should permit better data quality due to reduction of potential for error, as well as higher sample throughput and lower costs per sample.

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