Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 769–780
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of immunoaffinity solid phase microextraction probes for analysis of sub ng/mL concentrations of 7-aminoflunitrazepam in urine

H.L. Lord, M. Rajabi, S. Safari, J. Pawliszyn *

Department of Chemistry, University of Waterloo, Waterloo, Ont., Canada N2L 3G1

Received 24 June 2005; received in revised form 15 September 2005; accepted 1 October 2005
Available online 13 December 2005

Abstract

We report on the development of solid phase microextraction probes for drug analysis, prepared with antibodies specific for benzodiazepines covalently immobilized to the surface. In the technique, immobilized antibody probes are exposed to a sample containing the drug for 30 min. Extracted drugs are subsequently desorbed from the probes in 500 μL of methanolic desorption solution, which is dried, reconstituted in a small volume of injection solution and analysed by LC-MS/MS. The antibodies were characterized both before and after immobilization, to facilitate the rational selection of antibodies for such analyses. Polyclonal and monoclonal antibodies were compared as was the impact of affinity purification of the polyclonal antibody to isolate the drug-specific fraction. The probes were evaluated for utility in analyzing 7-aminoflunitrazepam at sub ng/mL concentrations in urine, which is expected to be found several days after a single oral dose of 2 mg of flunitrazepam. Such analyses are required in monitoring for abuse of this drug, both in terms of 'club drug' use and in cases of drug-facilitated sexual assault. In these cases drug concentrations in blood and urine are much lower than in chronic abuse cases and are difficult to analyse by conventional methods. The method developed has a limit of detection of 0.02 ng/mL, with accuracy ranging from 1% to 27% and precision (% R.S.D.) ranging from 2% to 10% between the lower and upper limits of quantitation for the analysis of 7-aminoflunitrazepam in urine. The dynamic range of the method is from 0.02 ng/mL, which is limited by the instrument sensitivity, to 0.5 ng/mL, which is approaching the capacity of the probes. This would allow for quantitative analysis of samples at concentrations below that measurable by many other methods for general benzodiazepines analysis from urine, and a highly selective screen for samples at higher concentrations. The method has similar limits of detection to the most sensitive literature methods specifically designed for such analysis but with the advantage of significantly simplified sample preparation. This simplification makes the technique more amenable for use by both professionals and non-professionals.

Keywords: Solid phase microextraction; Immunoaffinity analysis; LC-MS/MS; Flunitrazepam; Benzodiazepines; Antibody affinity; Polyclonal antibody; Monoclonal antibody

1. Introduction

1.1. Solid phase microextraction

Solid-phase microextraction (SPME) is a sample preparation and sample introduction method where analytes are extracted from a sample into a small volume of extraction phase having high affinity for the analyte. The primary advantage of the technique is the greatly simplified sample preparation prior to introduction to an analytical instrument. In the commercial device marketed by Supelco a polymer is coated on a fused silica fibre of 1 cm length × 100 μm diameter. The fibre is fastened into the end of a fine stainless steel tube contained in a syringe-like device. The device's plunger is depressed to expose the fibre to the sample matrix, retracted at the end of the sampling time, and then depressed again to expose the fibre to a desorption interface for analysis, typically by GC or HPLC. The extraction phase may also be coated on the inner wall of a capillary and sample passed through the capillary for extraction. In this configuration the technique is referred to as 'in-tube SPME' and has been used for automated sample preparation and introduction for HPLC analyses [1,2].

* Corresponding author.

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.10.003

To date, most of the significant forensic drugs have been successfully analysed by SPME-GC-MS, both as standards spiked into solutions for calibration and from biological matrices of clinical origin, including blood, urine, hair and saliva. The list of target compound classes includes amphetamines, narcotic analgesics, γ-hydroxybutyrate (GHB), benzodiazepines, Δ^9-tetrahydrocannabinol (THC), cocaine, barbiturates and tricyclic antidepressants. SPME-HPLC methods have also been published for both forensic and therapeutic drug monitoring applications [3,4]. Several methods have been published for the SPME analysis of benzodiazepines from biological matrices [5–8], although none of these reports the analysis of 7-aminoflunitrazepam (7-AF). Analysis was typically by GC with ECD or FID and detection limits were in the low ng/mL range. An in-tube SPME method has been published for analysis of 7-AF, with a detection limit of 24 pg/mL although this was for drug spiked into buffer solution [9]. With the improvements in technology seen with the most current LC-tandem mass spectrometry instruments the potential exists to greatly expand the drug classes amenable to SPME analysis, while maintaining acceptable sensitivity for extraction from biological matrices.

1.2. Requirements for flunitrazepam analysis

Flunitrazepam (Rohypnol®) as a benzodiazepine, is a sedative and hypnotic drug in the same class as Valium. It is however seven to 10 times more potent than Valium. It is produced by Hoffmann–La Roche and distributed for legal sale in many countries in the world, although it is not legally prescribed in Canada or the United States. Flunitrazepam (FN) is used legitimately as a pre-anaesthetic prior to surgery, and as a general sedative and sleep aid [10]. It has also gained notoriety as a drug of abuse because of its strong sedative and hypnotic effects.

As a drug of abuse, drug abusers frequently take it either on its own or in combination with alcohol or other illicit drugs. Along with such drugs as GHB, ketamine and 3,4-methylenedioxymethamphetamine (MDMA), Rohypnol® has gained popularity as a ‘club drug’. The use of ‘club drugs’ has increased significantly over the past decade [11]. Also of concern is the fact that since the early 1990s Rohypnol® has increasingly been identified as potentially being used to commit sexual assault [12–14]. Rohypnol® is available as a 2 mg tablet, which is normally sufficient to induce complete sedation within approximately 30 min. When combined with alcohol however, the effect is magnified, typically causing a victim to become drowsy and disoriented within minutes, with complete sedation occurring shortly thereafter. Blackouts normally last from 4 to 12 h. The drug also has amnesic and hypnotic effects. Upon waking, the victim will have little or no memory of what occurred, and will be unsure if any memories are real or not. Because the dose of Rohypnol® is so much lower than that of most other drugs in the class, the drug is a challenge to detect in a victim’s blood or urine.

Additionally, the drug is metabolized very quickly. In fact, the parent drug is converted to the metabolite so quickly that it may be undetectable in urine by the time the victim regains consciousness [14]. Analytical methods typically focus on analysis of the metabolite 7-aminoflunitrazepam (7-AF), which is present in urine at higher concentrations and for a longer time. Samples must be collected as soon as possible after an attack, in order for sufficient drug to be present for testing. Seventy-two hours is typically recommended where the analytical method has a limit of detection of 1 ng/mL [13]. Unfortunately, because of a variety of factors, including the fact that victims are often unsure of what if anything happened, most delay seeking help [10,15].

Numerous methods have been reported in the literature for the selective determination of flunitrazepam in either serum or urine by GC or LC [16–19] and 7-AF analysis is occasionally reported also [20]. These methods typically require significant sample pretreatment. Despite these efforts, pretreatments are often insufficiently selective, resulting in chromatographic interferences and elevated noise levels. Senstivities of the methods are typically not better than 1 ng/mL. To address these issues, a column-switching method employing an anti-benzodiazepine immunoaffinity column for on-line sample pre-treatment has been reported [21]. By this method sample pre-treatment and analysis was automated, total analysis time was under 40 min and the reported limit of detection was 1 ng/mL.

In the most comprehensive survey available of prevalence of drugs used in sexual assault cases EsSohly and Salmone found only about 0.5% of nearly 1200 urine samples from suspected drug-facilitated sexual assault cases to be positive for FN, as evidenced by the presence of 7-AF. There has however been speculation that the analytical method used may not have sufficient sensitivity, resulting in a significant number of false negative samples [22]. At the usual 2 mg dose of FN, 7-AF is normally detectable by the recommended GC-MS test (LOD 1 ng/mL) for 72 h. In practice though, illicit drugs are commonly aldulterated and if placed in a beverage, the victim may ingest only part of the dosage, resulting in lower than expected concentrations of the metabolite in the victim’s urine. For all of these reasons, methods capable of achieving much lower limits of detection for 7-AF (10–30 pg/mL) have been proposed [12,22,23]. By these methods the time for detection of 7-AF in urine has been extended to about 10 days. To achieve these low limits of detection samples were first treated enzymatically to convert glucuronide conjugates to 7-AF, subjected solid phase extraction to concentrate the metabolite and analysed by a high sensitivity instrument, either GC-MS with negative chemical ionization after derivatisation, or by LC-MS or LC-tandem MS with electrospray ionization.

The goal of the present work was to develop an analytical method with simplified sample preparation, which could achieve limits of detection comparable to the state-of-the art methods.

1.3. Immunoaffinity SPME

SPME probes with antibodies specific for the analyte(s) of interest have been investigated previously to overcome some difficulties observed with the use of conventional absorptive and adsorptive phases for analysis of drugs in biological samples [24]. Since most drug compounds are relatively more polar than the environmental pollutants with which the absorptive

polydimethylsiloxane (PDMS) phase is most useful, adsorptive phases have been preferred for drug analysis. While acceptable sensitivities have been observed for drug analyses with these phases, typically DVB, analysts must be aware of the potential for competition in extraction and displacement of the compound of interest by other compounds or matrix components with higher affinity for the phase or present in higher concentration. Immobilized antibody phases are also considered as adsorptive. Their high degree of selectivity for their target analyte, however, makes them less prone to the competition and displacement problems seen with general adsorbents. Adsorptive phases are also characterized by a non-linear adsorption isotherm as the capacity of the sorbent is approached. This is true for immobilized antibody surfaces as well.

In the previous report, the authors evaluated the performance of immobilized-antibody SPME probes for the analysis of theophylline in serum to address the limitations of competition and displacement and to seek improved limits of detection. The authors found the probes to be suitable for the analysis with no significant interference seen from either sample matrix or the presence of a large excess of the parent compound caffeine. The authors reported a limit of detection of 0.1 ng/mL. In the present work, we have addressed a primary limitation observed in that work, of limited density of binding of active antibodies, and investigated the use of the anti-benzodiazepine probes for the simplified analysis of 7-AF in urine. In order to characterize optimal antibodies for the technique, we have also investigated the use of monoclonal versus polyclonal antibodies, the extent of non-specific binding in the analysis and characterized both the free antibodies and the antibody-immobilized probes for affinity and the relationship of antibody affinity to analysis sensitivity and dynamic range.

2. Materials and methods

2.1. Materials

Benzodiazepine specific polyclonal antibodies raised in sheep were obtained from Cortex Biochemical (San Leandro, CA). Monoclonal antibodies were obtained from US Biological (Swampscott, MA). Both polyclonal and monoclonal antibodies were described by the supplier as cross-reactive to benzodiazepines as a class. Polyclonal antibody was received as immune serum and monoclonal antibody was received as purified IgG in PBS buffer containing 0.025% sodium azide and 40% glycerol. 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 protein G affinity column for initial antibody purification (Immunopure® (G) IgG purification kit) was obtained from Pierce Biotechnology (Rockford, IL). The oxazepam affinity column for final antibody purification was prepared in-house from a second affinity column kit from Pierce Biotechnology (Pharmalink™ Immobilization Kit). The centrifugal filtering devices for antibody preparation/concentration were Amicon® Ultra-4 (4 mL volume) with 30,000 MWCO high flow rate membranes from Millipore Corporation (Bedford, MA). The ultrafiltration microconcentrators for free antibody affinity tests were Amicon Ultrafree-MC (0.4 mL volume) with 30,000 MWCO low flow rate membranes from Millipore Corporation.

Benzodiazepines were obtained from Cerilliant (Round Rock, TX) as certified standards (1 mg/mL) in either methanol or acetonitrile. These were diluted as required with methanol to produce intermediate standards. Phosphate buffered saline (PBS) was prepared in house and consisted of potassium phosphate monobasic (1.8 mM), sodium phosphate dibasic (11.4 mM), potassium chloride (2.7 mM) and sodium chloride 0.14 M and was adjusted to pH 7.4. PBS was stored at 4 °C and used within 1 month of preparation. Aminopropyltriethoxysilane, Aldrich 99% (APTES) and glutaraldehyde grade II (25% aqueous solution) were purchased from Sigma–Aldrich (Oakville, Canada). The bottle of APTES and was layered with nitrogen after each use. Twenty-four hour urine samples were collected from a healthy female volunteer the day prior to an experiment and stored on ice until required. Methanol and acetonitrile were from Fisher Chemicals (Ottawa, Canada). All solvents were HPLC grade and other chemicals were ACS grade or higher. All water was obtained from a Nano Pure water system from Barnstead (Dubuque, IA) utilizing a deionized water feed and was collected at 18 MΩ or higher.

Borosilicate glass rods (4 mm × 10 cm) were obtained from the University of Waterloo glass blower. The pH meter was a Corning model 220 with a Corning combination electrode with calomel reference from Fisher Scientific (Ottawa, Canada). The conductivity meter was a VWR brand meter from VWR Scientific (Mississagua, Canada). The rotary shaker for sample agitation was a Jeiotech model SK-300 from Rose Scientific (Edmonton, Canada). Ninety-six-well plates and glass scintillation vials were obtained from VWR scientific. The 96-well plate dryer was machined and constructed in house (University of Waterloo Science Shops) from stainless steel. The manifold to distribute the gas to the wells was prepared to accept standard 'yellow' pipet tips (100–200 μL, VWR), which were replaceable in case of contamination.

2.2. Antibody purification

All antibodies were purified by using a protein G affinity column (Immunopure® (G) IgG purification kit) Pierce Biotechnology. IgG specific isolation was achieved by means of the supplier's proprietary 'binding buffer' and elution of the isolated IgG was achieved by eluting with the proprietary 'elution buffer'. Eluted IgG was transferred to PBS containing 0.05% sodium azide by use of the desalting columns included in the kit. A portion of the polyclonal antibody was further purified to enrich the benzodiazepine specific fraction using an oxazepam affinity column prepared in-house using a Pharmalink™ Immobilization Kit from Pierce Biotechnology. The column was prepared with 3 mg of oxazepam, dried from three 1 mg/mL ampules of oxazepam in methanol (Cerilliant) and dissolved in 1.5 mL ethanol prior to mixing with an equal volume of the kit's 'coupling buffer'. For fractionation, ca. 20 mg of the protein G purified polyclonal antibody was loaded onto the oxazepam affinity column and un-bound antibody was eluted with PBS buffer until

the absorbance of the eluent at 280 nm dropped below 0.1. Non-specifically bound antibody was removed by eluting sequentially with 0.1% Tween 20, 10% ethylene glycol, and PBS containing 0.3 and 0.5 M sodium chloride until A_{280} of the eluent dropped below 0.05. The column was subsequently re-equilibrated with regular PBS containing 0.05% sodium azide. The specific antibody 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 specific IgG were identified by monitoring A_{280} and these were pooled and immediately transferred to PBS containing 0.05% sodium azide by means of a centrifugal filter device (Amicon® Ultra-4) with a 30,000 MWCO membrane (Millipore). The selection of acidic phosphate buffer for protein elution was determined empirically after testing several different eluent systems. While it is expected that the antibody would be partially denatured by exposure to pH 2 buffer, the timely transfer to a neutral pH buffer restored antibody activity, as was determined by the assessment of antibody affinity described in the following section. The amount of IgG in the final solution was estimated by measuring absorbance at 280 nm and converting to concentration (mg/mL) using a molar absorptivity of $1.35\,mg^{-1}\,mL\,cm^{-1}$. Typically 7% of the applied antibody was recovered in the active fraction eluted with acidic phosphate buffer. Fractions with higher specific binding were obtained from the oxazepam affinity column by elution with higher strength eluents, but these fractions contained amounts of protein too low for immobilization to the glass rods. Purified antibody was stored in PBS + 0.05% sodium azide either at 4 °C for short-term storage or at −20 °C for long-term storage.

2.3. Characterization of the antibody preparations

Oxazepam rather than 7-AF was used to characterize the free antibodies as the hapten used in their preparation was protein-conjugated oxazepam. Cross-reactivity to 7-AF and some other benzodiazepines was subsequently evaluated through a comparison of their affinities (Section 3.2). After purification, free antibody preparations were characterized for valence, oxazepam affinity and specific binding by first incubating a known amount of protein (0.04 mg) with different concentrations of oxazepam in a 400 μL volume of PBS + 0.05% sodium azide. After 30 min of equilibration at room temperature ca. 40 μL of the buffer containing unbound drug was removed by ultrafiltration through a 30,000 MWCO membrane (100 × *g*, 13 min). Thirty microliters of filtrate was mixed with 90 μL of methanol containing IS (lorazepam, 75 ng/mL) and the concentration of un-bound drug was determinined by LC-MS/MS analysis of 20 μL of the resulting methanolic filtrate by the chromatographic method described below. Lorazepam was selected for use as an internal standard to control for any sample evaporation prior to injection and for variable injection volume. It was selected for its structural similarity to the analytes of interest and good chromatographic properties. Analyses were performed in triplicate and data were averaged. For the specific IgGs, protein:drug molar ratios of 1:1–5:1 were employed. For the non-specific IgGs ratios of 5:1–20:1 were used. It was determined that at high protein:drug ratios sufficient unbound drug remained for accurate analysis and at low protein:drug ratios a significant difference between initial and equilibrated drug concentrations could be determined. The same ratios could not be used for both specific and non-specific antibody due to the significant difference in the amounts of drug bound between the two proteins. The analysis was used to confirm activity in the IgG fraction eluted with acidic phosphate and to monitor degree of purification. Generic IgG, monoclonal 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 affinity of the IgG of interest was verified, antibodies were covalently immobilized to glass rods by glutaraldehyde cross-linking by the following method, which generally follows that of Yuan et al. [24] and Lin et al. [25]. The lower halves of the glass rods were first acid etched by immersing in piranha solution (36 mL H_2O_2 (30%) + 84 mL H_2SO_4 (86%)) for 1 h. Rods were then extensively rinsed with nanopure water and then with absolute ethanol. The lower halves of the rods were then silanized by immersing in ethanolic ATPES (5 mL APS, 5 mL deionized water, 90 mL abs. EtOH) for 24 h at room temperature. Rods were extensively rinsed with water and abs. ethanol and then placed in a 80 °C vacuum oven flushed with nitrogen, for 15 h. Rods were glutaraldehyde activated by immersing in a 2.5% solution of glutaraldehyde in PBS for 5 h. After extensively rinsing with nanopure water rods were immersed in the antibody solution (0.2–0.6 mg/mL in PBS) to a depth of 2.5 cm with gentle agitation for 10 h or overnight. It was previously determined that there was no significant difference in rod performance when prepared with antibody concentrations over this range. Also, although other authors recommend immobilizing with antibodies in a basic buffer such as 0.1 M carbonate pH 9.2 we found that there was not a significant difference in performance of rods immobilized with antibodies in basic carbonate buffer versus neutral PBS. Given the limited amount of protein available and the difficulty in transferring it to an alternate buffer, immobilizations were performed with antibody in PBS + 0.05% sodium azide. Afterward the rods were extensively rinsed with nanopure water and unreacted glutaraldehyde was deactivated by immersing in an aqueous ethanolamine solution (0.3 M adjusted to pH 7.5 with HCl). After deactivation rods were stored in PBS + 0.05% NaN_3 + 0.2 mg/mL $NaCNBH_3$ at 4 °C for 24–48 h to reduce the imide to amine and stabilize the covalent linkage. For long-term storage the rods were stored in PBS + 0.05% NaN_3 with the storage solution changed every 1–2 months. The rods were found to retain activity and utility for at least 6 months, although a gradual loss in capacity was observed over this time.

2.5. Extraction of samples

Prior to extraction rods were allowed to warm to room temperature on the lab bench. Samples were prepared just prior

to an experiment by spiking intermediate standards into either PBS or urine that had been warmed to room temperature prior to the experiment. Care was taken to ensure that final methanol concentration in the samples was well below 1%, a level that had been previously determined to not impact antibody binding of drug. Samples prepared in volume were aliquotted to 20 mL disposable glass scintillation vials (15 mL each). For extraction rods were briefly rinsed with water to remove sodium azide and set into individual sample vials. During extraction samples were shaken gently on the Jeiotech rotary shaker at 100 rpm for 30 min. After extraction rods were rinsed with a stream of nanopure water from a wash bottle for ca. 5 s each. This had been previously determined to minimize carryover of sample to the desorption solution. Rods 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. This volume of desorption solution was previously found to remove >95% of drug from the rods while maintaining antibody activity for subsequent use. Desorption solution was dispensed to the 96-well plates by means of an Eppendorf Repeater Plus positive displacement pipettor from Brinkman (Mssissauga, Canada). The accuracy of solvent dispensing obtained with the positive displacement pipettor relative to air displacement pipettors was found to be important in obtaining reproducible results. The plate containing the rods was returned to the shaker for a 30 min desorption. After desorption rods were rinsed briefly with nanopure water and returned to the storage bottles. Rods were allowed to re-nature at 4 °C for a minimum of 24 h between experiments. Plates were dried under a stream of ultra high purity (UHP) grade nitrogen by means of the 96-well plate dryer described above. Just prior to chromatographic analysis a solution of 75% methanol, 25% water (25–75 μL) was dispensed to each well. The solvent was selected to both provide sufficient dissolution of the dried samples and good chromatographic peak shape. 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 consisted of a Shimadzu gradient LC system with a model SCL 10 AVP system controller, two model LC 10 AVP dual piston pumps and a model DGU 14A on-line mobile phase degasser purchased from Mandel Scientific (Guelph, ON), a CTC analytics model HTS PAL autosampler from Leap Scientific (Carrboro, NC) with a 20 μL injection loop 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 purchased from Waters Corporation (Milford, MA). Mobile phases were as follows: (A) acetonitrile:water (10:90) with 0.1% acetic acid; (B) acetonitrile:water (90:10) with 0.1% acetic acid. Mobile phase flow was 0.5 mL/min and the gradient used was 0% B for the first 0.5 min. This was ramped to 90% B over 2.0 min, held for 1.5 min and finally returned to 0% B for 1 min. A 20 μL injection volume was used for experimental samples. The HPLC effluent was analysed after ESI in positive ion mode with selected reaction monitoring. MS settings were as follows: nebulizer flow (N_2), 8; curtain gas flow (N_2), 12; CAD gas (Ar), 12; nebulizer voltage, 4500 V; drying gas (N_2), 7 L/min at 250 °C. All nitrogen was from Praxair (UHP grade, Toronto, ON) and supplied from in-house distribuion lines from a central tank. Transitions monitored were: 7-aminoflunitrazepam, 284.3/135.3; oxazepam, 286.9/241.0; lorazepam, 321.1/275.1. Compound specific mass spectrometer settings were determined for each compound separately by infusion of a 1 μg/mL methanol:water (1:1) solution at 20 μL/min using a model 100 digital syringe pump from kd Scientific, purchased from VWR (Mississagua, Canada). Flow dependent parameters were determined after combining the 20 μL/min infusion to a 0.48 mL/min flow of a 1:1 mixture of mobile phases A and B using a chromatographic tee. Mass spectrometer response sensitivity and linearity were monitored before and after each set of experimental samples by injection of 10 μL of a series of standards (0.2–200 ng/mL) prepared in 75% methanol, 25% water containing IS. For samples from the affinity tests, which contained 25% PBS, LC effluent was directed to waste for the first 1 min of run time to prevent salt from the buffer from entering the MS. The valve used to automatically control the bypass was a Waters model EV 750 electronic switching valve with a Rheodyne PEEK 6 port valve purchased from Waters (Milford, MA), connected to event contacts on the Shimadzu system controller. Analyst version 1.4 software (Applied Biosystems) was used to control all components of the system and for data collection and analysis. Chromatographic hold-up time was ca. 20 s and the first analytical peak did not elute until after 1.5 min. During this divert time a make-up flow of 10% acetonitrile, 90% water (0.5 mL/min) was supplied to the MS from a separate isocratic pump (Tosohaas model TSK 6010, Philadelphia, PA).

3. Results and discussion

3.1. Characterization of purified free antibodies

Prior to immobilization antibodies were characterized for activity towards oxazepam, which when conjugated to keyhole limpet hemocyanin, was used as the immunogen in their preparation. The results of Scatchard analysis of the three specific antibodies are shown in Fig. 1. As can be seen from the figures, the linearity of the data improved as the homogeneity of the protein increased. The monoclonal IgG, which is expected to be highly homogeneous shows the best linearity. The improved linearity of the fractionated polyclonal over that of the polyclonal recovered from the protein G column verifies the increase in homogeneity achieved by the oxazepam affinity chromatography. Affinity (K), valence (n) obtained from the Scatchard analysis, along with specific binding data for each protein are given in Table 1. From the calculated affinity values, we can estimate free drug concentrations that would produce 50% saturation of the antigenic sites, which is the range appropriate for quantitative analysis. Based on Eq. (1) $[H] = 1/K$ at

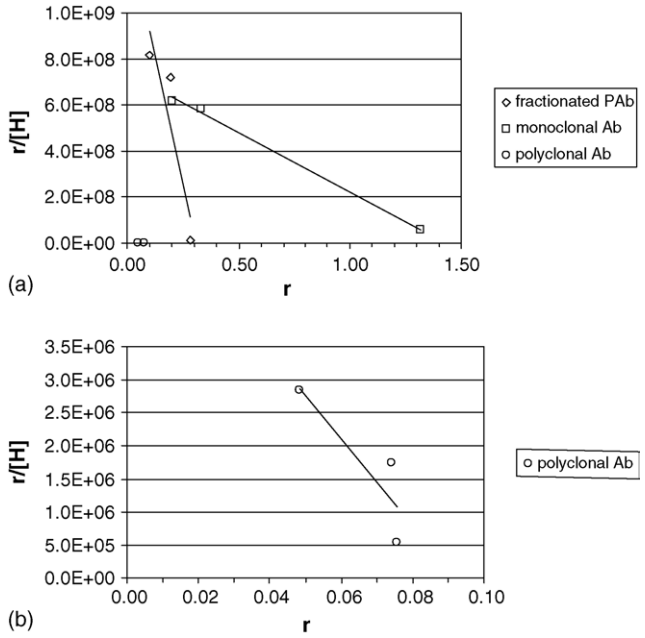

Fig. 1. Scatchard analysis of antibodies in solution. (b) is an expansion of the lower left region of (a), to allow visualization of the polyclonal Ab data.

half-saturation.

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

Total drug [H_{tot}] is then estimated as 2[H] at half-saturation and a 1:1 molar ratio of drug to protein. Thus we expect these antibodies to be suitable for extraction of drugs in the low to sub ng/mL range. These are also summarized in Table 1.

3.2. Characterization of rods with immobilized antibodies

Four sets of 21 rods were prepared, each with one of the four proteins immobilized to it. This allowed the collection of seven data points in triplicate. Characterization of the antibody-immobilized rods involved determining equilibration time profiles, calibration curves, limits of detection and affinities of the immobilized antibodies using samples in PBS buffer. Characterizations were carried out with oxazepam as this was the compound used to develop the antibodies. Cross-reactivities for 7-aminoflunitrazepam and several other benzodiazepines were

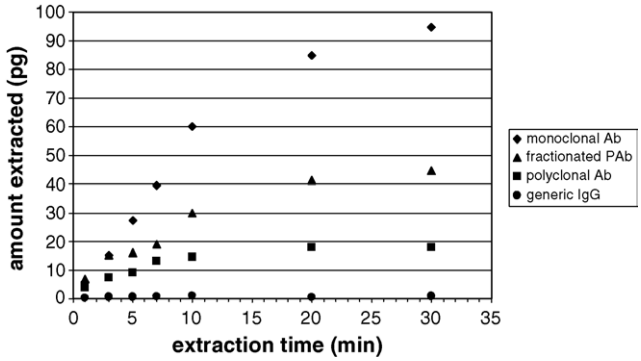

Fig. 2. Oxazepam equilibration time profiles. Sample: PBS containing oxazepam at 0.05 ng/mL, LOD = 1.2 pg extracted.

then evaluated by comparing calibrations, affinities and limits of detection.

3.2.1. Extraction time profiles

These were determined for the four proteins immobilized to glass rods using 0.05 ng/mL samples of oxazepam in buffer. The resulting profiles are shown in Fig. 2. As can be seen from the figure, equilbration time is related to the affinity of the probe. The probes that extract the highest amount of drug exhibit the longer equilibration times. Equilibration times are estimated as: MAb: >30 min; fractionated PAb: 20 min; PAb: 10 min. The probes with generic IgG extracted amounts of drug that were at or below the limit of detection for the experiment (1.2 pg extracted). The data for generic IgG demonstrate that non-specific binding does not impact the data from the specific probes. Equilibration times for other benzodiazepines are expected to be similar.

3.2.2. Effect of agitation during extraction

In the extraction method described only a minimal agitation is employed. Normally with SPME extractions the extraction rate (pg extracted per second) increases significantly with vigorous agitation, which is related to a corresponding reduction in the thickness of the boundary layer surrounding the extraction phase. This parameter is normally optimized in method development. In the case of the immunoaffinity probes, only a moderate increase in extraction rate was observed between static extraction (0.032 pg/s) and extraction employing minimal agitation (0.035 pg/s). No further enhancement in extraction rate was observed at higher rates of agitation. This observation

Table 1
Summary of affinitiy assays of free IgG

	K (M^{-1})	n	r	sb (ng/mg)	[H_{tot}] (M)	[H_{tot}] (ng/mL)
Generic IgG	1.00E+07	0.023	0.013	38		
Polyclonal Ab	6.54E+07	0.092	0.076	150	3.06E−08	8.78
Fractionated PAb	4.30E+09	0.314	0.286	575	4.65E−10	0.13
Monoclonal Ab	5.14E+08	1.432	1.317	2520	3.89E−09	1.12

The r term is used for the Scatchard analysis and is the molar ratio of bound to free antibody. The n term is antibody valence calculated from the Scatchard analysis. The values for specific binding (sb) were those observed at the lowest drug concentration testable, given the sensitivity limits of the mass spectrometer, which was the maximal specific binding measured. Generic IgG for this experiment was the IgG that eluted through the oxazepam affinity column in the unretained fraction. [H_{tot}] is calculated from K as an indication of sample concentrations expected to be quantifiable (produce 50% saturation) for a given IgG.

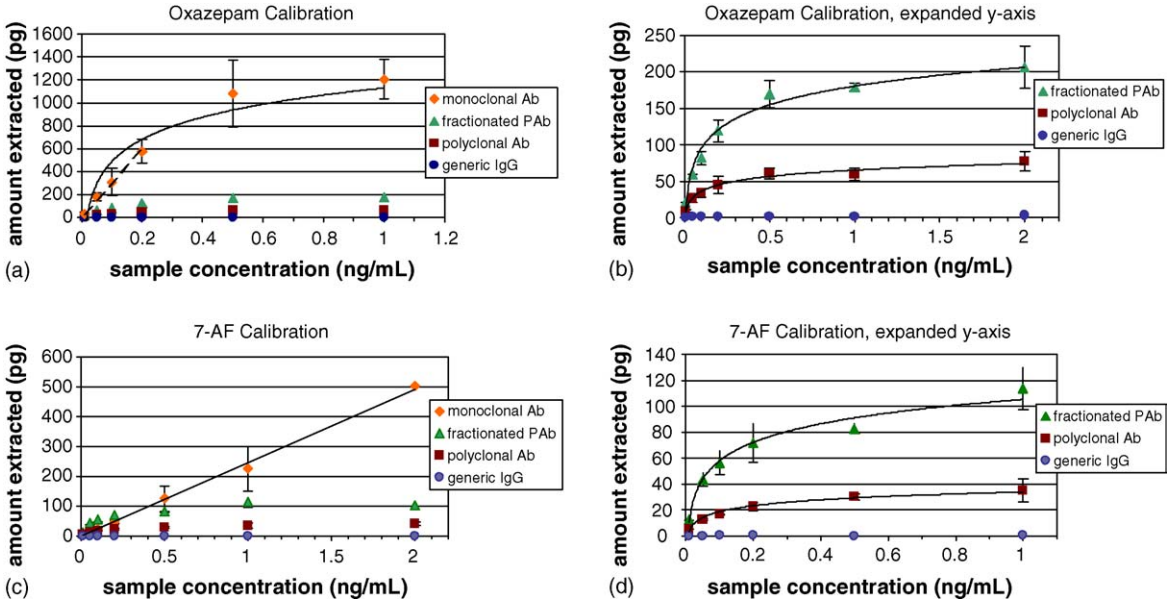

Fig. 3. Calibration curves for extraction of drugs from buffer. Correlation coefficients and limits of detection calculated from these data are given in Table 2. (b) is an expansion of the lower part of (a), to allow the polyclonal data to be seen. (d) is a similar expansion of (c). In (a), the monoclonal data may be used to produce either a linear regression over a narrow range of sample concentrations or a logarithmic regression to higher concentrations. Non-linear calibration data were fit to the Sips equation.

indicates that at higher agitation rates the extraction is controlled by antibody reaction kinetics rather than mass transfer across the boundary layer. Reproducibility of extraction was, however, somewhat better under conditions of minimal agitation than with static samples. For these reasons conditions of minimal agitation were selected to optimise extraction reproducibility and minimize the extraction equilibration time.

3.2.3. Extraction calibrations in buffer

Calibrations for both oxazepam and 7-aminoflunitrazepam are shown in Fig. 3. From the data, we can conclude that for polyclonal antibody, calibrations are non-linear rather than linear and half-saturation occurs at very low ng/mL sample concentrations, as was indicated from the Scatchard analysis for free PAb and is expected from SPME theory for adsorptive phases [26]. The monoclonal antibody appears to extract in a linear fashion to at least 1 ng/mL for 7-AF and to 0.2 ng/mL for oxazepam, indicating a lower affinity than for the polyclonal IgG rods. We know that linear calibration can be expected for adsorptive phases only when the ratio of occupied sites to total sites is negligible, or when the product KC^*_{sA} is much less than 1, where K is the coating affinity for the analyte and C^*_{sA} is the equilibrium free analyte concentration in solution [27]. This is typically understood as analyte binding of less than 10% of capacity or an $KC^*_{sA} < 0.01$. Unfortunately, for the polyclonal antibodies this range of sample concentrations is below the sensitivity of the mass spectrometer used. Thus it is necessary to use non-linear calibrations for the range of sample concentrations shown in Fig. 3. Limits of detection may also be calculated from these data and in all cases they are below 10 pg/mL. Limits of detection were calculated based on three times the standard deviation of the 0.05 ng/mL point. These data along with correlation coefficients and regression equations are summarized in Table 2.

Table 2
Summary of limits of detection, and correlation coefficients for oxazepam and 7-AF extraction from buffer

	Oxazepam		7-AF	
	LOD (ng/mL)	R^2	LOD (ng/mL)	R^2
Polyclonal Ab	0.007	0.9781	0.001	0.9818
Fractionated PAb	0.005	0.9774	0.003	0.9750
Monoclonal Ab	0.009	0.9911	0.005	0.9971

3.2.4. Evaluation of immobilized antibody affinities

As was discussed above, MAb affinities are expected to be significantly lower than those for the polyclonals, although this was not clearly seen in the Scatchard analysis. Affinities of the immobilized antibodies may be calculated from the calibration data, according to the calculations provided by Gorecki [27]. Briefly, the amount of analyte extracted by the fibre is given by:

$$C^{\infty}_{fA} = \frac{C_{f\max} K C^{\infty}_{sA}}{1 + K C^{\infty}_{sA}} \quad (2)$$

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. The reciprocal of this equation yields:

$$\frac{1}{C^{\infty}_{fA}} = \frac{1}{C_{f\max}} + \frac{1}{C_{f\max} K C^{\infty}_{sA}} \quad (3)$$

If the denominator terms in Eq. (3) are multiplied by V_f, which is a term representing the bulk concentration of the antibody active

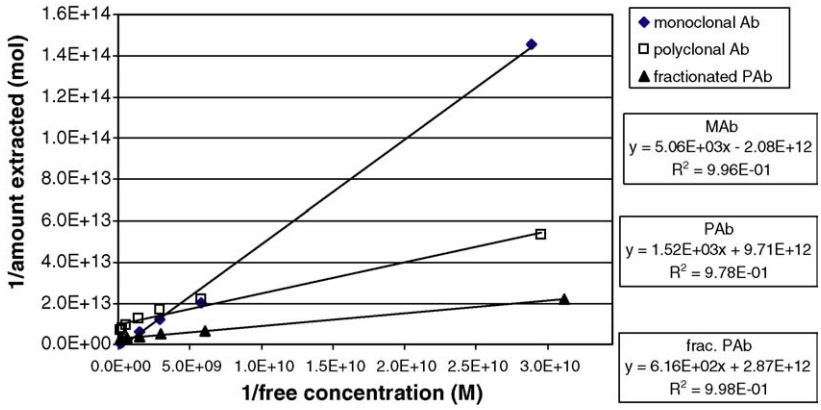

Fig. 4. Reciprocal Langmuir Analysis of calibration data from Fig. 3c and d. These data were used to produce the values of affinity (K) and capacity ($n_{f\,max}$) given in Table 3.

sites on the probe surface, Eq. (3) becomes:

$$\frac{1}{n_{fA}^{\infty}} = \frac{1}{n_{f\,max}} + \frac{1}{n_{f\,max} K C_{sA}^{\infty}} \quad (4)$$

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. The form of the equation given in Eq. (4) is more convenient to use with affinity probe calibration data, where amount of analyte adsorbed is plotted versus sample concentration of analyte. Where sample depletion may not be negligible, analyte concentration in the sample at equilibrium is calculated by subtracting the amount of drug extracted from the total amount of drug originally added to the sample. From Eq. (4) we see that a plot of $\frac{1}{n_{fA}^{\infty}}$ versus $\frac{1}{C_{sA}^{\infty}}$ yields a straight line with a slope of $\frac{1}{n_{f\,max}K}$ and a y-intercept of $\frac{1}{n_{f\,max}}$. 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 calibration curve, providing internal verification that the estimate of K is accurate. This method of determining $n_{f\,max}$ and K from the immobilized antibody probes is referred to below as an 'Reciprocal Langmuir Analysis'.

The results of this analysis for 7-AF are shown in Fig. 4. For comparison purposes the affinities for a range of benzodiazepines, calculated similarly, are shown in Table 3. As was seen for the Scatchard data in Fig. 1, more homogeneous proteins produce more linear data in this analysis. By comparing Tables 1 and 3, we can see that estimates of affinity (K) are in general lower for the free antibodies than the bound antibodies. Other authors have noted that if anything, a loss in affinity may be observed after covalent immobilization [28,29]. This discrepancy may be explained by the fact that drug concentrations used for the Scatchard analysis of free antibody were more than two orders of magnitude higher than the concentration required for half saturation. This was necessary to ensure sufficient free drug remained to allow accurate quantification. At these concentrations the latent affinity of IgG for drug, which we see from Table 1 for generic IgG, would be expected to contribute to measured affinity, as its affinity is ca. two orders of magnitude lower than the antibody–antigen binding affinity from Table 3. We expect the affinity measurements shown in Table 3 to be more accurate since specific antibody was not oversaturated during the analysis. It can also be seen that better agreement between free and bound antibody affinities are achieved as homogeneity increases, as measured by valence (n). From Table 3, we see that, in general, for all antibodies, affinities for 7-AF are slightly lower than for the other benzodiazepines tested, but not so low that analysis would be unsuccessful.

3.2.5. Evaluation of immunoaffinity probe capacities

From the Reciprocal Langmuir Analysis it is also possible to calculate $n_{f\,max}$, which is the mass of analyte extracted at saturation and hence the probe capacity. Capacities ($n_{f\,max}$) are also summarized in Table 3. From this and the calibration equations calculated from the data we may also estimate the sample concentrations resulting in 10%, 50% and 80% saturation. This provides an indication of half-saturation sample concentrations and maximum sample concentrations appropriate for non-linear (80%) and linear (10%) calibrations. These data are summarized in Table 4. From $n_{f\,max}$, we can also estimate binding density

Table 3
Summary of affinities (K) and capacities ($n_{f\,max}$) calculated from the Reciprocal Langmuir Analysis

	Polyclonal Ab		Fractionated PAb		Monoclonal Ab	
	K (M^{-1})	$n_{f\,max}$ (pg)	K (M^{-1})	$n_{f\,max}$ (pg)	K (M^{-1})	$n_{f\,max}$ (pg)
7-AF	2.0E+09	40.8	3.1E+09	108.7	6.6E+06	10290
Diazepam	2.5E+10	47.5	2.0E+10	142.5	1.5E+09	713
Nordiazepam	1.8E+10	69.2	1.4E+10	191.3	N/D	N/D
Oxazepam	1.1E+10	59.4	1.2E+10	124.8	8.2E+08	1821

Table 4
Summary of estimated sample concentrations and amounts extracted at 10%, 50% and 80% saturation of active sites, based on extraction from buffer

	Polyclonal Ab		Fractionated PAb		Monoclonal Ab	
	pg	ng/mL	pg	ng/mL	pg	ng/mL
Oxazepam						
10%	5.9	0.009	12.5	0.011	182	0.061
50%	29.7	0.059	62.4	0.043	911	0.307
80%	47.5	0.244	99.8	0.116	1457	0.491
7-AF						
10%	4.1	0.011	10.9	0.010	1029	4.2
50%	20.4	0.129	54.4	0.084	5145	20.9
80%	32.6	0.816	87.0	0.408	8233	33.4

of active antigenic sites, which was identified in the previous report of immunoaffinity SPME as a limitation of the method [24]. The 4 mm diameter probe, coated to a length of 2.5 cm, has a surface area of immobilized antibody of 3.1 cm^2. $n_{f\,max}$ for oxazepam range from 60 to 1800 pg extracted at saturation. This corresponds to 0.2–6.3 pmol of oxazepam. Given a 1:1 ratio of drug to antigenic site at saturation, this also corresponds to 0.2–6.3 pmol of antigenic sites, or 0.1–3.2 pmol of antibodies. At a molecular weight for IgG of 150,000, this corresponds to 5–150 ng IgG/cm^2. This is a significant improvement over the binding density observed previously and is in line with literature values of 40–200 ng/cm^2 [30,31].

3.3. Effect of urine matrix on probe performance

The data from probe performance in buffer indicates that the probes should be appropriate for monitoring of sub ng/mL concentrations of 7-AF, which is in line with other high sensitivity methods in the literature. The potential strength of the SPME method, however, is significantly simplified sample preparation, making faster testing or testing by non-specialists potentially feasible. For this reason it was of interest to evaluate the performance of the probes in urine. In addition to typical urine components such as inorganic ions, creatinine, and urea, urine typically has a higher ionic strength and lower pH than PBS, and is also variable in character [32]. It was previously determined that probe performance for extraction of diazepam was not significantly effected by variation in ionic strength from 0.08 to 0.2 MNaCl (conductivities 0.01–0.02 S), or variation in pH from 5.4 to 9.4 for extraction from aqueous solution (data not shown). The impact of variations expected for urine matrices were tested for the extraction of 7-AF for these probes and the results are shown in Fig. 5. From these data we can see that while there was only a slight matrix effect observed for the polyclonal data (reduction in amount extracted by ca. 10–20%) there was a significant reduction in the performance of the monoclonal probes in urine, on the order of 90%. In all cases, however, there was no significant difference seen in performance of the probes over the range of pH and ionic strengths tested, although pH 7.0 appeared optimal. It was observed that when urine pH was adjusted above pH 7, a significant precipitate formed. While this additional phase in the solution did not appear to impact the amount extracted

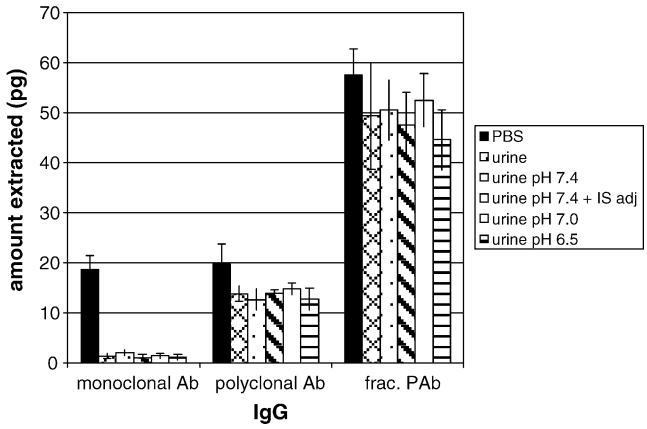

Fig. 5. Evaluation of the effect of urine pH and ionic strength modification, where ionic strength is measured in terms of conductivity. Sample: 0.2 ng/mL 7-AF in urine or PBS. The range of conductivities tested corresponds to NaCl concentrations ranging from 0.12 to 0.18 M. It was previously determined that for diazepam extraction, no significant difference in amount extracted was observed for any of the IgGs in the range of 10.2–18.8 mS (0.08–0.2 M NaCl). The first two bars refer to extraction from PBS and unmodified urine respectively. The third bar is for extraction from urine that was modified to have the same pH as PBS. The fourth bar is for extraction from urine that was modified to have the same pH and ionic strength as PBS. The last two bars are for urine that was pH adjusted as shown, but with no ionic strength adjustment (although the pH adjustment changed the ionic strength from that of unmodified urine). Conductivities of all samples (mS) were as follows: PBS, 15.4; urine, 14.8; urine pH 7.4, 17.8; urine pH 7.4 + IS adj., 15.4; urine pH 7.0, 14.5; urine pH 6.5, 14.2.

by the probe, it was decided to adjust urine pH to 7.0 and not adjust ionic strength unless it was out of the range of 0.08–0.2 M (conductivities 0.01–0.02 S).

3.4. Probe calibration in urine

For calibration from urine, concentrations were tested from 0.02 to 0.5 ng/mL. The range was chosen to span sample concentrations from the LOD to a concentration producing not more than 80% saturation of the antibodies. Calibrations are shown in Fig. 6, as non-linear data and in a semi-log plot to produce linear data. As was expected from the matrix tests, the monoclonal antibody probes performed poorly and were not useful for analysis of unknowns. The reasons for this were not investigated. It may be that some urine constituent significantly competes with the isotypic antigenic site of MAb but not PAb where the inherent heterogeneity in antigenic sites provides a redundancy, or possibly that the lack of post-translational modifications including addition of carbohydrate groups, known to be inherent with MAb production, renders the proteins more susceptible to interference of protein structure by exogenous compounds. Limits of detection and quantitation, as well as calibration and affinity data for probe extraction from urine are shown in Table 5. The antibodies retained 7-AF affinity for extraction from urine, although capacity (as indicated by $n_{f\,max}$) was reduced somewhat. Limits of detection were less than 20 pg/mL for both polyclonal probes, with the fractionated polyclonal performing somewhat better.

Fig. 7 shows representative chromatograms for the fractionated PAb rod extraction from both blank urine and urine spiked with 7-aminoflunitrazepam at a level near the LLOQ.

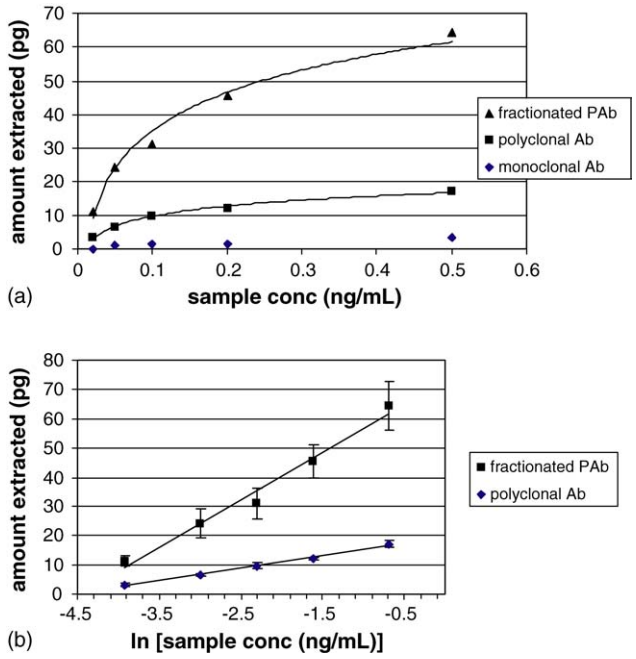

Fig. 6. Urine calibrations: (a) data presented as a non-linear plot, (b) data presented as a semi-logarithmic plot to show linearized data. Either may be used to estimate concentrations of unknowns. The regression data and limits of detection calculated from these data are given in Table 5.

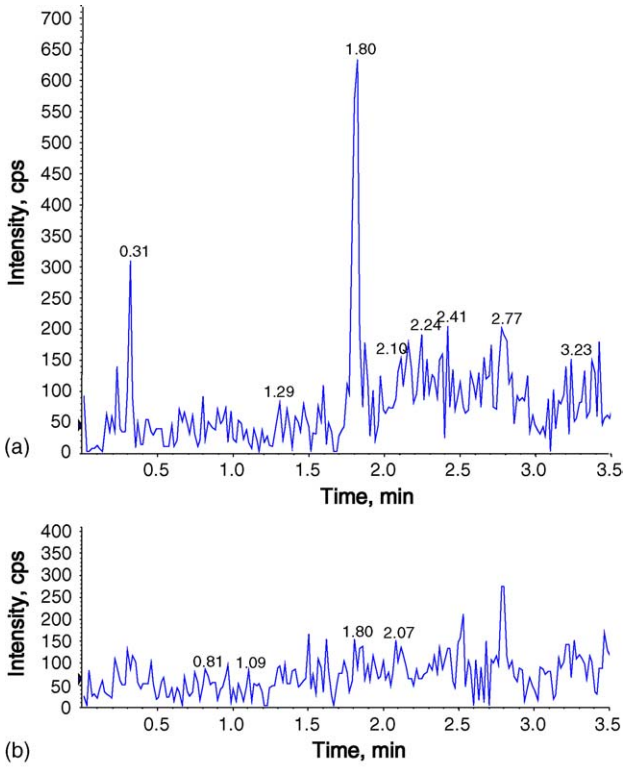

Fig. 7. Representative SRM chromatograms for extraction of 7-AF from urine using immobilized-antibody SPME probes with the fractionated PAb. Panel (a), extraction from urine with 7-AF at 0.04 ng/mL; panel (b), extraction of blank urine with the same probes. 7-AF elutes at 1.8 min.

The average level of noise calculated by the software is shown on the *y*-axes as a triangle. Some baseline drift is evident in the traces after about 1.7 min, due to the impact of the gradient elution. As can be seen no interference peaks are present from the blank extraction at the 7-AF retention time (1.8 min).

3.5. Estimates of accuracy and precision of analysis

Accuracy and precision of the method were determined by analysis of urine samples prepared separately, which were spiked with 7-AF at levels near the lower and upper limits of quantitation (LLOQ and ULOQ). This allowed for an evaluation of accuracy and precision through the entire dynamic range. Both probes performed better at the LLOQ concentration than the ULOQ, likely because the slope of the calibration curve is less at the ULOQ, resulting in inherently higher error. In all cases precision was less than 10% (measured as relative standard deviation (R.S.D.)) and inaccuracy in terms of deviation of the estimated concentration from nominal concentration ranged from 1% for fractionated PAb at the LLOQ to Ca. 15–25% at the ULOQ. These data are summarized in Table 5. The probes allow for good quantitative assessment of 7-AF concentrations and the instrumental analysis provides for high specificity in identification of the presence of 7-AF in urine.

Table 5
Summary of limits of detection and quantification, affinities, correlations and estimates of unknowns for extraction of 7-AF from urine

	PAb	Frac. PAb
LOD (ng/mL)	0.018	0.016
LOQ (ng/mL)	0.060	0.034
K (M^{-1})	2.8E+09	2.9E+09
n_{max} (pg)	20	70
R^2	0.9930	0.9815
0.04 estimate	0.036	0.039
0.04 accuracy	90%	99%
0.04 precision	2.1%	7.7%
0.4 estimate	0.455	0.292
0.4 accuracy	114%	73%
0.4 precision	9.9%	9.3%

The low concentration unknown (0.04 ng/mL) is approximately at the LLOQ for the analysis and the high concentration unknown (0.4 ng/mL) is at the ULOQ for the fractionated PAb and at about half the ULOQ for the polyclonal Ab (see Table 4)

3.5.1. Options to enhance method performance

A limitation of the immunoaffinity SPME technique may be its narrow dynamic range. While it has been shown accurate for analysis of samples with concentrations below 0.5 ng/mL, samples above this concentration are not expected to be accurately quantified. For these samples the technique may be beneficial in providing a convenient but sensitive pre-screen to select samples for analysis by one of the higher LOD methods with which the literature abounds.

Antibodies specific to 7-AF may be sourced, but the benefit of using such antibodies with this technique may or may

not be significant. They could potentially have either higher affinity or higher specificity for 7-AF than those used here. As was demonstrated, antibodies with higher affinity produce probes with both lower LLOQ and ULOQ. This may or may not be desirable, depending on the nature of the analysis. Probes with higher specificity (or lower cross-reactivity to other benzodiazepines) could potentially be used to accurately measure 7-AF in the presence of other benzodiazepines. It would be of interest to determine if other MAb would retain their activity in urine matrix. This would provide the possibility of producing a consistent probe in the long-term as MAb may be produced reproducibly indefinitely, whereas PAb cannot.

For optimal sensitivity in practice, the impact of enzyme pre-treatment to convert glucuronidated 7-AF back to 7-AF, should be studied. Although the supplier indicates the antibodies should be equally effective in extracting glucuronidated versus non-glucuronidated 7-AF, enzyme treatment has been found to be important in improving sensitivity for other antibody-based assays for 7-AF [33]. It is of course critical for SPE concentration of total 7-AF from urine, reported in most of the methods in the literature to date. Ultimately, the sensitivity of this assay is directly related to the sensitivity of the mass spectrometer employed. Because mass spectrometers of more recent models than the one used in this work are known to have sensitivities one to two orders of magnitude better, it should be possible to improve the sensitivity of this method accordingly, which would then extend the dynamic range.

It is often of interest in urine analysis to express observed drug concentrations relative to the creatinine concentration, to compensate for the variable dilution seen in urine and normalize drug analysis to metabolic rate. Where sample volume is high the sample is typically split, with part submitted for drug analysis and part for creatinine analysis. An advantage of the SPME method proposed is that the analysis procedure is non-destructive for the sample. Apart from a slight increase in sample pH and selective partial removal of the analytes of interest, the original urine sample is unchanged and may be subjected to further analyses, including creatinine analysis. This may be advantageous where sample volume is limited.

4. Conclusion

The immobilized antibody probes described have been shown to be useful for the simplified quantitative analysis of sub ng/mL concentrations of 7-aminoflunitrazepam in urine. Because of the highly cross-reactive nature of the antibodies selected for this work, the probes are expected to also be appropriate for identifying other benzodiazepines in a sample, but with the limitation that compounds with higher affinity (e.g. diazepam) may out compete 7-aminoflunitrazepam, particularly if the latter were present at much lower concentrations, making quantitative analysis of 7-AF problematic in the presence of other benzodiazepines. This may also be an advantage where a survey for the presence of any members of the class of benzodiazepines is desired.

Antibodies for other ‘club drugs’ and suspected sexual assault drugs are generally available, making it potentially feasible to generate a suite of probes specific for the various drug classes, but with specificity and sensitivity not seen in other simplified ‘dip stick’ like tests proposed to date.

Acknowledgments

The authors gratefully acknowledge funding for this project from the National Science and Engineering Research Council (NSERC) Canada, Supelco and Leap Scientific.

References

[1] J. Pawliszyn, Solid Phase Microextraction: Theory and Practice, Wiley-VCH, New York, 1997, pp. 15–22.
[2] H. Lord, J. Pawliszyn, J. Chromatogr. A 885 (2000) 153–193.
[3] H.L. Lord, J. Pawliszyn, J. Chromatogr. A 902 (2000) 17–63.
[4] H. Kataoka, H.L. Lord, Sampling and sample preparation for clinical and pharmaceutical analysis, in: J. Pawliszyn (Ed.), Sampling and Sample Preparation for Field and Laboratory, Elsevier, Amsterdam, 2002, pp. 779–836.
[5] F. Guan, H. Seno, A. Ishii, K. Watanabe, T. Kumazawa, H. Hattori, O. Suzuki, J. Anal. Toxicol. 23 (1999) 54–61.
[6] K.J. Reubsaet, H.R. Norli, P. Hemmersbach, K.E. Rasmussen, J. Pharmaceut, Biomed. Anal. 18 (1998) 667–680.
[7] Y. Luo, L. Pan, J. Pawliszyn, J. Microcol. Sep. 10 (1998) 193–201.
[8] K. Jinno, M. Taniguchi, M. Hayashida, J. Pharmaceut. Biomed. Anal. 17 (1998) 1081–1091.
[9] H. Yuan, Z. Mester, H. Lord, J. Pawliszyn, J. Anal. Toxicol. 24 (2000) 718–725.
[10] L. Slaughter, J. Reprod. Med. 45 (2000) 425–430.
[11] P.M. Gahlinger, Am. Family Physician 69 (2004) 2619–2626.
[12] K.M. Smith, J. Am. Pharm. Assoc. 39 (1999) 519–525.
[13] M.A. ElSohly, S.J. Salamone, J. Anal. Toxicol. 23 (1999) 141–146.
[14] A. Negrusz, C.M. Moore, T.L. Stockham, K.R. Poiser, J.L. Kern, R. Palaparthy, N.L.T. Le, P.G. Janicak, N.A. Levy, J. Forensic Sci. 45 (2000) 1031–1040.
[15] M.J. McGregor, Women Health 37 (2003) 71–80.
[16] R. Jochemsen, D.D. Breimer, J. Chromatogr. 227 (1982) 199–206.
[17] J. Gjerde, E. Dahlin, A.S. Christophersen, J. Pharm. Biomed. Anal. 10 (1992) 317–322.
[18] P. Lillsunde, T. Seppala, J. Chromatogr. 533 (1990) 97–110.
[19] J. Guichard, G. Panteix, J. Dubost, P. Baltassat, C. Roche, J. Chromatogr. 612 (1993) 269–275.
[20] Y.C. Sumirtapura, C. Aubert, P. Coassolo, J.P. Cano, J. Chromatogr. 232 (1982) 111–118.
[21] I. Deinl, C. Franzelius, L. Angermaier, G. Mahr, G. Machbert, J. Anal. Toxicol. 23 (1999) 598–601.
[22] A. Negrusz, R.E. Gaensslen, Anal. Bioanal. Chem. 376 (2003) 1192–1197.
[23] N. Jourdil, J. Bessard, F. Vincent, H. Eysseric, G. Bessard, J. Chromatogr. B. 788 (2003) 207–219.
[24] H. Yuan, W.M. Mullett, J. Pawliszyn, Analyst 126 (2001) 1456–1461.
[25] J.-N. Lin, J. Herron, J.D. Andrade, M. Brizgys, IEEE Trans. Biomed. Eng. 35 (1988) 466–471.
[26] J. Pawliszyn, Solid Phase Microextraction: Theory and Practice, Wiley, NY, 1997, p. 132.
[27] T. Gorecki, Solid versus liquid coatings, in: Janusz Pawliszyn (Ed.), Applications of Solid-Phase Microextraction, Royal Society of Chemistry, Cambridge, UK, 1998, pp. 92–108.
[28] N. Delaunay, V. Pichon, M.-C. Hennion, J. Chromatogr. B. 745 (2000) 15–37.

[29] S.R. Mikkelsen, E. Corton, Bioanalytical Chemistry, Wiley-Interscience, Hoboken, NJ, 2004, p. 71.
[30] M.L. Williamson, D.H. Atha, D.H. Reeder, P.B. Sundaram, Anal. Lett. 22 (1989) 803–816.
[31] J.-N. Lin, J. Herron, J.D. Andrade, M. Brizgys, IEEE Trans. Biomed. Eng. 35 (1988) 466–471.
[32] E.B. Mason, Human Physiology, The Benjamin/Cummings Publishing Company Inc., Don Mills, ON, 1983, p. 483.
[33] S.J. Salamone, S. Honasoge, C. Brenner, A.J. McNally, J. Passarelli, K. Goc-Szkutnicka, R. Brenneisen, M.A. ElSohly, S. Feng, J. Anal. Toxicol. 21 (1997) 341–345.