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Bio-compatible in-tube solid-phase microextraction capillary for the direct extraction and high-performance liquid chromatographic determination of drugs in human serum

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Abstract

A restricted access material (RAM), alkyl-diol-silica (ADS), was used to prepare a highly bio-compatible solid-phase microextraction (SPME) capillary for the automated and direct in-tube extraction of several benzodiazepines from human serum. The bifunctionality of the ADS extraction phase prevented fouling of the capillary by protein adsorption while simultaneously trapping the analytes in the hydrophobic porous interior. This the first report of a restricted access material utilized as an extraction phase for in-tube SPME. The approach simplified the required apparatus in comparison to existing RAM column switching procedures, and more importantly eliminated the excessive use of extraction solvents. The biocompatibility of the ADS material also overcame the existing problems with in-tube SPME that requires an ultrafiltration or other deproteinization step prior to handling biological samples, therefore further minimizing the sample preparation requirements. The calculated oxazepam, temazepam, nordazepam and diazepam detection limits were 26, 29, 22 and 24 ng/ml in serum, respectively. The method was linear over the range of 50–50 000 ng/ml with an average linear coefficient (R^2) value of 0.9998. The injection repeatability and intra-assay precision of the method were evaluated with five injections of a 10-μg/ml serum sample (spiked with all compounds), resulting in an average RSD<7%. The ADS extraction column was robust, providing many direct injections of biological fluids for the extraction and subsequent determination of benzodiazepines.

Keywords: In-tube solid-phase microextraction; Solid-phase microextraction; Restricted access media; Alkyl-diol silica columns; Benzodiazepines

1. Introduction

The complexity of biological samples demands a powerful sample preparation technique and is usually the most critical and time-consuming step for drug analysis in biological matrices [1]. Although, extraction strategies such as solid-phase extraction (SPE) and liquid–liquid extraction (LLE) have been extensively used [2], it has become widely recognized that these sample preparation approaches can suffer from lengthy extraction times, excess use of solvents and poor automation capabilities.

Solid-phase microextraction (SPME) is a relatively new approach to sample preparation, initially developed by one of the authors for the determination of volatile and semi-volatile organic pollutants

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in water [3]. A recent literature review of SPME for drugs and poisons from biological samples concluded the main advantages of SPME are high sensitivity, solventless extraction, small sample volume, simplicity, and speed [4]. Successful automation and interfacing of SPME to high-performance liquid chromatography (HPLC) has been accomplished by in-tube SPME, which utilizes an extraction phase coated on the inside of a capillary [5]. After positioning of the extraction capillary between the injection loop and injection needle of an autosampler, the sample is repeatedly drawn and ejected through the capillary for analyte partitioning into the capillary's extraction phase.

However, commercially available SPME coatings lack biocompatibility and therefore the direct SPME extraction of drugs from biological samples often require additional sample preparation, such as ultracentrifugation, to eliminate the protein component of the sample. Direct exposure of the extraction coating to biological samples, is complicated by the presence and adsorption of interferents such as proteins [6] and has consequently limited the wide application of SPME for bio-analysis [7].

One promising class of biocompatible extraction phases are the restricted access materials (RAMs), which fractionate a sample into the protein matrix and the analyte component on the basis of size [8–11]. Simultaneous with this size exclusion process, low-molecular-mass compounds are extracted and enriched, via partition, into the phase's interior. One recently developed example of a bio-compatible material for direct drug extraction and analysis is alkyl-diol-silica (ADS) and several applications are found in the literature [12–14]. A column switching format was configured using multiple pumps and switching valves, for the direct and multiple injections of untreated biofluids.

The objective of this research was to develop a simple, on-line and biocompatible in-tube SPME method, based on a restricted access material, for the determination of a class of model drug compounds in serum samples. Benzodiazepines were evaluated as they represent a class of drug compounds administered for a wide range of clinical disorders [15] and their extensive use and potential abuse demands an accurate and rapid sample preparation and analysis method. The existing extraction methods include LLE [16], SPE [17–19] or SPME [20–22] and have been the subject of a recent review article [23]. However, the developed in-tube ADS SPME approach was favorable to existing methods since the on-line and biocompatible extraction capillary enabled direct extraction and analysis of the sample, minimizing the sample handling requirements for biological samples. The developed approach was easily automated (less chance of sample loss or contamination), overcame the existing problems of biocompatibility for in-tube SPME phases, and utilizes less instrumentation (requires a single HPLC instrument) than traditional RAM column switching procedures. This is the first report of a restricted access material applied as a biocompatible in-tube SPME phase for the direct injection and simultaneous extraction of several benzodiazepines in biological samples.

2. Experimental

2.1. Chemicals and materials

All solvents were HPLC grade or better and purchased from J.T. Baker (Greisheim, Germany). The benzodiazepines were purchased from Radian International (Austin, TX, USA) as 1 mg/ml methanol solutions and stored at 4 °C. Deionized water, from a Millipore Milli-Q water system (Eschborn, Germany) was used for all experiments. Prepackaged phosphate-buffered saline (PBS, pH 7.4) was purchased from Sigma–Aldrich (Mississauga, Canada) and prepared according to the manufacturer's instructions. The LiChrospher RP-18 ADS, 25 μm material was supplied by Merck (Darmstadt, Germany).

2.2. Preparation and conditioning of in-tube ADS SPME capillary

The ADS particles were slurried in 2-propanol and packed into a 50 mm length of polyether ether ketone (PEEK) tubing (1.59 mm O.D.×0.76 mm I.D. from Upchurch Scientific, Oak Harbor, WA,

USA). The capillary column was capped at both ends by a 1/16 in. (1 in.=2.54 cm) zero-volume union fitted with a 2-μm frit (Chromatographic Specialties, Brockville, Canada). The ADS capillary was successively pre-conditioned with 10 ml of methanol and water, at a flow-rate of 0.25 ml/min, prior to its first use.

2.3. Instrumentation and analytical conditions

The in-tube ADS SPME configuration is shown in Fig. 1. An Agilent (Palo Alto, CA, USA) HPLC system (Model 1100) complete with autosampler and multiple-wavelength UV detector was used. The ADS capillary was connected between the injection needle and the injection loop of the autosampler. Vials (2 ml) filled with 1 ml of sample were set into the autosampler for analysis. Extraction of each sample was possible by repeated aspirating (draw) and dispensing (eject) of the sample through the capillary. Desorption of the extracted analyte was then possible by redirecting the appropriate mobile phase through the ADS capillary by switching the six port injection valve from the load to inject position for transport to the analytical column. All measurements were performed with a UV detector wavelength of 230 nm. The chromatographic column was a LiChrospher 100 RP-18e (15.0 cm×4.0 mm I.D., 5.0 μm particle size) from Merck. An inline filter (2 μm frit) and a LiChrospher 100 RP-18e (4.0 cm× 4.0 mm I.D., 5.0 μm particle size) guard column from Merck were installed at the inlet of the switching valve and the chromatographic column, respectively. Elution of the extracted compounds from the ADS column and separation by the reversed-phase HPLC column was accomplished with an optimized mobile phase gradient (reported below) at a flow-rate of 0.4 ml/min.

The void volume of the ADS capillary was determined by replacing the analytical column with the ADS capillary and measuring the retention volume of an injected acetone sample. Several experimental parameters, such as the volume of sample to be draw/ejected, the number and speed of draw/eject cycles and mobile phase composition, were evaluated as part of the optimization for the developed method.

2.4. Preparation of serum samples

Serum samples were collected from a drug-free healthy volunteer. Any precipitated material was removed by centrifuging the sample at 10 000 *g* for 10 min. The benzodiazepines were directly spiked into the supernatant of the biological samples over a range of 50–50 000 ng/ml. The samples were diluted 10 times with a PBS–methanol (95:5, v/v) mixture to adjust the pH to 7.4.

3. Results and discussion

3.1. In-tube ADS SPME

The theoretical considerations of in-tube SPME have been previously discussed [3] and will therefore not be dealt with here. In summary, the extraction process of in-tube SPME involves the partitioning of analytes into the extraction phase contained inside the capillary. Positioning the extraction capillary in the path of an autosampler's injection loop, as shown in Fig. 1A, provided a simple and automated way to expose the sample to the extraction phase. In this configuration, the autosampler was able to perform and repeat the extraction cycle by drawing and ejecting the sample through the ADS capillary. In principle, since the drawn and ejected sample volumes were equal, no or little sample matrix was introduced onto the HPLC system during the injection (see Fig. 1B).

The biocompatibility of the extraction material, ADS, was important to permit the direct, untreated and multiple exposure of complicated matrices such as biological fluids. The material possessed two different chemical surfaces and a pore size that restricted large molecules such as proteins from accessing the inner surface [24]. In combination with the size-exclusion process, the presence of hydrophilic electroneutral diol groups bound to the external surface of the spherical particles further protected the extraction phase from contamination by surface adsorption of proteins. The inner surface of the porous ADS material was modified with a C_{18} alkyl hydrophobic bonded phase that was responsible for simultaneous extraction of the target compounds. The volume of sample for each draw/eject extraction

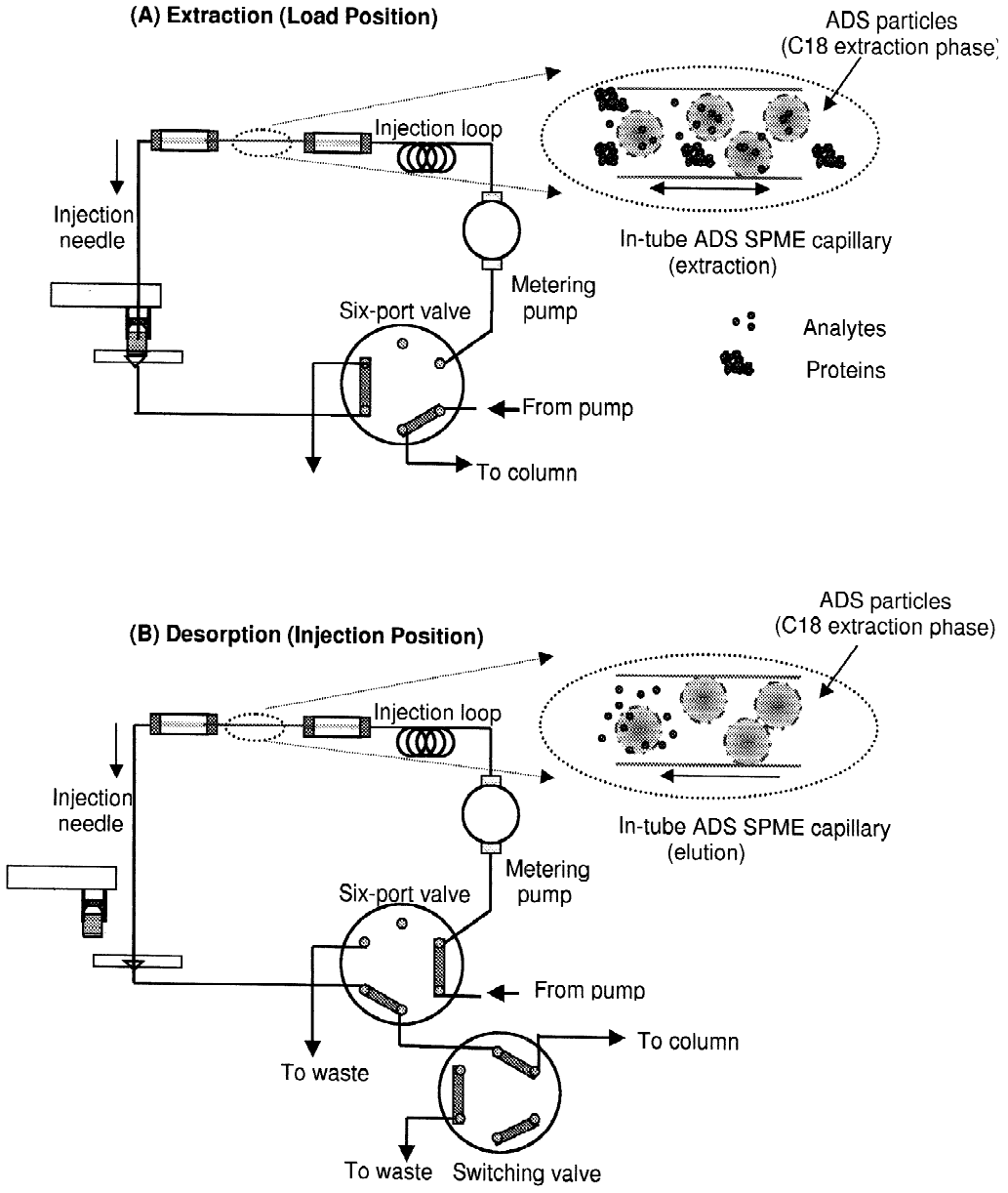

Fig. 1. Schematic representation of in-tube ADS SPME configuration. (A) Load position (extraction); (B) injection position (elution).

cycle was determined from the void volume (20 μl) of the ADS capillary and this value was used for all experiments. Although larger extraction volumes can be used, peak broadening was previously observed [25]. Since no extraction solvent is employed during in-tube SPME, it was critical to ensure the ADS capillary possessed the appropriate chemical composition to ensure high affinity of the benzodiazepines with the hydrophobic bonded phase, while also remaining compatible with plasma proteins. In previous serum extraction studies, the ADS capillary was equilibrated with a mobile phase containing <15%

of organic solvent to prevent the serum proteins from precipitating [26]. The low percentage of an organic modifier was also important to resolve protein bound drugs (providing a higher analyte recovery from the serum sample), prevent microbiological growth in the extraction system and to speed up the reconditioning.

Several strategies were evaluated for ensuring the in-tube ADS SPME capillary was properly conditioned prior to sample extraction. Since the total extraction phase volume was relatively small, conditioning of the capillary was possible under the control of the autosampler and the various approaches evaluated are summarized in Table 1. The autosampler was initially programmed to repeatedly draw and eject a 20-μl aliquot of conditioning solvent (water–methanol, 95:5, v/v), through the ADS extraction capillary. However as shown in Table 1, simply drawing one 20-μl aliquot of solvent and eliminating the ejection step was shown to be much more effective as indicated by a larger amount of diazepam extracted. Ejecting the conditioning solvent resulted in the capillary being reconditioned with the residual mobile phase present in the sample loop. Further enhancements in the reproducibility of the extraction efficiency were possible by flushing the ADS capillary with mobile phase of water–methanol (95:5, v/v). In this approach, the larger volume of solvent (400 μl) ensured the conditioning of the ADS capillary was complete. After elution of the analytes from the analytical column, the mobile phase was switched to water–methanol (95:5, v/v) to ensure the ADS capillary was properly conditioned prior to the next sample extraction.

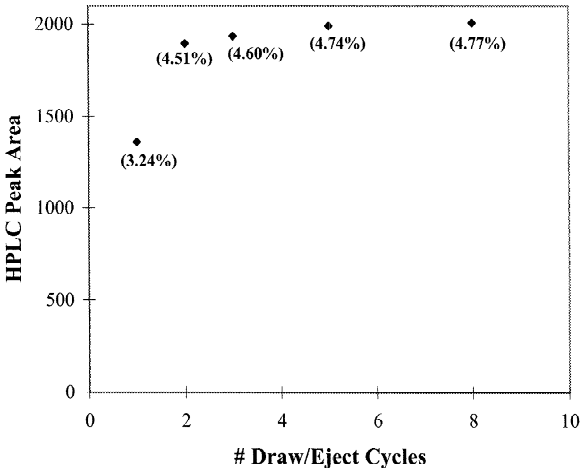

Fig. 2. In-tube SPME extraction recovery profile of 10 μg/ml diazepam with ADS capillary.% Recovery (as shown in parentheses) was calculated as mass extracted/total mass·100%. Conditioning solvent=water–methanol (95:5, v/v); sample solvent=water; SPME conditions: number of sample draw/eject cycles=5; draw/eject volume=20 μl; draw/eject rate=20 μl/min. Desorption mobile phase=water–methanol (34:66, v/v); detection wavelength=230 nm.

3.2. Equilibrium extraction profile

An equilibrium time extraction profile was monitored by increasing the number of draw eject steps for a 10 μg/ml diazepam standard solution. As shown in Fig. 2, the amount of diazepam extracted (corresponding to the resulting peak areas) increased greatly and rapidly when the number of extraction cycles (draw/eject cycles) was increased from 1 to 4. Increasing the number of extraction cycles beyond

Table 1
Effect of in-tube ADS capillary conditioning on the extraction efficiency of diazepam

Conditioning step		Diazepam peak area	RSD[a] (%)
Draw cycle No. (20 μl)	Eject cycle No. (20 μl)		
1	1	562	7.72
2	2	509	8.94
5	5	530	9.56
1	0	1885	6.86
400 μl of conditioning solvent		1909	4.31

[a] Calculated from three injections. Conditioning solvent=water–methanol (95:5, v/v). 10 μg/ml diazepam standard solution (in water). SPME conditions: number of sample draw/eject cycles=5; draw/eject volume=20 μl; draw/eject rate=50 μl/min. Desorption mobile phase=water–methanol (34:66, v/v); detection wavelength=230 nm.

this point does not result in a proportional increase in the amount extracted and therefore represents the equilibrium extraction value. Since the equilibrium value represents the total amount of analyte that can be extracted with the ADS capillary for that concentration, the number of cycles required to reach it is important to ensure the highest possible sensitivity while also minimizing the total time required for sample extraction.

Several experimental parameters can affect this equilibrium value, such as speed of the draw/eject cycle and modifications to the sample matrix. For example, changes in salt concentration and pH of the sample can affect the extraction efficiency. The optimal SPME extraction of benzodiazepines has been observed near physiology pH when neutral extraction coatings were employed [27,28]. This pH result has also been confirmed with the ADS material, while the extraction effect of salt addition to the sample was shown to be minimal [24,29,30]. Therefore, the pH of all samples was adjusted to 7.4 with PBS–methanol (95:5, v/v) to ensure a reproducible extraction, while no salt addition was performed to minimize the sample preparation requirements. As previously shown, the presence of an organic modifier, such as 5% methanol, added to the sample was useful to achieve release of the protein associated drugs like benzodiazepines, from the protein complex [24,30].

3.3. In-tube ADS SPME desorption and HPLC separation of benzodiazepines

After extraction of the analytes, on-line elution was simply accomplished by redirecting the mobile phase through the ADS capillary as shown in Fig. 1B. The hydrophobic interactions between the C_{18} extraction phase, located in the pores of the ADS material, and the extracted benzodiazepines can be effectively reduced by decreasing the polarity of the mobile phase, resulting in the rapid elution of the compounds from the ADS capillary. However, it is important to ensure the selected mobile phase will not only provide quantitative transfer of the extracted benzodiazepines from the ADS capillary but also still allow their separation on the analytical column.

Optimization of the mobile phase polarity for the elution and HPLC separation of the analytes was accomplished by performing direct injections of a benzodiazepine standard mixture into the HPLC system while using various volume ratios of water–methanol for the mobile phase. As expected, decreasing the polarity of the mobile phase with a higher percentage of methanol produced shorter elution times for the benzodiazepines on the C_{18} analytical column, but also sacrificed the chromatographic resolution. Good separation (resolution=1.5) of the compounds was possible with a mobile phase composition of water–methanol (37:63, v/v). This mobile phase composition completely eluted the extracted compounds from the ADS capillary and still provided adequate chromatographic resolution by the analytical column. The total run time was further reduced by employing a gradient elution. As described in Fig. 3A, a 1.0 μg/ml in-tube ADS SPME standard chromatogram, the ratio of water–methanol was increased slightly to (34:66, v/v) over the time interval of 2–8 min and held at this value until the end of the run.

The elution efficacy of the selected mobile phase was evaluated by measuring the presence of sample carryover. Also shown in Fig. 3B, a blank sample was extracted after performing an in-tube ADS

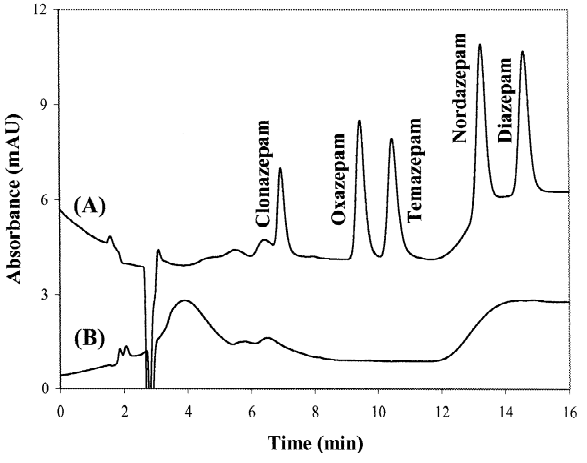

Fig. 3. In-tube ADS SPME of benzodiazepine standard sample (A) and blank water sample (B). Conditioning solvent=water–methanol (95:5, v/v); sample solvent=water; SPME conditions: number of sample draw/eject cycles=4; draw/eject volume=20 μl; draw/eject rate=15 μl/min. HPLC conditions: 0.4 ml/min gradient elution with water (solvent A) and methanol (solvent B); 0.0–2.0 min 5% B, 2.0–8.0 min 63% B, 8.0–19.0 min 66% B, 19.0–20.0 min 5% B; detection wavelength=230 nm.

SPME–HPLC analysis of a 1.0 μg/ml benzodiazepine sample. The absence of analyte peaks in the chromatogram confirms the complete elution of the extracted benzodiazepine analytes from the previous sample. Optimization of the extraction, elution and separation conditions was complete and a direct and on-line extraction of benzodiazepines analysis from biological fluids, such as serum, was now possible.

3.4. Serum analysis by in-tube ADS SPME

The successful extraction of drugs from biological fluids and matrices presents several challenges. They often contain proteins, salts, acids, bases and numerous other organic compounds with similar chemistry. Traditionally, SPE or LLE have been employed, but as is well recognized, these techniques can suffer from lengthy extraction times, excess use of solvents and poor automation capabilities and therefore alternative extraction approaches are desirable. SPME has overcome some of these difficulties, with its solventless and convenient format, however the number of commercially available extraction coatings is limited and suffer from poor biocompatibilty. Previous examples of SPME for benzodiazepines required additional sample pretreatment steps such ultrafiltration, LLE or the use of additional extraction agents [21,31,32]. However, the elimination of sample interferents such as proteins, can be greatly eliminated with an in-tube ADS SPME capillary, which simultaneously fractionates the protein component from the hydrophobic analytes.

Using the previously determined extraction and elution conditions, a blank serum sample was extracted using the in-tube SPME capillary. The resultant chromatogram is shown in Fig. 4A. Somewhat unexpectedly, the presence of residual proteins in the capillary was detected, as shown by the initial large absorbance peak. An increase in the HPLC column back pressure with subsequent injections was also observed, further indicating the presence of proteins precipitating on the analytical column.

One explanation for the observed result was the occurrence of sample mixing with the mobile phase during the draw/eject extraction cycles. To confirm this hypothesis, experiments were performed to quantify any effect of mixing during in-tube SPME. The ADS capillary was replaced with a chemically

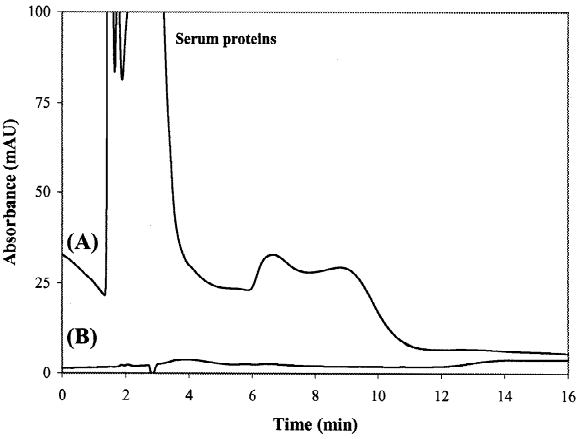

Fig. 4. In-tube ADS SPME of blank serum sample. Conditioning solvent=water–methanol (95:5, v/v); SPME conditions: number of sample draw/eject cycles=4; draw/eject volume=20 μl; draw/eject rate=15 μl/min. HPLC conditions: 0.4 ml/min gradient elution with water (solvent A) and methanol (solvent B); 0.0–2.0 min 5% B, 2.0–8.0 min 63% B, 8.0–19.0 min 66% B, 19.0–20.0 min 5% B; detection wavelength=230 nm.

inert PEEK capillary of similar dimensions to function as a control extraction capillary [33,34]. A 0.5% (v/v) solution of acetone (in water) was used as the sample. Diluted acetone was chosen as the test sample due to its high UV molar absorptivity, and compatibility with the analytical column. The acetone sample was analyzed by in-tube SPME using 10 draw/eject cycles and rate of 100 μl/min. Since the peak capillary did not possess any extraction coating, in the absence of sample mixing, there should be no measurable signal. However, after the in-tube SPME analysis, the presence of an acetone peak was detected. The absolute mass of acetone mixed as a result of the in-tube SPME analysis was calculated from a HPLC acetone mass calibration curve, determined by direct injections (data not shown). A mass ratio of acetone present from mixing over the total amount of acetone in the sample exposed to the PEEK capillary during SPME analysis was calculated to be 2.9%. Although, this value may appear to be small, in the presence of highly concentrated protein serum samples, it becomes unacceptable.

To compensate for the effects of mixing a rapid wash step was incorporated in the in-tube ADS SPME–HPLC method. A second switching valve, located on the autosampler (see Fig. 1B), was used to

direct a very small volume of wash solvent (400 μl) through the extraction capillary to waste, prior to switching the valve for elution of the analytes. As shown in Fig. 4B, the incorporation of the wash step was successful in removing any residual sample proteins from the extraction capillary. The composition of the wash solvent was critical to remove the residual sample matrix but not elute the extracted analytes. A polar solvent mixture such as water–methanol (95:5, v/v) was selected as it was previously shown to be unable to desorb the extracted benzodiazepines from the hydrophobic pores of the ADS material [30]. A mobile phase gradient was employed, incorporating both the wash and elution mobile phase conditions. The total time required for the analysis was ~11 min for extraction of the sample, followed by a 1 min wash of the capillary and a 15 min chromatographic run time.

Serum samples were spiked over a range of concentrations (0.05–50 μg/ml) with four benzodiazepine compounds. The successful extraction and elution of the benzodiazepines from the ADS capillary, followed by analytical separation of all compounds in the serum sample was accomplished under the optimized in-tube ADS SPME–HPLC method conditions. Baseline separation of all compounds was observed and no interfering compounds were present in the blank serum chromatogram, confirming the selectivity of the hydrophobic C_{18} extraction phase. The ADS material coupled to in-tube SPME was successful in eliminating the potential interference from this complicated biological sample. Analysis of a blank serum sample, after performing many spiked serum samples analysis, was also used to confirm the absence of sample carryover. Serum calibration curves were constructed for the benzodiazepines and as shown in Table 2, excellent linearity was observed for all analytes (average $R^2 = 0.9998$). The recovery of the analytes from spiked serum was calculated by comparison of the in-tube ADS SPME–HPLC peak areas to the standard solutions (in water) and was found to be >90% for all analytes.

The limit of detection (LOD) and limit of quantification (LOQ) for each compound in serum was determined at a concentration where the signal/noise ratios were equal to 3 and 10, respectively, and these calculated concentrations are included in Table 2. The metabolism of the clinically prescribed benzodiazepines over various dosage regimes, results in a wide concentration ranges being reported in the literature. However, diazepam concentrations over the range of 100–1000 ng/ml have been reported for serum samples [35]. Therefore, the detection limit and linear dynamic range of the developed in-tube ADS SPME–HPLC is suitable for the clinical analysis of this benzodiazepine. A further reduction of the limits of detection (with simultaneous increase in the selectivity) can be achieved by using HPLC–mass spectrometry (in the reaction monitoring mode).

The reproducibility of the developed method was determined with five injections of a 10-μg/ml sample and is shown in Table 2. The injection repeatability was calculated as an RSD for each benzodiazepine HPLC peak area in serum and the average value for all compounds was determined to be 4.6%. The intra-assay precision was determined with repeated analysis of a sample that has been independently prepared, over 1 day, yielding an average RSD of 6.3%.

The stability of the ADS extraction material has been previously reported under similar experimental

Table 2
Linear regression and injection repeatability data for benzodiazepine serum calibration curves

Compound	Regression line[a]			LOD (ng/ml)	LOQ (ng/ml)	RSD (%) (n=5)
	Slope	Intercept	R^2 value			
Oxazepam	125.5	14.7	1.0	26	86	3.7
Temazepam	130.4	13.1	0.9998	29	98	5.2
Nordazepam	218.0	−27.6	0.9999	22	74	5.1
Diazepam	217.1	−4.5	0.9999	24	81	4.6

[a] Concentration range 50–50 000 ng/ml; number of data points 6. SPME conditions: number of sample draw/eject cycles=4; draw/eject volume=20 μl; draw/eject rate=15 μl/min. HPLC conditions: 0.4 ml/min gradient elution with water (solvent A) and methanol (solvent B); 0.0–2.0 min 5% B, 2.0–8.0 min 63% B, 8.0–19.0 min 66% B, 19.0–20.0 min 5% B; detection wavelength=230 nm.

conditions and was shown to be functional for over 2000 50-μl injections of plasma [36]. The present in-tube ADS SPME configuration was validated with over 100 extractions of serum and only minimal system pressure increase was observed. The stability and robustness of the method were also investigated by varying the percentage of methanol in the conditioning solvent and elution mobile phase. As previously mentioned, too much organic content in the conditioning solvent can lead to irreversible protein precipitation on the column, however, adequate extraction, elution and separation of each benzodiazepine compound from the serum matrix was still possible with ±2% of methanol in the solvent.

4. Conclusions

The in-tube ADS SPME capillary provided a simple and on-line approach for the direct injection and repeated extraction of benzodiazepines in complicated biological matrices, such as serum. In comparison to existing extraction procedures for benzodiazepine determination, the procedure was simple and easily automated with minimal sample preparation requirements and thereby provided a safer handling of potentially bio-hazardous material. The biocompatibility of the RAM material overcame the existing disadvantages of existing in-tube SPME phases, while the convenient format and minimal solvent requirements of SPME represented an significant improvement over the more the established RAM column switching approaches. Only a single pump and autosampler was required for the repeated ejection and analysis of drugs in a biological matrix.

The well understood extraction chemistry of the C_{18} extraction phase simplified the in-tube ADS SPME method development. The isolation and separation of analytes can be further simplified with the modification of the interior extraction phase to render the sorbent more selective [37] and the use of restricted access materials even more versatile. Additional experiments in our laboratory are underway to further validate the approach as a useful tool for screening and determination of a wide range in analytes in complicated biological matrices.

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