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Automation of Solid-Phase Microextraction in High-Throughput Format and Applications to Drug Analysis

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The automation of solid-phase microextraction (SPME) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) was accomplished using a 96 multiwell plate format, a SPME multifiber device, two orbital shakers, and a three-arm robotic system. Extensive optimization of the proposed setup was performed including coating selection, optimization of the fiber coating procedure, confirmation of uniform agitation in all wells, and the selection of the optimal calibration method. The system allows the use of pre-equilibrium extraction times with no deterioration in method precision due to reproducible timing of extraction and desorption steps and reproducible positioning of all fibers within the wells. The applicability of the system for the extraction of several common drugs is demonstrated. The optimized multifiber SPME-LC-MS/MS was subsequently fully validated for the high-throughput analysis of diazepam, lorazepam, nordiazepam, and oxazepam in human whole blood. The proposed method allowed the automated sample preparation of 96 samples in 100 min, which represents the highest throughput of any SPME technique to date, while achieving excellent accuracy (87−113%), precision (≤20% RSD), and sensitivity (limit of quantitation 4 ng/mL). Automated SPME provides unique advantages over automated solid-phase extraction (SPE) including lower cost, the ability to quantitatively determine free and total drug concentrations in a single biofluid sample, and the ability to directly process whole blood samples with absolutely no sample pretreatment required.

Solid-phase microextraction (SPME) is a simple, fast, sensitive, and convenient equilibrium-based sample preparation technique that allows the integration of sampling and sample preparation steps.[1] SPME has been applied successfully to the analysis of a variety of drugs when coupled to liquid chromatography (LC) methods with ultraviolet or mass spectrometric (MS) detection.[2−4] Although these SPME-LC methods are found to be sensitive, accurate, and precise, they suffer from low-throughput and lack of automation. This limits their widespread use in laboratories that routinely need to analyze a large number of similar samples. To date, the need to automate SPME-LC analyses has been addressed primarily through the development of in-tube SPME.[5] For in-tube SPME, a high-performance liquid chromatography autosampler is programmed to perform the extraction and desorption steps of SPME with minimal user intervention. Successful applications of in-tube SPME include clinical applications such as the analysis of β-blockers,[6−9] amphetamines,[6] cortisol,[10] tricyclic antidepressants,[11] and benzodiazepines[12−14] in biological and pharmaceutical samples. Although in-tube SPME offers a high degree of automation, there are two main limitations of the technique. Narrow capillary columns are used as the extraction phase, so it is necessary to filter, dilute, or pretreat bioanalytical samples prior to in-tube SPME in order to prevent blockage of the extraction column, thus adding extra steps to the analysis.[14,15] More importantly, the samples are processed and analyzed serially, so although in-tube SPME is a fully automated technique, its throughput is limited. This limited throughput is primarily due to long extraction and desorption times, which are a result of slow kinetics of mass transfer between (i) extraction phase and sample solution and/or (ii) extraction phase and desorption solvent.

One way to address the problem of slow kinetics in the liquid phase and thus drastically improve the throughput of SPME is to perform the extraction and desorption steps of many similar samples in parallel, as originally proposed by O'Reilly et al.[16] The

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potential of this parallel extraction approach using a multiwell plate format was initially demonstrated by Wang et al.,[17] who used it to successfully increase the throughput of peptide analysis by SPME-matrix assisted laser desorption ionization mass spectrometry. To enable parallel SPME extraction using the fiber configuration and solvent desorption, Hutchinson et al.[18] recently evaluated different SPME geometries and agitation methods to conclude that the multifiber SPME using hollow-membrane polydimethylsiloxane (PDMS) coating and orbital agitation was the best platform for the automation of SPME on a 96 multiwell plate format.

Further development of this automated parallel SPME system would enable applications such as clinical studies and routine therapeutic drug monitoring while offering numerous advantages over the sample preparation methods currently employed for such high-throughput bioanalyses.[19−31] Very few automated solid-phase extraction (SPE) methods for the analysis of whole blood are reported in current literature, and sample pretreatment steps such as dilution, filtration, centrifugation, and/or protein precipitation with organic solvents or acids are necessary prior to the application of the whole blood sample to the SPE cartridge.[26,32] In comparison with automated liquid−liquid extraction (LLE) or SPE, automated SPME is simpler, minimizes solvent consumption, and can be used directly in complex biofluids with absolutely no sample pretreatment. Automated SPME is also faster than current online SPE methods which typically require 1.5−10 min per sample,[26] and its speed is comparable to 96-well plate automated (and semiautomated) LLE and SPE methods. In contrast to traditional techniques, which provide information on the total (free and protein-bound) drug concentration only, SPME can directly determine both the pharmacologically active free drug concentration as well as total drug concentration in the same experiment as long as appropriate calibration curves are constructed.[33−35] Similarly, SPME can also be used for the determination of free concentration of drug in liposome formulations.[36]

The objective of this work was to build on the original study by Hutchinson et al.[18] and present a fully optimized and automated multifiber SPME-LC-MS/MS system for the first time. PDMS coating was found unsuitable for drug analysis due to very slow kinetics and lack of intrafiber reproducibility, so an alternative simple and reproducible fiber preparation procedure based on SPE-type sorbents is reported. The system is applicable to the analysis of various drugs in complex matrixes such as whole blood. As an example application, the performance of the optimized system was evaluated for high-throughput analysis of benzodiazepine concentrations in whole blood. Benzodiazepines were chosen as model analytes because of their widespread use as tranquilizers, hypnotics, muscle relaxants, and anticonvulsants. The proposed high-throughput SPME-LC-MS/MS method met current regulatory requirements for quantitative bioanalytical method validation set by the Food and Drug Administration (FDA)[37] and allowed the automated sample preparation of 96 samples in 100 min. This study shows for the fist time the applicability of SPME to high-throughput automated quantitative bioanalysis and the capability of the technique to directly handle whole blood samples with no sample pretreatment steps.

EXPERIMENTAL SECTION

Chemicals and Materials. Diazepam, nordiazepam, oxazepam, dextromethorphan, pseudoephedrine, and diazepam-d_5 were purchased from Cerilliant (Round Rock, TX) as 1 mg/mL methanolic solutions, while lorazepam was purchased as a 1 mg/mL solution in acetonitrile. Working standards were prepared weekly from these stock solutions using acetonitrile/water (1:1 v/v) as the diluent and kept refrigerated when not in use. Acetonitrile (HPLC grade), methanol (HPLC grade), and glacial acetic acid were purchased from Fisher Scientific (Ottawa, Canada). Sodium chloride, potassium chloride, and potassium phosphate were purchased from EMD Chemicals (Gibbstown, NJ). Sodium phosphate, acetaminophen, and Carbowax/Templated Resin (CW/TPR) SPME fiber were purchased from Sigma-Aldrich (Oakville, Canada). C18 and C16-amide-coated silica particles were obtained as research samples from Supelco (Bellefonte, PA). Type 316 stainless steel wire was purchased from Small Parts Inc. (Miami Lakes, FL). Polypropylene 96-deep-well plates and BD K_2 EDTA 18 mg Plus Vacutainer blood collection tubes were purchased from VWR International (Mississauga, Canada). HelixMark polydimethylsiloxane-based hollow tubing with 0.31 mm i.d. and 0.64 mm o.d. was purchased from Helix Medical (Carpinteria, CA). Locktite 349 glue was purchased from R.S. Hughes Company (Plymouth, MI). Phosphate-buffered saline solution (PBS, pH 7.4) was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate in 1 L of purified water and adjusting the pH to 7.4, if necessary. The pin-tool replicator (model AFIX96FP3) and floating

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pins (FP8, 0.356 mm diameter), which were used in the studies with PDMS coatings, were purchased from V&P Scientific (San Diego, CA, United States).

Automation of Multifiber SPME-LC-MS/MS. The automated SPME system was obtained from Professional Analytical System Technology (PAS, Magdala, Germany) and consisted of a three-arm robotic autosampler that was fully controlled with Concept software and two orbital agitators. One XYZ arm of the autosampler was used to hold, transport, and position the 96-fiber SPME device for the extraction and desorption steps of SPME. The second arm was equipped with a N_2 blow-down device in order to perform solvent evaporation and analyte preconcentration steps if enhanced sensitivity was required. Finally, an *XYZ* arm equipped with a 250 μL syringe was used to dispense preset volumes of reconstitution/desorption solvents or internal standard solutions into the individual wells of the 96-well plate, as well as to perform sample injection into the HPLC port of the analytical system. In summary, the use of this autosampler allowed for the automation of all sample preparation steps, including the addition of an internal standard, multifiber SPME extraction, and desorption for a preset amount of time with controlled agitation, evaporation of the solvent, reconstitution, and injection of the final sample solution from individual wells into the HPLC injection port.

A multifiber SPME device was constructed with 96 SPME fibers (Figure 1b) that were positioned to fit in the centers of each well of a 96-well plate (Figure 1c). As part of the optimization study, three different types of extraction phases were compared: (1) PDMS-based, (2) C18-coated silica particle, and (3) RP-amide C16 (RPA)-coated silica particle extraction phases.

For PDMS coatings, a hollow fiber tubing with a small internal diameter, made from PDMS and proprietary filler, was cut into 2 mm pieces and placed over stainless wire. The thickness of the extraction phase for these fibers was 165 μm, as determined by the thickness of the tubing. A commercially available pin-tool replicator was used as the support to immobilize the PDMS coatings, as described previously by Hutchinson et al.[18] The pin-tool replicator was directly attached to the PAS autosampler arm using appropriate screws. For C18 and RPA coatings, a new multifiber SPME device was custom-built by the University of Waterloo Machine Shop to accommodate 0.061 in. fibers (Figure 1b). This multifiber SPME device was built as described elsewhere in detail,[38] except that silicone PCR-Mat (Ultident Scientific, St. Laurent, Canada) was used to hold the fibers between the two plates of the device instead of foam. This improved device addressed two main shortcomings of the commercially available pin-tool device: (1) it allowed the use of thick 0.061 in. wires which significantly increased the surface area of coating and (2) it improved the robustness of the automated system by eliminating the problem of fiber bending, which was occasionally encountered during the robotic manipulations of the pin-tool replicator.

The desorption volume used in the application presented in this paper was 800−1000 μL, so the above PAS system could not be used to accurately dispense desorption solvent. For these experiments, the desorption solvent was either dispensed manually using an air displacement Fisherbrand 200−1000 μL pipet or using Ultraspense 2000 (KD Scientific, Holliston, MA), an automated 96/384-well microplate reagent dispenser. The use of the automated dispenser was found to be equivalent to manual pipetting in terms of accuracy and precision (Table S-1 in Supporting Information) but improved automation and offered significant time (about 10-fold) and labor savings.

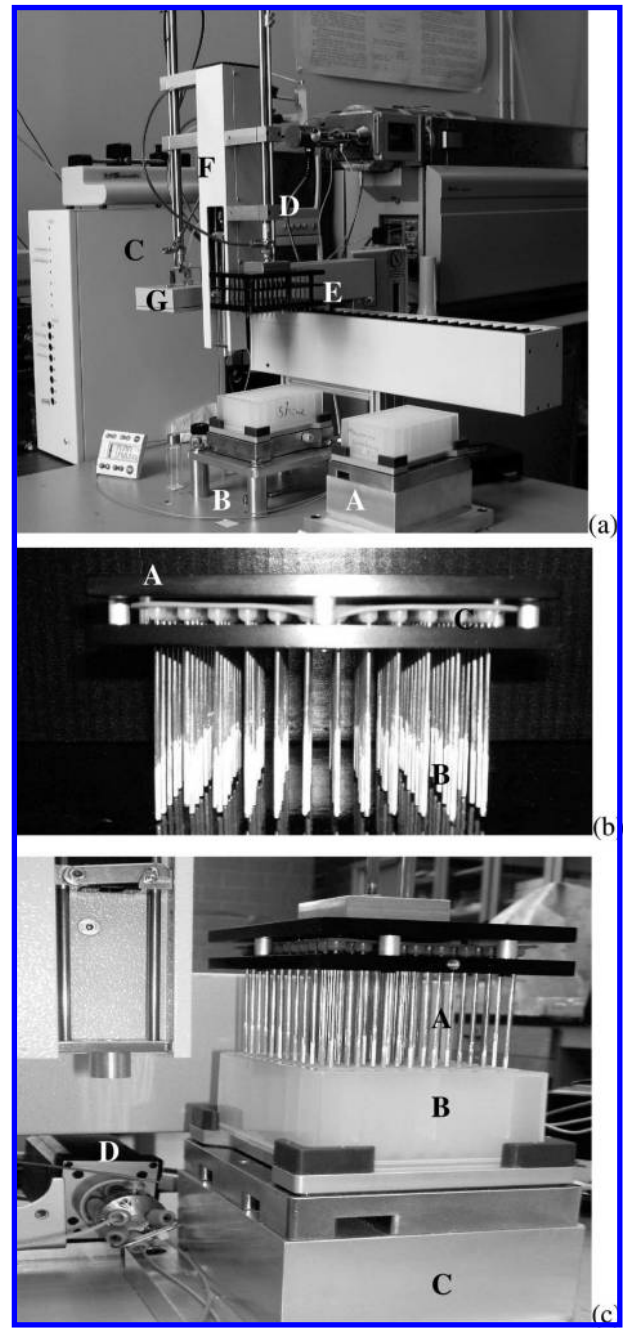

Figure 1. (a) Photo showing PAS robotic system. The main components of the system are orbital agitators used for extraction and desorption (A, B), system controller (C), arm used to manipulate the SPME multifiber device (D), SPME multifiber device (E), syringe arm (F), and the arm used for simultaneous nitrogen evaporation from all wells (G). HPLC 6-port valve is positioned between two orbital agitators as shown in part c.(b) Photo showing custom-made multifiber SPME device (A) constructed using 0.061 in. stainless steel wires and RPA extraction phase (B). Part C shows the PCR silicone mat used to hold the fibers in the device. (c) Photo showing the details of the SPME multifiber device (A), 96-well plate(B), agitator (C), and HPLC six-port valve (D) employed in the PAS robotic system.

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Table 1. Summary of MS/MS Parameters

analyte	pK_a[41−44]	log P[41]	Q1 mass (amu)	Q3 mass (amu)	DP (V)	FP(V)	EP(V)	CE (V)	CXP (V)
diazepam	3.4	2.82	285.0	153.9	92	120	7.5	39	10
lorazepam	13	2.39	321.1	275.1	101	360	10	31	20
oxazepam	12.4	2.24	287.1	241.1	61	160	10	31	18
nordiazepam	11.8	2.93	271.1	140.0	66	170	10	39	10
diazepam-d_5	N/A[a]	N/A[a]	290.2	154.1	92	120	7.5	39	10
pseudoephedrine	10.3	0.89	165.8	115.2	15	70	10	30	8
acetaminophen	9.38	0.46	152.0	110.0	92	120	7.5	39	10
dextromethorphan	8.3	3.97	271.4	216.1	30	100	7	30	15

[a] N/A = not applicable.

Preparation of Extraction Phases Based on C18 and RPA Coated Silica Particles. The preparation procedure for C18 and RPA SPME extraction phases used in this work was based on the procedure reported by Mullett and Pawliszyn[39] but further optimized to improve interfiber reproducibility. Stainless steel wire of 0.061 in. diameter was cut into 8 cm pieces. Prior to coating, the surface was prepared by sanding, followed by 20 min sonication in acetone, and 2 h sonication in 6 M nitric acid. The wires were rinsed thoroughly with purified water and then methanol and then allowed to air-dry. The wires were then coated with a thin layer of Locktite 349 glue and rolled in 5 μm coated silica particles (C18 or RPA) to obtain good surface coverage. Coated fibers were then cured under an ultraviolet lamp for 1 h. The length of coating was 15 mm. Before initial use, the fibers were conditioned by overnight exposure to a methanol/water (1:1 v/v) solution, followed by a 30-min exposure to purified water, and a 30-min exposure to acetonitrile/water (1:1 v/v) mixture. For subsequent uses, the fibers were only conditioned for 30 min using the methanol/water (1:1 v/v) mixture.

LC-MS/MS Analysis. All analyses were performed using a LC-MS/MS system consisting of CTC-HTS PAL autosampler with cooled sample tray, Shimadzu 10AVP LC with dual pumps (Shimadzu LC10ADvp) and system controller (SCL10Avp), and a Applied Biosystems API3000 tandem mass spectrometer equipped with TurboIonSpray source. Analyst software (version 1.4.1) was used for data acquisition and processing. The column used for the separation of the analytes was Symmetry Shield RP18 with dimensions of 2.1 mm × 50 mm and 5 μm particles (Waters, Millford, MA). Chromatographic conditions used for the separation of benzodiazepines are reported elsewhere.[40] Sample injection volume was 20 μL. Samples were injected in duplicate and kept at 5 °C on the autosampler while waiting for analysis. Total chromatographic run-time was 5.0 min and included the re-equilibration of the analytical column. A bypass pump and a Waters switching valve were used to divert the flow of column effluent for the first 1.0 min of benzodiazepine run time. MS conditions used were nebulizer gas = 6, curtain gas = 10, CAD gas = 12, ionspray voltage 5300 V, and source temperature set to 400 °C. The same MS parameters were used for the analysis of pseudoephedrine, acetaminophen, and dextromethorphan except CAD gas =4 and ionspray voltage = 4000 V. All of the compounds were analyzed in positive ion MRM mode using instrument settings described in Table 1.

SPME Procedure for High-Throughput Analysis of Benzodiazepines in Whole Blood. Whole blood was collected from a healthy volunteer using BD K_2 EDTA 18 mg Plus Vacutainer tubes, and samples were kept on ice until use. Standards and validation samples were prepared by spiking an appropriate amount of each drug standard ranging from 1 to 1000 ng/mL in such a way as to keep the organic solvent concentration at exactly 1% methanol. The internal standard (diazepam-d_5) was then spiked into all samples and standards at the final concentration of 400 ng/mL, and 800 μL of appropriate whole blood sample or standard solution was pipetted into each well. The plate was then placed on the automated system; the 96-fiber SPME top plate was lowered to expose the fiber coating to the well contents. The extraction was performed for 30 min with agitation set to 850 rpm. Fibers were rinsed for 30 s using purified water to eliminate any blood cells from the surface of the fibers. After rinsing, the fibers were desorbed at 850 rpm for 30 min into a new 96-well plate prefilled with 800 μL of desorption solvent (acetonitrile/water, 1:1 v/v). These extracts were then analyzed directly using LC-MS/MS. The calibration was performed using $1/x$ weighted linear regression in SigmaPlot 2004 for Windows (version 9.0) software.

Scanning Electron Microscopy (SEM). LEO 1530 field emission SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) was used to acquire SEM images of the coating using an acceleration voltage of 15 kV. Carbon conductive tape and specimen mounts (Ted Pella, Redding, CA) were used to hold the samples. Prior to analysis, fibers were dried for 1 h in the oven at 140 °C and then sputtered with ~10 nm of gold.

RESULTS AND DISCUSSION

Optimization of Automated SPME-LC-MS/MS System. The evaluation of the performance of the proposed automated SPME-LC-MS/MS system included the evaluation of uniformity of agitation in all wells, fiber-to-fiber reproducibility, carryover of analyte in the extraction phase, possibility of cross-contamination across the wells during agitation, fiber robustness, selection of the optimal calibration method, and evaluation of suitability of the system for use with pre-equilibrium extraction times. For the automation of SPME to be successful in parallel extraction format, excellent reproducibility of the amount of analyte extracted by

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different fibers is necessary. Otherwise, the calibration and quantitative results obtained by the use of multiple fibers would not be accurate or precise. Thus, coating selection and reproducibility of the coating procedure were also the major factors affecting system performance and were investigated in detail in the current study. The comparison of performance of three different types of coatings: PDMS, C18-coated silica particles, and RPA coated silica particles is reported and shows that the best quantitative results were obtained when thin coatings with short extraction times were employed.

Investigation of the Uniformity of Agitation Using Orbital Shaking and PDMS Coatings. An extensive investigation to evaluate the uniformity of agitation using orbital shaking was carried out using PDMS coatings proposed by Hutchinson et al.[18] The thick 165 μm PDMS coating was ideally suited to the study of the uniformity of agitation because of its large extraction capacity. The uniformity of agitation was initially examined by plotting the dependence of the amount extracted to the well position by examining the residual plots of the amount extracted versus the well position over eight independent extractions (Figure S-3 in Supporting Information). No trends in these residual plots were found, indicating uniformity of agitation. The uniformity of agitation within wells was further confirmed by constructing extraction time profiles for all 96 fibers. In SPME, any differences in agitation among the wells would result in some wells requiring longer extraction times in order to reach equilibrium. The time required to reach equilibrium is defined as the time when no further increase in the amount extracted occurs within experimental error and was determined to be 20 h for diazepam in this study. The extraction time profiles for each of 96 fibers indicated that the equilibrium was reached in all cases confirming uniformity of agitation when a speed of 850 rpm was used. The extraction time profiles of six representative fibers are shown in Figure 2a.

On the basis of the residual plots and extraction time profiles, it was concluded that an agitation of 850 rpm using orbital shaking is uniform and there is no need to apply well position correction factors to this automated multifiber SPME system. However, the use of a 20 h extraction time is impractically long, so the suitability of using pre-equilibrium SPME conditions was examined. As shown in Figure 2b, for all extraction times longer than 15 min, excellent reproducibility was achieved using pre-equilibrium conditions as shown by % RSD of 11 ± 2%. The higher RSD for 15-min extraction time is attributed to the time required to establish uniform mixing in the wells and/or remove any air bubbles present in the wells. The results show that automated SPME can be used with pre-equilibrium extraction times with no sacrifice of method precision as long as sufficient time is allowed to establish uniform agitation. This is accomplished through use of parallel automated extraction which allows very accurate computer-controlled timing of extraction and desorption steps as well as very reproducible software-controlled positioning of fibers in all of the wells.

Investigation of Well Cross-Talk Using PDMS Coatings. The cross-talk between wells is another important factor to examine during the evaluation of automated systems based on multiwell plate technology. In order to evaluate if there is any well cross-contamination, a blank buffer solution was placed in random well locations across the plate (26 locations as shown in

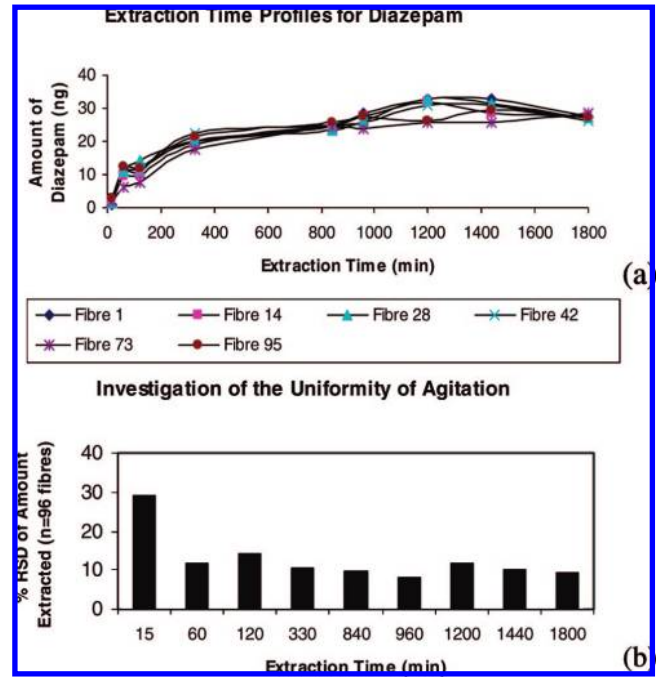

Figure 2. (a) Representative extraction time profiles for the extraction of diazepam from a 100 ng/mL standard solution for six different fibers and well positions (data for all 96 fibers was collected as discussed in text but shown for only six fibers for clarity). (b) Dependence of % RSD for the amount extracted of diazepam (n = 96 fibers) on the extraction time.

Figure S-4), while the remaining wells contained a high concentration diazepam standard (300 ng/mL). This plate was then analyzed using optimized SPME-LC-MS/MS method. The diazepam peak was not detected in the chromatograms of any of the wells containing blank solution thus confirming that cross-contamination across wells does not occur using optimum agitation conditions.

Summary of Overall Performance of PDMS Coatings. In the initial experiments, PDMS-based hollow tubing of 165 μm thickness was used as the SPME extraction phase because of its low cost and good robustness and reproducibility. For this PDMS coating, the fiber-to-fiber reproducibility is limited by the uniformity of the tubing thickness and the ability to cut the tubing into pieces of equal length. The robustness, extraction capacity, reusability (>50 times with no loss of extraction efficiency), and interfiber reproducibility (Table 2) of the PDMS hollow fiber coatings were all found to be satisfactory for their use in the automated multifiber SPME system. However, intrafiber reproducibility for a significant number (about one-third) of the fibers exceeded 10%, which is unacceptable for quantitative analysis (Figure S-2). Fiber carryover, loss of extraction phase, well cross-talk, and possible differences in agitation across the plate were investigated in detail and eliminated as possible causes for the lack of reproducibility observed for some fibers. On the basis of this data, it was concluded that large RSDs for the extraction using the same fiber are most likely caused by unequal evaporative losses from different wells and changes in extraction temperature due to very long extraction and desorption times. The use of internal standard to help compensate for the possible evaporative losses was examined but did not yield an improvement in reproducibility (Table 2). Therefore, as summarized in Table 2, PDMS coating was found unsuitable for drug analysis using the

Table 2. Comparison of Performance of PDMS and RPA Coatings for the Extraction of Diazepam from PBS buffer, pH 7.4 Using Optimized SPME Conditions

analytical parameter	PDMS	RPA (0.061 in.)
extraction capacity for diazepam	~30%	~30%
carryover	0.3%	2%
intrafiber reproducibility (n = 5 extractions, mean of 96 fibers)	9% RSD	7% RSD
intrafiber reproducibility (n = 5 extractions, range for 96 fibers)	RSD range: 1−23%	RSD range: 6−10%
interfiber reproducibility (n = 96 fibers, no internal std)	10% RSD	12% RSD
interfiber reproducibility (n = 96 fibers, with internal std)	15% RSD	7% RSD
equilibration time	20 h	30 min

proposed SPME system due to very slow kinetics for extraction and desorption, which are impractical for high-throughput applications, and unacceptable intrafiber reproducibility exhibited by a significant number of the PDMS fibers.

SPME Fibers Based on Coated Silica Particles: Optimization of Coating Procedure and Evaluation of Fiber Performance. The materials that are typically used in reverse-phase LC such as octadecyl (C18) porous silica particles were previously shown to perform well for SPME extraction phases due to their good extraction capacity and reasonable extraction kinetics for more polar analytes such as drugs and metabolites.[35,39,45−47] These extraction phases address some of the problems encountered with PDMS coatings so their suitability for use with the automated multifiber SPME system was evaluated. The coatings were prepared by the attachment of the coated silica particles to the stainless steel surface using a strong adhesive with good chemical resistance.

During initial experiments, the performance of different types of coated silica particles was compared for four benzodiazepine drugs: diazepam, nordiazepam, oxazepam, and lorazepam. Figure 3a shows the results of this comparison for diazepam, but the results for other analytes tested showed similar trends. Two consecutive extractions were performed using 12 fibers of each coating type. The coatings were compared in terms of interfiber reproducibility, extraction efficiency, and adhesion of the coating upon repeated use. C18 silica particles of 10 μm diameter were found to have the highest extraction efficiency for benzodiazepines among the particles tested. However, the results for the second extraction using this coating show significant decrease in the amount extracted and significant deterioration of interfiber reproducibility, both of which are indicative of a significant loss of coating. SPME coatings based on 5 μm particles showed significantly improved adhesion as shown by excellent extraction reproducibility for the two extractions. Among these coatings, the Ascentis RPA coating was found to have the best interfiber reproducibility and was selected for all further experiments. This coating has carbon loading of 19.5%, surface area of 450 m^2/g, and its structure includes an octadecyl carbon chain modified with a stable embedded amide group.

Prior to further RPA coating development, intrafiber reproducibility was examined to ensure the performance for repeated extractions using the same fiber was acceptable. On the basis of the results of 14 extractions, the use of lorazepam as an internal standard was found to significantly improve reproducibility from 9–19% RSD to 3−11% RSD for all compounds as summarized in Table S-3. No fiber exceeded an RSD of 13% indicating better performance than the PDMS coating. Figure 3b illustrates that fibers are reusable with no loss of coating upon repeated use.

Stainless steel wires of different dimensions were evaluated in terms of their compatibility with the PAS system, reproducibility of fiber coating, and extraction capacity in order to determine which was most suitable for use with automation. The results of this experiment are shown in Figure 3c. As predicted by SPME theory, the extraction capacity of the fibers increased as the surface area of the solid support increased. The best fiber-to-fiber reproducibility as indicated by the lowest RSD was achieved by using the thickest stainless steel wires (0.061 in.) as support for immobilizing the SPME coating, thereby indicating a more uniform surface coverage was achieved with thicker fiber cores. It was not feasible to increase the fiber diameter further due to the limited dimensions of the wells, so the use of thin films to further increase surface area and extraction capacity of automated SPME devices was subsequently examined.[38] In addition to the above fiber coating optimization experiments, acid etching time was also investigated and optimized (Figure S-5). The optimum coating conditions are summarized in the Experimental Section.

Comparison of Calibration Methods. The use of lorazepam as an internal standard was found to significantly improve intrafiber reproducibility as discussed in the previous section. A more detailed study was then conducted to evaluate whether the use of an internal standard was also able to compensate for interfiber variability in extraction capacity and whether the use of a deuterated internal standard provides additional improvements. In addition, the use of the standard-in-fiber calibration approach for automated SPME-LC methods was investigated and compared to the internal, fiber constant, and external standard calibration methods as a way to compensate for interfiber variation. Briefly, with the use of the standard-in-fiber calibration approach, a known amount of standard (usually a deuterated analogue) is added to the system under investigation from the extraction phase, while the analyte is simultaneously being extracted into the extraction phase.[48−51] This calibration approach has proven particularly useful for increasing the accuracy of the analysis by successfully correcting for matrix effects and as the calibration

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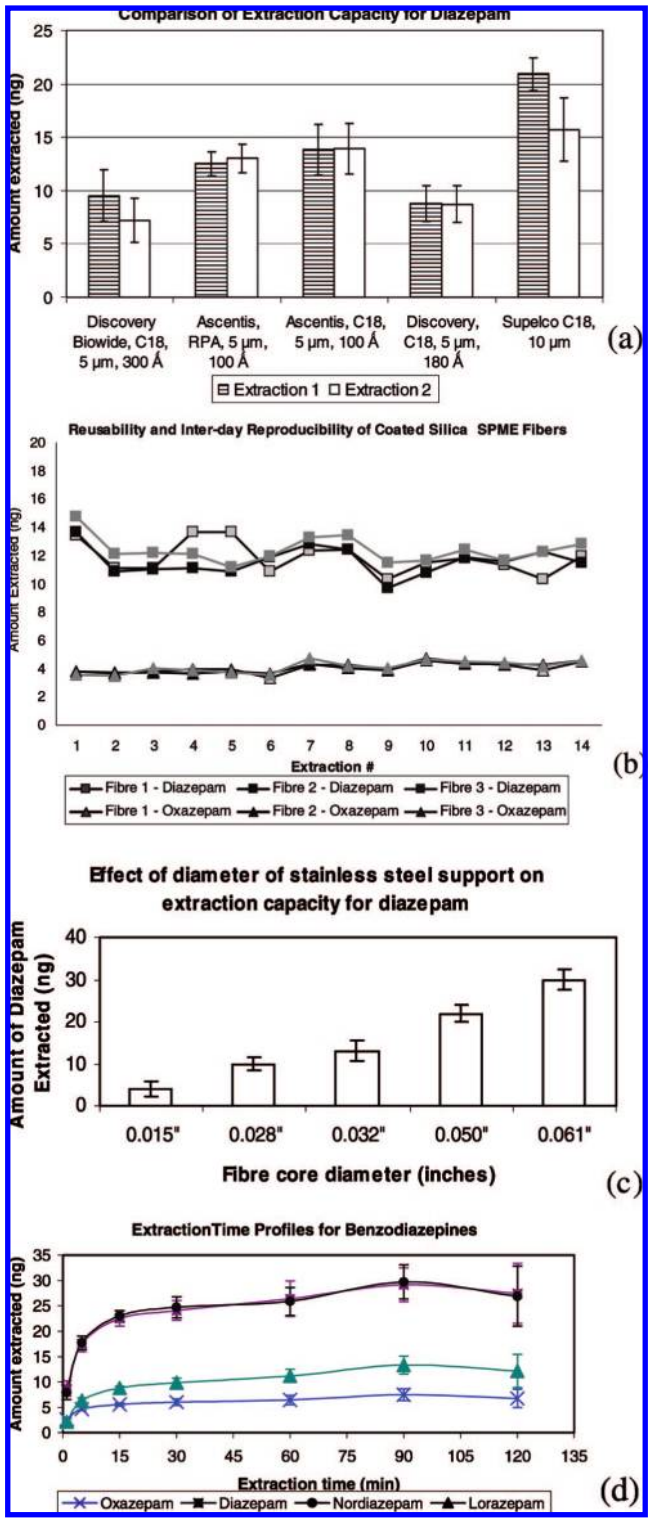

Figure 3. (a) Evaluation of different coated silica particles for use as SPME extraction phases. The amount shown is the mean amount extracted by 12 fibers tested. The results for two consecutive extractions are shown as an indication of coating adhesion. (b) Diagram showing reusability of RPA 5 μm coatings over 14 extractions. The amounts of diazepam and oxazepam extracted are shown over time for three representative fibers. Data points for lorazepam and nordiazepam are omitted for clarity. (c) The effect of stainless steel support diameter on the extraction capacity for diazepam. The bars show the mean amount extracted by a batch of 12 fibers. (d) Extraction time profile for diazepam, nordiazepam, oxazepam, and lorazepam. Extraction time profile was constructed using 100 ng/mL benzodiazepine standard in PBS pH 7.4 using SPME conditions as described in the Experimental Section.

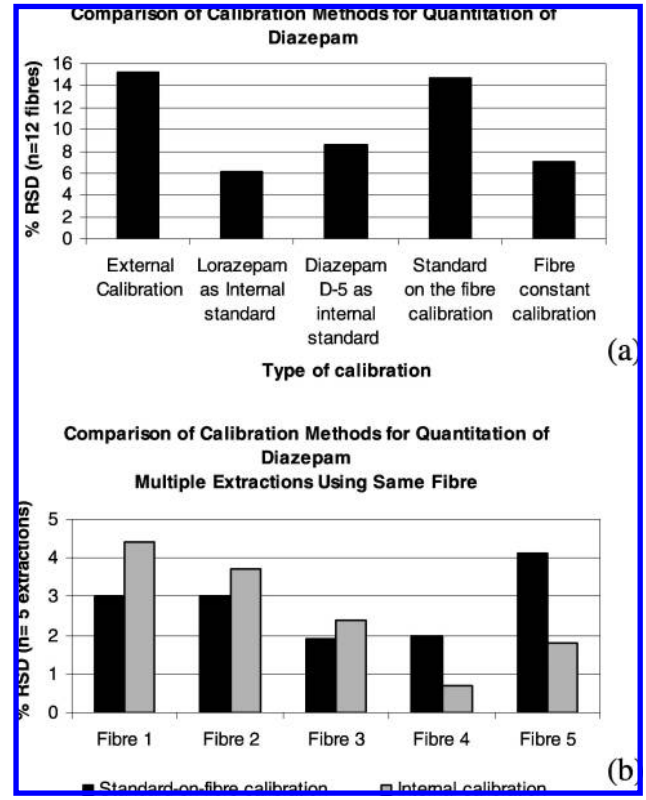

Figure 4. (a) Comparison of external standard, internal standard, fiber constant, and standard-on-the fiber calibration methods for the use with automated multifiber SPME. The dependence of % RSD (n = 12 fibers) of the amount extracted on the choice of calibration method is shown. (b) Comparison of internal standard and standard-on-the-fiber calibration methods for the use with automated SPME using a single fiber for repeated extractions. The dependence of % RSD (n = 5 extractions) of the amount extracted on the choice of calibration method is shown. All extractions were performed from 100 ng/mL diazepam standard solution in PBS buffer pH 7.4 using optimal SPME conditions.

approach of choice for the calibration in nonuniform agitation conditions.[49]

Figure 4a compares the results obtained for 12 randomly selected fibers for the extraction of diazepam using each of five calibration methods tested (1) external calibration, (2) lorazepam as the internal standard, (3) diazepam-d_5 as the internal standard, (4) standard-on-the-fiber calibration, and (5) calibration using the fiber constant. The best results were obtained with either internal standard calibration or the calibration using fiber constants. Among these two calibration methods, internal standard calibration is simpler and less time-consuming because it does not require any additional experiments. Both lorazepam and deuterated diazepam were found to perform equally well when used as internal standards for the quantitation of diazepam. On the basis of the results obtained, the internal standard calibration method was selected as the best and simplest calibration method for automated multifiber SPME. In subsequent experiments, the use of internal standard calibration reduced RSDs for all four compounds to ≤9% when all 96 fibers were tested using the extraction from the same standard solution (Table S-4).

The use of standard-in-fiber calibration did not improve quantitative results when compared with the results obtained using external calibration in contrast to expectations. Theoretically, this

approach should be able to compensate well for the differences in extraction capacity and extraction conditions, so the observed result may be caused by the compounding of experimental uncertainty because multiple parameters must be determined experimentally in order to apply standard-in-fiber calibration. The uncertainty of electrospray LC-MS/MS analysis is higher than for electron impact GC/MS where standard-in-fiber calibration is routinely used. This possibility was further examined by comparing the performance of internal standard calibration and standard-in-fiber calibration when performing repeated analyses using the same fiber. The results of this experiment are shown in Figure 4b. Both internal standard and standard-in-fiber calibration methods were found to perform equivalently, indicating that factors other than instrumental performance caused high RSD in the multiple fiber experiment. Further investigation into improving standard-in-fiber calibration for use with high-throughput SPME applications is currently underway.

Summary of Overall Performance of RPA Coatings for Drug Analysis. With the use of optimized fibers, the extraction time profiles were constructed for all four benzodiazepines using extraction times ranging from 1 to 120 min (Figure 3d). Equilibrium was reached in 30 min for all benzodiazepines tested, which confirms that the kinetics of the extraction for these SPME fibers are much faster than those for PDMS-based fibers. The surface morphology and coating thickness of the RPA coating were investigated using SEM (Figure 5). Figure 5a shows that uniform coverage of silica particles on the surface of SPME fibers was achieved. Figure 5b demonstrates monolayer coverage of RPA particles on the surface of stainless steel support. The coating thickness of RPA coating is estimated to be 5−6 μm which explains the fast extraction kinetics.

Furthermore, the optimized desorption conditions were determined to be acetonitrile/water (1:1, v/v), with 30-min desorption time with 850 rpm agitation. With the use of these conditions, the carryover was determined to be 1% to 2% for the four benzodiazepines. However, this carryover was completely eliminated during the preconditioning step. Therefore, the 30-min preconditioning step of the fibers in methanol/water (1:1, v/v) was necessary prior to each use of fibers in order to (1) wet the fiber surface in preparation for the extraction and (2) eliminate any carryover of analyte from previous extraction.

In summary, all properties of silica-particle based coatings were found to be acceptable for use in quantitative drug analysis using benzodiazepines as model drugs. These coatings eliminated the need for long equilibration times and improved intrafiber reproducibility in comparison to PDMS coatings. (Table 2). The absolute recovery of diazepam was 30% using either coating which is excellent for a microextraction method where absolute recoveries as low as 0.1% may be encountered.[52] Poor PDMS performance was caused by the length of the extraction step, and therefore thin coatings and/or short extraction times are recommended for use with automated multifiber SPME. Thick coatings with slow kinetics should only be employed if the analyte-coating distribution constant is sufficiently high to allow the use of short pre-equilibrium extraction times. The comparison of intra- and interfiber reproducibility for RPA coatings presented in Table 2 shows that the addition of internal standard at the beginning of the sample preparation procedure can eliminate the SPME sample preparation procedure as a significant cause of variance. Further narrowing of variance is limited by the performance of electrospray LC-MS instrumentation over long analysis times.

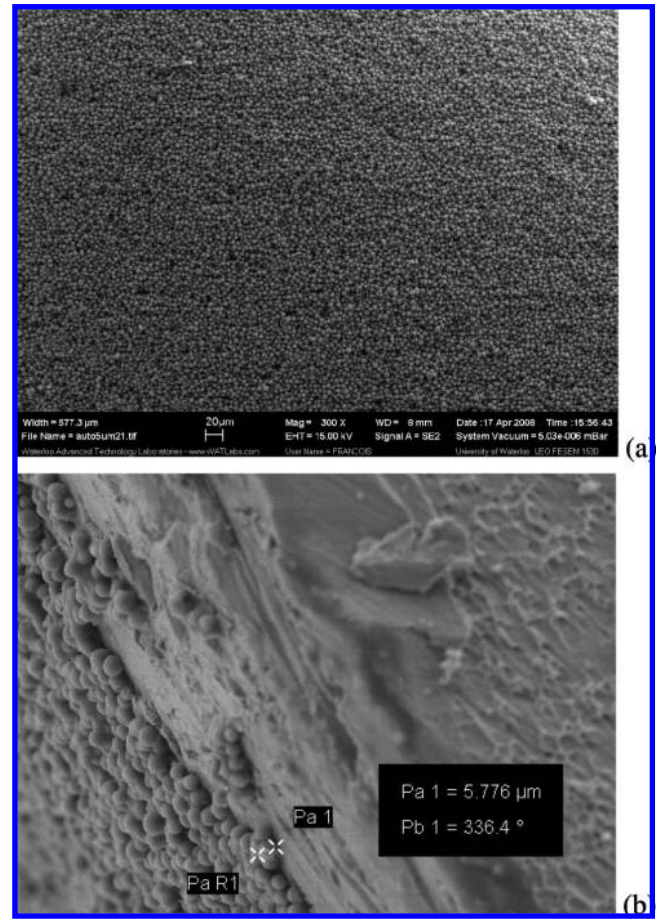

Figure 5. SEM images of 0.061 in. SPME fiber coated with 5 μm RPA particles and prepared according to the procedure described in the Experimental Section. (a) Surface morphology using magnification = 300×. (b) Estimation of coating thickness using magnification = 600×.

The utility of RPA coating for the extraction of more polar analytes such as acetaminophen, pseudoephedrine, and dextromethorpan was examined and compared to commercially available Carbowax/Templated Resin (50 μm coating thickness) SPME fiber. As shown in Figure 6, commercial CW/TPR and RPA coatings exhibited comparable extraction efficiency for acetaminophen, while proposed RPA coating had better extraction capacity for pseudoepehedrine and dextromethorphan. This preliminary data indicates that the RPA coating is applicable to extraction of drugs with properties substantially different from those of benzodiazepines. The coating was shown to work well for the extraction of compounds with pK_a values ranging from 3.4 to 13 and log P values ranging from 0.5 to 4 as summarized in Table 1. It is important to note that for the extraction of acidic and basic compounds, additional enhancements in sensitivity can be achieved by adjusting sample pH to suppress analyte ionization, since SPME coatings typically extract the nonionized form of the analyte. Second, the extraction efficiency for an analyte can also be enhanced by careful coating selection based on the analyte properties. The proposed coating procedure may therefore be

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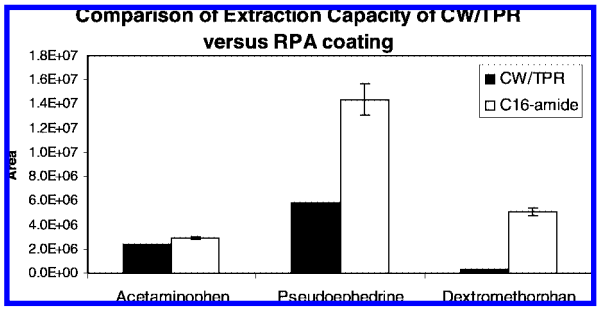

Figure 6. Comparison of extraction capacity of commercial CW/TPR coating and 0.061 in. fiber coated with 5 μm C16-amide (RPA) coating. Extraction was performed in duplicate from 1.0 mL of cough syrup for 2 min using an agitation of 850 rpm. Analytes were desorbed for 15 min using acetonitrile/water (1:1).

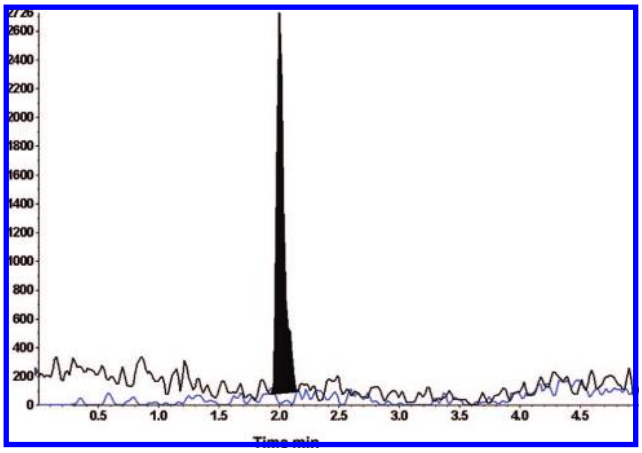

Figure 7. Example XIC (287.1 → 241.1) chromatograms of blank whole blood sample and 4 ng/mL oxazepam standard (lower limit of quantitation (LLOQ) concentration) in whole blood.

used to immobilize SPE-type sorbents other than C18 and RPA in order to further improve distribution constant between the coating and a particular target analyte.

Application: Validation of High-Throughput Analysis of Benzodiazepines in Whole Blood. The optimized method for the high-throughput analysis of benzodiazepines in whole blood was fully validated according to FDA guidelines for the method validation of bioanalytical methods to demonstrate the suitability of automated SPME-LC-MS/MS for quantitative bioanalysis.[37] The specificity of the method was evaluated by analyzing two lots of blank whole blood samples, and no interferences were detected in any of the chromatograms. Example chromatograms of blank whole blood and 4 ng/mL benzodiazepine standard in whole blood are shown in Figure 7. Postpreparative stability of samples was demonstrated up to 72 h when stored in a refrigerated autosampler by reanalysis against the original calibration curve.

The limit of detection (LOD) for the proposed method was determined according to 3× the signal-to-noise criteria and was found to range from 0.8 ng/mL for oxazepam to 3 ng/mL for diazepam. Lower limit of quantitation (LLOQ) for the proposed method was determined as the lowest concentration of the analyte in whole blood that can be reproducibly determined with the accuracy of ±20% from the nominal concentration and the RSD of five replicate determinations not exceeding 20%. A concentration of 4 ng/mL of all four benzodiazepines tested met this criteria as shown in Table 3. This LLOQ is acceptable for therapeutic drug monitoring of these drugs, so the addition of solvent evaporation/reconstitution steps was not necessary. The method was linear in the range of 4−1000 ng/mL for diazepam and nordiazepam and in the range of 4−500 ng/mL for oxazepam and lorazepam. All six independent calibration curves constructed during the validation using independent sets of multiple fibers showed excellent agreement. For example, for diazepam the average slope of six calibration curves in two different lots of whole blood was found to be 0.00243 ± 0.00005. This value meets the recent recommendation that the precision of calibration line slopes expressed as % RSD should not exceed 3−4% if the method is to be considered free from relative matrix effects.[26] The linear regression coefficients (R^2) for the six calibration curves ranged from 0.997 to 0.999.

The accuracy and intrabatch repeatability of the proposed method was evaluated at four concentration levels (10, 25, 100, and 500 ng/mL) using five replicate samples at each level. The relative recoveries and RSDs obtained are shown in Table 4. For all compounds and all levels, precision of results does not exceed 20% RSD (range 2−20% RSD) and mean relative recovery does not deviate more than 9% from nominal concentration. RSD value for 25 ng/mL concentration of oxazepam (20% RSD) exceeds 15% RSD FDA precision requirement. It is suspected that fifth fiber in this set of data (recovery of 121%) represents an analytical outlier. The use of the median/median absolute difference method as recommended by the Analytical Methods Committee when the presence of outliers is suspected, yields more robust statistics for the data set with median = 87 and median absolute difference = 8.[53] According to this method, therefore, the estimated precision of the data set is 9% indicating acceptable method performance. The large difference between mean-based (20% RSD) and more robust median-based statistics (9% RSD) supports the conclusion that the oxazepam value for the fifth fiber is an outlier. This conclusion is further supported by the fact that 10 (low QC) and 4 ng/mL (LLOQ) samples all meet accuracy and precision criteria for oxazepam (Tables 3 and 4). In addition, a 25 ng/mL concentration of oxazepam also shows acceptable interday precision (Table 5).

Interbatch accuracy and reproducibility were evaluated using five independent extractions of the same spiked whole blood sample at four different concentration levels and using five fibers at each level. The results of this study are reported in Table 5. Relative recovery did not deviate more than ±15% from nominal concentration, and intrabatch reproducibility ranged from 1% to 12% RSD, which is acceptable for quantitative bioanalysis.

In summary, the proposed method meets FDA method validation requirements[37] for linearity, precision, and accuracy and establishes for the first time the utility of automated high-throughput SPME in bioanalysis. Excellent quantitative results were obtained for the analysis of diazepam and nordiazepam concentrations in whole blood in the range of 4−1000 ng/mL and in the range of 4−500 ng/mL for oxazepam and lorazepam. The sample preparation procedure was simple, automated, and applicable to the extraction of whole blood samples with absolutely no sample pretreatment steps required. The validated method

(53) *Analytical Methods Committee*, Royal Society of Chemistry AMC Technical Brief No. 6, 2001.

Table 3. Automated SPME-LC-MS/MS Method Validation Results, LLOQ and Linear Range

	diazepam	oxazepam	nordiazepam	lorazepam
LLOQ (ng/mL)	4	4	4	4
mean % accuracy ($n = 5$)	102	111	105	102
% RSD ($n = 5$)	11	17	9	14
signal-to-noise ratio	5	19	16	16
dynamic linear range (ng/mL)	4−1000	4−500	4−1000	4−500

Table 4. Automated SPME-LC-MS/MS Method Validation Results, Accuracy and Intrabatch Reproducibility

concentration (ng/mL)	relative recovery (%)			
	diazepam	oxazepam	nordiazepam	lorazepam
	116	93	86	98
	103	81	89	88
10 ng/mL	105	114	106	123
	120	94	117	115
	100	82	94	101
mean ($n = 5$)	109	93	98	105
% RSD ($n = 5$)	8	14	13	13
	84	75	103	84
	102	79	99	113
25 ng/mL	96	92	102	94
	99	87	113	93
	87	121[a]	114	100
mean ($n = 5$)	94	87[a]	106	97
% RSD ($n = 5$)	8	9[a]	6	11
	102	108	97	99
	103	98	99	109
100 ng/mL	109	94	100	105
	108	104	108	116
	92	86	95	99
mean ($n = 5$)	103	98	100	106
% RSD ($n = 5$)	6	9	5	7
	97	87	106	83
	101	102	96	102
500 ng/mL	97	96	96	94
	100	100	92	110
	101	87	99	100
mean ($n = 5$)	99	94	98	98
% RSD ($n = 5$)	2	8	5	10

[a] Oxazepam recovery for the fifth fiber for 25 ng/mL concentration of oxazepam is a suspected outlier. More robust statistics, median and median absolute difference, were calculated for this oxazepam concentration data set as discussed in the text.

Table 5. Automated SPME-LC-MS/MS Method Validation Results, Summary of Accuracy and Interbatch Reproducibility Results[a]

concentration (ng/mL)	fiber	mean relative recovery (%) ($n = 5$ analyses)			
		diazepam	oxazepam	nordiazepam	lorazepam
	1	115 (1)	99 (8)	89 (6)	94 (11)
	2	102 (2)	89 (10)	91 (6)	85 (12)
10	3	115 (1)	112 (7)	115 (4)	115 (10)
	4	104 (2)	101 (8)	108 (5)	109 (10)
	5	99 (2)	90 (10)	96 (6)	96 (11)
	6	113 (3)	97 (8)	100 (4)	99 (7)
	7	97 (3)	98 (8)	93 (1)	87 (9)
25	8	102 (4)	107 (8)	106 (4)	103 (8)
	9	96 (4)	101(8)	104 (4)	107 (10)
	10	100 (3)	96 (7)	95 (4)	96 (8)
	11	112 (5)	115 (10)	101 (4)	109 (12)
	12	104 (6)	96 (10)	100 (3)	100 (11)
100	13	97 (5)	106 (10)	101 (4)	115 (11)
	14	101 (5)	113 (10)	101 (4)	91 (11)
	15	113 (5)	101 (10)	107 (3)	115 (9)
	16	105 (6)	104 (12)	103 (4)	103 (10)
	17	97 (5)	95 (10)	92 (2)	94 (8)
500	18	102 (3)	103 (11)	94 (4)	106 (10)
	19	99 (5)	104 (10)	90 (3)	97 (10)
	20	95 (5)	91 (12)	96 (3)	104 (8)

[a] Values in parentheses refer to RSD obtained for five independent analyses performed on different days.

requires about 100 min (30 min preconditioning, 30 min extraction, 30 min desorption, and about 10 min for dispensing of samples/desorption solvent) for the preparation of 96 samples, which represents the highest throughput of any SPME technique to date. Existing manual SPME methods for the extraction of diazepam from biofluids require between 5 to 180 min of extraction time per sample,[54] so the current method offers at least a 5−180-fold improvement in sample throughput. Xie et al. recently compared the performance of manual multiple-fiber SPME and LLE in multiwell plate format,and also concluded that the performance of SPME was satisfactory for clinical sample analysis with comparable speed to automated LLE as long as a SPME system capable of extracting up to 96 samples is used.[55]

CONCLUSION

One of the problems commonly encountered with manual pre-equilibrium SPME methods is deterioration in method precision caused by imprecise timing of extraction and desorption steps and the difficulty in positioning the fiber reproducibly for all samples and standards. Automated multifiber SPME effectively addresses these issues, and no loss in method precision was observed when a system was used with pre-equilibrium extraction times.

A simple, flexible, low-cost, and reproducible procedure for the preparation of SPME coatings for use with automated multifiber SPME is reported and is expected to be applicable to a wide variety of SPE-type sorbents other than C18 and RPA silica-particle sorbents used in current study. The selectivity of the coatings can be further increased by using immunoaffinity[56−58] or molecularly imprinted polymer coatings.[59] The automation of the coating procedure and/or commercial production of coatings for this system is expected to further improve interfiber reproducibility and consequently system performance.

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The applicability of automated SPME for high-throughput bioanalytical analyses of clinical and forensic interest was shown by presenting full validation data for the analysis of four benzodiazepines in whole blood. The proposed benzodiazepine method allows the processing of >1200 whole blood samples per day if multiple LC-MS/MS systems are available to perform the chromatographic analysis or if faster UPLC chromatographic method is employed. Additional strategies to further enhance throughput using this system are currently under study in our laboratory and include development of thinner coatings with faster extraction/desorption kinetics, development of coatings which do not require a preconditioning step, and the use of thin-film geometry to further enhance the extraction rate.[38] Enhanced recoveries can be achieved using thin film microextraction due to increased surface area and the use of larger sample volumes since this geometry takes up less well volume than fiber geometry.

The speed of current state-of-the-art SPME is comparable to automated SPE and LLE methods, but SPME provides lower cost per analysis (lower cost of autosampler and reusable extraction phases) and requires no pretreatment of whole blood samples. In addition to whole blood samples, automated SPME should also be applicable to other complex matrixes containing particulate matter and unprocessed samples such as tissue and plant homogenates. SPME also enables quantitative determination of both free and total drug concentration in the same experiment using a single sample of biofluid thus eliminating the need to perform separate ultrafiltration experiments to determine the unbound drug fraction.[33−35] Such and other applications of the system for various analyte classes are currently underway in our laboratory.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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