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Quantitative in Vivo Microsampling for Pharmacokinetic Studies Based on an Integrated Solid-Phase Microextraction System

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An integrated microsampling approach based on solid-phase microextraction (SPME) was developed to provide a complete solution to highly efficient and accurate pharmacokinetic studies. The microsampling system included SPME probes that are made of poly(ethylene glycol) (PEG) and C18-bonded silica, a fast and efficient sampling strategy with accurate kinetic calibration, and a high-throughput desorption device based on a modified 96-well plate. The sampling system greatly improved the quantitative capability of SPME in two ways. First, the use of the C18-bonded silica/PEG fibers minimized the competition effect from analogues of the target analytes in a complicated sample matrix such as blood or plasma samples, which is a common problem associated with solid coating SPME fibers for quantitative analysis. Moreover, the C18-bonded silica/PEG fibers provide high sensitivity and a large dynamic range that covers the possible sample concentration range during diazepam administration and elimination. Second, the kinetic calibration method offers more accurate quantitation than the calibration curve method for in vivo SPME, because it compensates for convection and matrix effects during sampling. Therefore, it is especially suitable as a fast sampling technique for pre-equilibrium SPME. Furthermore, with the high-throughput desorption device, the integrated system offers compactness and high efficiency. Its feasibility for in vivo sampling was demonstrated by monitoring diazepam pharmacokinetics and validated by conventional chemical assays and equilibrium SPME. In addition, we propose a simple method to determine the aparent distribution constant between an SPME fiber and a blood matix (K_{fs}) and the distribution constant between an SPME fiber and a pure PBS buffer sample matrix (K_{fb}). As a result, both total and free concentrations of the drug and its metabolites can be detected simultaneously. Accordingly, the binding constants to the blood matrix can be obtained, which are of special significance for clinical diagnosis and drug discovery.

In vivo sampling possesses special significance for the study of chemical processes, such as drug metabolism, in the normal biochemical environment of a living system, as Lipinski described in 2004, "whether the aim is to discover drugs or to gain knowledge of biological systems, the nature and properties of a chemical tool cannot be considered independently of the system it is to be tested in."[1]

Currently, microdialysis and ultrafiltration are the main approaches for in vivo sampling.[2,3] However, pumps and other appliances are required, which makes these approaches more suitable for laboratory use than for field sampling. In addition, the sampling process may affect the dissociation equilibrium between the bound and free analytes, thus interfering with the biological system under study. Solid-phase microextraction (SPME), a simple but effective sampling and sample preparation method,[4,5] has been introduced for in vivo pharmacokinetic studies,[6,7] due to its unique advantages for rapid sampling. Specifically, since a small fraction of the free compounds is extracted (negligible depletion), it does not result in significant disturbance to the system under study;[8−11] therefore, it provides a simple means to study the chemical equilibrium, to determine the free and total concentration of the analytes,[12,13] or to investigate the partition constant of the analyte to sample matrix.[14−16] Additionally, the

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SPME device can be easily miniaturized for in-vein use. These qualities make the SPME suitable for in vivo sampling in a living system.

However, in order to achieve accurate quantification for an in vivo sampling, besides biocompatibility and sterilizability, the SPME probes should have a large dynamic range to cover the expected sample concentration ranges under study. In addition, the probe should exhibit a high sensitivity for the detection of the target drug at low concentrations. More important, competition and displacement effects between similar molecules from the sample matrix should be prevented; otherwise, it would result in low signal intensity, which leads to an underestimate of the target analyte concentration. Furthermore, the extraction time should be as short as possible so that more time points can be obtained to provide detailed information. Last but not least, accurate calibration is needed to deliver reliable results.

The previously published procedures for in vivo pharmacokinetics were mainly based on equilibrium extraction with the use of adsorption-type polypyrrole (PPY) probes, and the in vivo concentrations were calibrated by the calibration curve method.[6,7] Since the blood used to prepare standard solutions was not from the dogs in the in vivo SPME experiments, and the extraction conditions, such as temperature and matrix agitation, in the in vitro calibrations were not the same as those for in vivo SPME, the results are considered approximate. In addition, competition effects should be considered for in vivo pharmacokinetic studies due to the similarity between the drug and its metabolite molecules.

To address these issues, an integrated microsampling approach based on SPME was developed. By using C18-bonded silica/poly(ethylene glycol) (PEG) SPME probes, fast and efficient sampling strategies with accurate calibration, and a high-throughput desorption device based on a modified 96-well plate, the sampling system significantly improved the quantitative capability, portability, and efficiency of the method.

EXPERIMENTAL SECTION

The experimental work included two stages. First, the C18-bonded silica/PEG probes were prepared and characterized. Then, the in vivo pre-equilibrium sampling of the probes for pharmacokinetic studies was conducted; meanwhile, conventional blood analysis and equilibrium SPME were carried out to validate the in vivo pre-equilibrium extraction. During the experiment, a flow system to simulate the blood circulation and a high-throughput desorption device based on a modified 96-well plate were developed.

Preparation and Characterization of SPME Probes. C18-bonded silica/PEG SPME probes and the needle housed in vivo sampling devices were prepared using the method described elsewhere.[7] The C18 particles (10 μm) and PEG were from Supelco (Bellefonte, PA). All of the fibers with the same extracted amount in a fixed 30-min extraction in a standard solution (50 μg/L diazepam in phosphate-buffered saline (PBS) were used for in vivo experiments. The C18-bonded silica/PEG fibers that exhibited the same capacity (extracted amount of analyte in the equilibrium extraction) were assumed to have the same volume, since extraction capacity is proportional to the fiber volume. This was verified by scanning electron microscopy.

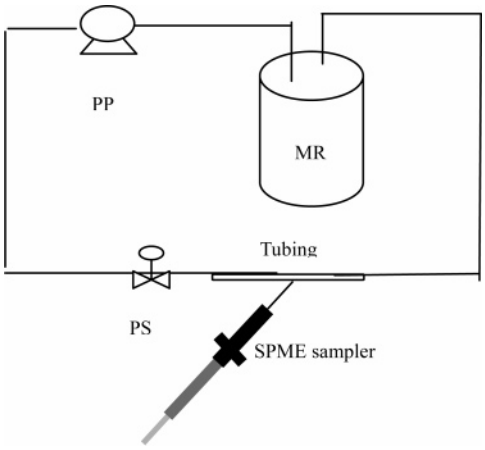

Figure 1. Schematic of the flow system for the in vitro experiments. A peristaltic pump (PP) acting as an artificial heart provided the power for fluid delivery at a precisely controlled linear velocity from the matrix reservoir (MR) to the tubing. The SPME sampler was placed into the tubing with the aid of an in-dwelling intravenous catheter assembly. A pressure sensor (PS) was used to measure the actual pressure in the tubing before extraction.

A systematic in vitro investigation was conducted to determine the extraction behavior of the probes used in this study, including the extraction time profile, the desorption time profile, the matrix effects of plasma and whole blood, the competition effect, and the dynamic range in a controllable flow system. The schematic of the flow system is shown in Figure 1. The experimental conditions were optimized for the in vivo experiments.

Only the fibers used in the dog experiments for the pre-equilibrium extraction were preloaded with deuterated standards. The fibers used for the equilibrium extraction were not preloaded. The deuterated standards for diazepam, nordiazepam, and oxazepam were obtained from Cambridge Isotope Laboratories (Andover, MA). The loading solution was prepared by spiking three deuterated standards (diazepam-d_5, nordiazepam-d_5, oxazepam-d_5) into 25 mL of sterile deionized water at 50 μg/L. Then the sterilized probes were exposed to the loading solution for 30 min and stored in sterile Falcon tubes.

Parallel Desorption Device Based on a 96-Well Plate. In order to improve the efficiency of the SPME process, a high-throughput device for extraction and desorption based on a 96-well plate was fabricated. The device was only used for transportation and parallel desorption in this work, but it could also be used for high-throughput in vitro extraction. The device included a 96-well plate (VWR Canada, ON, Canada), an inner cover made from a silicon compression mat (Ultident, St-Laurent, Canada), and an outer cover made of poly(tetrafluoroethylene) (PTFE) with four clips to fasten the plate (Figure 1). The PTFE cover was machined in the machine shop of the University of Waterloo (Waterloo, ON, Canada) to the required dimensions (8.0 cm $\times$ 12.4 cm $\times$ 0.50 cm). A total of 96 holes were drilled through the cover to align with the center of a 96-well plate, and four stainless steel clips were installed on the four edges of the PTFE block. The silicon compression mat was placed in between the 96-well plate and the PTFE cover. After tightening the clips, the silicon mat ensured that the SPME device remained vertical while preventing leakage of the desorbing solvent during desorption.

After sampling, the probe was withdrawn into the needle and introduced into the well of the plate by the needle, which pierced

the two layers of plastic covers. Then, the plate device with probes was placed on dry ice for transportation. For desorption, the clips were loosened and the two layers of covers with probes were fixed to another 96-well plate with pure methanol in each well. The probe holder was pushed and the needle was withdrawn to expose the probe in methanol for desorption. A total of 2 min with agitation (115 rpm shaking speed) was adequate to desorb 99% of the analyte from the probes.

LC−MS/MS Analysis. A CTC-PAL autosampler/Shimadzu 10 AVP LC/MDS Sciex API 3000 tandem MS system was used for the analysis of the drugs and their deuterated standards. The assay conditions were the same as described previously,[6] except that the transitions monitored for the deuterated standards were m/z 290.2/154.1 for diazepam-d_5, m/z 276.1/140.0 for nordiazepam-d_5, and m/z 292.1/246.1 for oxazepam-d_5, respectively.

Animal Experiments. All animal experiment procedures were approved by the Animal Care Committees at the University of Guelph (ACCUG; Guelph, ON, Canada), the experiments were performed in the Central Animal Facility of the University of Guelph, and diazepam was dosed at 0.5 mg/kg as described previously,[6,7] except for some minor modifications. First, sterilization was conducted by immersing the samplers in 8% formaldehyde and 70% ethanol for 18 h based on an ACCUG-approved surgical protocol. Steam autoclaving was not used in this project because serious damage to the C18-bonded silica/PEG coatings was determined experimentally from the autoclave procedure. Second, at each time point, two extractions were conducted. The probe without standard was introduced for 5 min for equilibrium extraction immediately after blood drawing, followed by a 2-min extraction in the same place with a probe preloaded with deuterated standards for pre-equilibrium extraction. After extraction, the probe was briefly rinsed with deionized water and then put into a well on the 96-well desorption plate placed in a box filled with dry ice.

Conventional Blood Analysis. In order to compare the SPME data with a conventional blood assay, blood drawing was performed after each SPME extraction and prior to the diazepam injection for calibration. A 500-μL aliquot of acetonitrile was added to 100 μL of whole blood in a 1.5-mL microcentrifuge tube, followed by a brief vortex spin and centrifugation (5000 rpm, 10 min). Then, 400 μL of supernatant was removed for evaporation under nitrogen gas. Samples were reconstituted in 50 μL of methanol/water (1/1, containing 10 ppb lorazepam as the internal standard to calibrate for sample loss during instrumental analysis). The linear range was 5−2000 μg/L. A six-point calibration (n = 3) from 5 to 2000 μg/L for diazepam, nordiazepam, and oxazepam was performed at 25 °C, based on the aforementioned approach. The standard solutions were prepared by spiking standards into the dog blood collected before drug administration.

RESULTS AND DISCUSSION

SPME has been used for the quantitative analysis of contaminants in groundwater[17−20] and flavors in beverages and food.[21−23] However, for in vivo pharmacokinetic studies, quantitative analysis is often more challenging due to the large concentration range associated with metabolism, the complex matrix effect, and the need for fast sampling. Nevertheless, besides its good biocompatibility,[7,24] the newly developed C18-bonded silica/PEG probe provided satisfactory performance for quantitative analysis.

Preconditions of the SPME Probe for Quantitative Analysis. To carry out a quantitative analysis, first and foremost, there must be a linearly proportional relationship between the amount of analyte extracted by the SPME probe and the initial sample concentration of the analyte. Moreover, the linear range of the extraction should be broad enough to cover the expected concentration range of analytes in blood, especially during the early stage of pharmacokinetic studies where blood concentrations are very high. In this work, the C18-bonded silica/PEG probe exhibited a relatively broad dynamic range (0.5−2000 μg/mL) for the three target analytes (diazepam, nordiazepam, oxazepam), which ensured detection over the sample concentration range in whole blood during the pharmacokinetic study.

Second, the competition effect should be considered with any SPME method, especially for in vivo pharmacokinetic studies, where competition can occur from the metabolites and the drug, due to the similarity of their molecular structures. It might also be from the complicated matrix effect in whole blood samples due to the presence of endogenous compounds. Usually the competition effect is the main problem associated with porous solid coating SPME, because, according to the developed theory,[25−27] the number of effective surface sites where adsorption can occur is limited. Thus, the extraction of analytes by solid coating SPME is a competitive process in which several similar molecules compete for one binding site and a molecule with a higher affinity for the surface can replace a molecule with a lower affinity. As a result, both the amount of target analyte extracted and the linear range of the extraction decrease, and the decrement is dependent on the concentration of the competitors.

Since the competition effect is common in complicated sample matrixes such as whole blood, in vitro calibration experiments were conducted to evaluate the competition between similar analytes, including diazepam, nordiazepam, and oxazepam for C18-bonded silica/PEG fibers. The calibration curve of diazepam extracted by C18-bonded silica/PEG fibers from the standard solutions prepared by spiking only the diazepam standard in serial dilutions in the whole blood was compared with the curve produced from the equal spiking of the three analyte standards in whole blood. It was found that the slope of the diazepam standard curve was almost identical in the two situations, illustrating that there was no competition effect during equilibrium extraction (10 min) even at high analyte concentrations (2000 μg/L), which was an important advantage of the C18-bonded silica/PEG fiber for quantitative analysis. However, when the PPY fibers were evaluated in a parallel study, it was found that the competition was significant, and dependent on the extraction time and the concentration of the competing molecules. Specifically, we found

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that competition still occurred on PPY fibers at 50 ng/mL oxazepam and diazepam during a sampling time of 10 s. The difference between these two types of fibers is ascribed to their different extraction mechanism. Although some resourceful work has illustrated that an SPME fiber made from bonded silica particles and epoxy glue is porous and the extraction of benzene, toluene, and xylene is based on adsorption,[28] our work indicated that the drug extraction based on C18-bonded silica/PEG fibers was predominantly absorptive. For example, the desorption time of the drug in methanol was ~2 min, but the drugs on an adsorptive fiber such as PPY fiber can be desorbed thoroughly in pure methanol in a few seconds, as the desorption and adsorption occur on the fiber surface. Furthermore, no competition effect can also be served as evidence. More work is required to investigate the extraction mechanism, but the findings from this study demonstrate the feasibility of the C18-bonded silica/PEG probes for quantitative microsampling.

Sensitivity and the Extraction Strategy. The sensitivity of the probe is another factor that should be considered for quantitative analysis, since it determines the detection limit of the method and the accuracy during the late phase of the pharmacokinetics. Here, the sensitivity of the probe is defined as the amount of analyte extracted in a given time, which is determined by the kinetics of the absorption, shown by eq 1,[29] where n is the

$$n/n_e = 1 - \exp(-at) \tag{1}$$

amount of analyte in the extraction phase after sampling time t, n_e is the amount of analyte in the extraction phase at equilibrium, a is the time constant that is dependent on the volume of the extraction phase and sample, mass-transfer coefficients, distribution coefficients, and the surface area of the extraction phase.

According to eq 1, before equilibrium is reached, the longer the sampling time t and the larger the value of time constant a, the higher the sensitivity. For a given sampling system, including a given probe, sample matrix, and target analyte, the time constant is fixed, so that the extracted amount n is determined by the sampling time. Therefore, the equilibrium extraction method provides the highest sensitivity due to the largest amount of analyte that can be extracted from the sample matrix. More fundamentally, the sensitivity of the probe at equilibrium is determined by the interaction between the analyte molecules, SPME coating materials, and sample matrix, which is described by the equations for the partitioning equilibrium:[5]

$$K_{fs} = \frac{C_f}{C_s} \Rightarrow K_{fs} = \frac{n_e/V_f}{C_s} \Rightarrow n_e = K_{fs}V_fC_s \tag{2}$$

where K_{fs} is the partitioning coefficient of the analyte between the coating surface and the sample matrix, C_f is the concentration of the analyte on the surface of the fiber coating, and C_s is the concentration of the analyte in the sample matrix. Equation 2 shows that n_e is determined by the K value and the fiber volume V_f, when the sample concentration is fixed. Thus, the K value determines both the fiber selectivity and sensitivity. The sensitivity can also be used to evaluate the preconcentration capability of the extraction phase. With C18-bonded silica/PEG fibers, when a 5-min extraction was applied, the limit of detection for diazepam, nordiazepam, and oxazepam was 1.7, 1.4, and 2.8 μg/L, respectively, which permitted the detection of the drug concentrations during the late phase of the metabolism.

Despite the high sensitivity, the equilibrium extraction method is not applicable in some cases. For example, when the equilibrium time is too long, it is not practical for dynamic monitoring due to the loss of temporal resolution. Moreover, it is not applicable for adsorption-type coatings, also called solid coatings, where the extracted amount of analyte under equilibrium could be nonlinear with the initial concentration of the analyte in the sample at high concentrations.[25]

To address these issues, there are two options. First, a fiber could be developed that would provide a short equilibrium time, such as the C18-bonded silica/PEG fibers, for which the equilibrium time was ~5 min during static extraction in whole blood. Another solution is to use the pre-equilibrium extraction method, according to the finding that the amount of analyte extracted by the fiber before equilibrium, n, is linearly dependent on the initial sample concentration, C_0.[29,30] For pre-equilibrium extraction, the sensitivity is determined by both the a value and n_e when the sampling time is fixed. For the C18-bonded silica/PEG fibers, it was found that the limit of detection for diazepam, nordiazepam, and oxazepam was 2.8, 2.5, and 4.7 μg/L, respectively, when a 2-min extraction was applied. These results illustrated that the sensitivity of the pre-equilibrium extraction was somewhat lower than that of the equilibrium extraction method, but it was still sensitive enough for pharmacokinetic studies.

External Calibration versus Kinetic Calibration. Theoretically, the calibration curve method, also referred to as the external calibration method, applies to both pre-equilibrium extraction and equilibrium extraction because the amount of analyte extracted by the fiber is linearly dependent on the initial sample concentration in these two cases. However, for in vivo pharmacokinetic studies, care should be taken to ensure that the experimental conditions for calibration are the same as for the in vivo sampling. This can often be challenging because the flow rate of the blood during an in vivo experiment is not known. Thus, it is impossible to accurately replicate the agitation of the sample matrix in a calibration experiment. Nevertheless, the calibration for equilibrium extraction was still feasible because the total amount of drug extracted at equilibrium was the same regardless of the flow rate. This was an important advantage for in vivo analysis since it allowed for reproducible results even if the blood flow varied at different time points during the experiment. The blank blood, which was drawn from the same beagles before drug dosing, was used to prepare the standard solutions, in order to keep the matrix effect constant during the in vivo sampling and calibration. As the K value is temperature dependent, calibration was conducted at 37 °C. The calibration curve is provided in Figure 3.

Since the calibration was based on equilibrium extraction in the whole blood, the calibration curves provided information about two important parameters for the extraction: the volume of the fiber, V_f, and the fiber coating/sample distribution coefficients of the analytes, K_{fs}. The regression slope of each trend line is the

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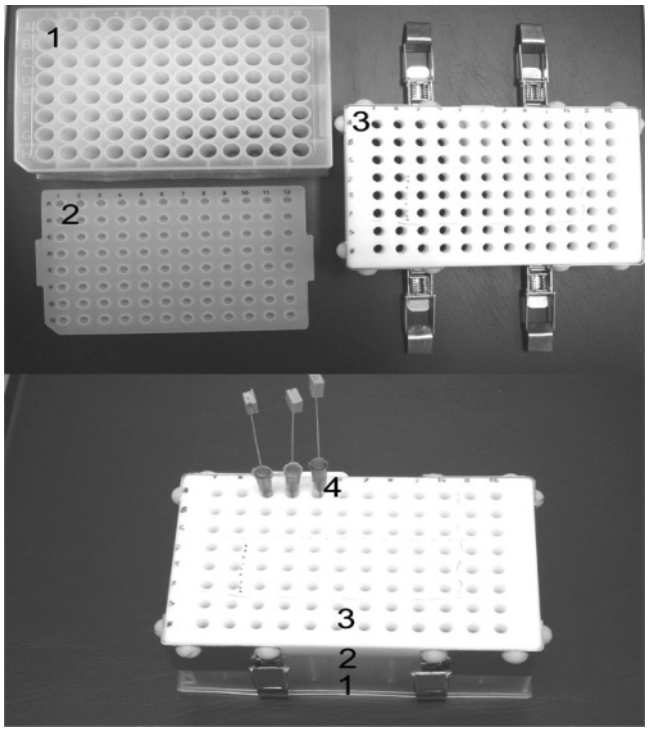

Figure 2. Modified 96-well plate for on-site sampling: (1) 96-well plate, (2) silicon compression mat, (3) PTFE block with stainless steel clip, and (4) sampling device.

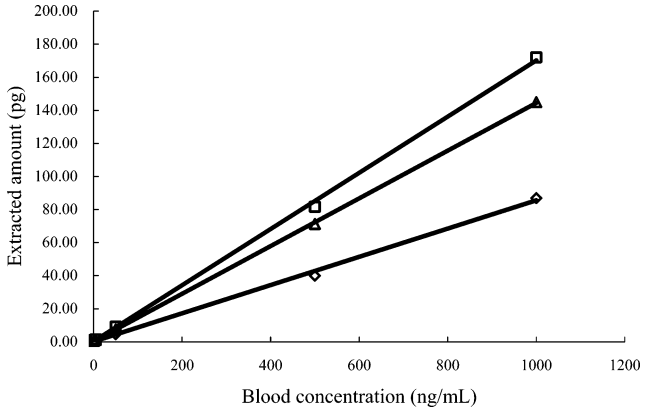

Figure 3. SPME calibration with C18-bonded silica/PEG fibers for diazepam (◇), nordiazepam (□), and oxazepam (Δ). A six-point calibration (n = 3), from 0.5 to 1000 μg/L, was conducted. The regression slope for each analyte presents the product of V_f and its K_{fs}. The standard solutions were prepared by spiking standards into the dog blood obtained from the same beagles that were used for the in vivo SPME experiments before drug injection. Static extraction was performed until equilibrium was reached (10 min) at 37 °C.

product of V_f and K_{fs} for the corresponding analyte if negligible depletion occurs, according to eq 2, where K_{fs} is the C18-bonded silica/PEG coating/blood sample distribution coefficients of the drugs. This is a simple but important method to determine the apparent distribution coefficients (since their magnitude is dependent on the concentration of the binding matrix) of the analytes between the fiber coating and the sample matrix, K_{fs}. Using the calibration curve from Figure 3, the obtained sample concentration, C_0, is the total concentration of the drug in whole blood, since the K_{fs} is the distribution coefficient of the drug between the fiber coating and the blood matrix. The free fraction of the drug, which is the active portion of the dose in blood, can be obtained based on the distribution coefficients of the analytes between the fiber coating and the buffer matrix, K_{fb}, which can be obtained from the calibration curve with standard solutions prepared by spiking known amounts of the target drugs into PBS, pH 7.4.

The calibration curve method is capable of providing relatively accurate data for equilibrium extraction, in spite of the inconvenient and tedious procedures associated with the preparation of the standard solutions and controlling the experimental conditions. However, it is not appropriate for pre-equilibrium extraction for in vivo sampling because the flow rate of the blood during the in vivo experiment is not known and the amount of analyte extracted at pre-equilibrium would be significantly affected by the sample agitation.

To address the inherent weakness of the calibration curve method, the kinetic calibration method,[31,32] also referred to as the in-fiber standardization technique,[33] was developed. The method is based on the isotropic relationship between the absorption process of analytes from a sample matrix to the extraction phase and the desorption process of preloaded standards from the extraction phase to the sample matrix. Thus, the extraction of analytes can be calibrated by determining the desorption of the preloaded standards (isotope-labeled compounds are usually used as standards). This method is simpler than the calibration curve method. More importantly, it compensates for the effects of agitation and the sample matrix. The method provides a means of accurate quantification of target analytes, especially during in vivo sampling, where standard addition and external standard calibration methods are not practical.[31−35]

The isotropic relationship of the absorption and desorption processes is expressed by eq 3,[31] where Q is the amount of

$$n/n_e + Q/q_0 = 1 \tag{3}$$

standard remaining in the extraction phase after exposure of the extraction phase to the sample matrix for the sampling time, t, and q_0 is the amount of standard that is preloaded in the extraction phase. In eq 3, n_e can be easily calculated since n, q_0, and Q are detectable. Afterward, the initial concentration of target analyte, C_0, can be calculated according to eq 4 for on-site or in vivo sampling, where $V_s \gg K_{fs}V_f$ is always satisfied.

$$n_e = C_0 K_{fs} V_f \tag{4}$$

In order to verify the feasibility of the kinetic calibration for blood sampling with a C18-bonded silica/PEG probe, in vitro experiments were conducted to study the isotropism of the absorption and desorption of the deuterated and nondeuterated drugs in plasma. C18-bonded silica/PEG fibers preloaded with deuterated standards were exposed to a flowing standard solution (flow rate 7.2 cm/s) for different times to study the time profile for

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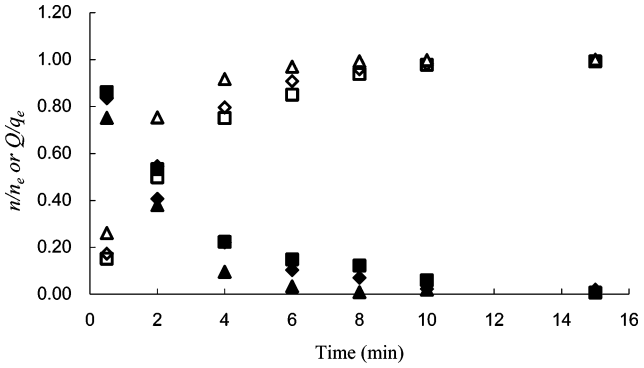

Figure 4. Absorption and desorption time profiles for diazepam (◇), nordiazepam (□), and oxazepam (Δ) and their deuterated analogues, diazepam-d_5 (◆), nordiazepam-d_5 (■), and oxazepam-d_5 (▲).

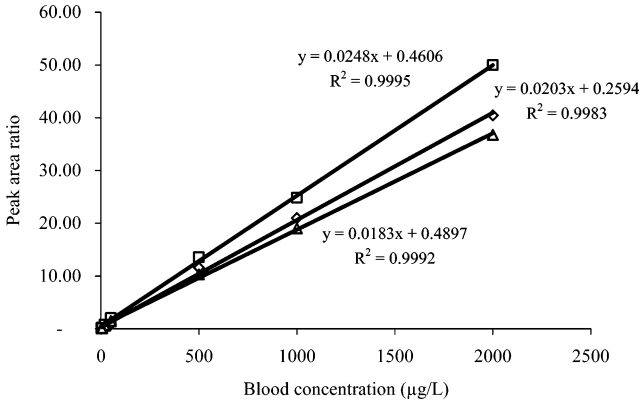

Figure 5. Calibration for the conventional blood analysis. A six-point calibration (n = 3), from 5 to 2000 μg/L, was performed for diazepam (◇), nordiazepam (□), and oxazepam (Δ) based on a chemical assay at 25 °C. The standard solutions were prepared by spiking standards into the dog blood obtained from the same beagles that were used for the in vivo SPME experiments before drug injection.

absorption and desorption. Figure 4 provides the Q/q_0 values, which were calculated from the desorption time profiles and the n/n_e values, which were calculated from the absorption time profiles. The sum of Q/q_0 and n/n_e is close to 1 at each point (0.97−1.15), which demonstrates the isotropism of the absorption and desorption processes, as described by eq 3.

To calculate the sample concentrations, C_0, the volume of the fiber, V_f, and the fiber coating/sample distribution coefficients of the analytes, K_{fs}, need to be determined in eq 4. As previously mentioned, the product of V_f and K_{fs} can be obtained directly from the regression slope of the calibration curve for equilibrium extraction, shown as Figure 3. It should be noted that the calibration curves were used to determine $V_f \times K_{fs}$ rather than calibrating the in vivo SPME, where the effect from agitation on the pre-equilibrium extraction could not be compensated.

Similarly, the total and free concentrations of the drug in whole blood can be obtained by using $V_f \times K_{fs}$ and $V_f \times K_{fb}$ from the calibration curves in standard solutions by whole blood and PBS buffer, respectively. With both the total and free concentrations, the free fraction, a parameter suitable for evaluation of the binding constant of the drugs to the blood matrix, K_b, can be calculated by eq 5, where C_{free} is a free concentration and C_{total} is a total

$$K_b = \frac{C_{free}}{C_{total}} \Rightarrow K_b = \frac{n_e/K_{fb}V_f}{n_e/K_{fs}V_f} \Rightarrow K_b = \frac{slope_1}{slope_2} \qquad (5)$$

concentration, $slope_1$ is the regression slope of the calibration curve in a PBS standard solution, and $slope_2$ is the regression slope of the calibration curve in a blood standard solution. Therefore, the free fraction can be obtained by comparing the regression slopes of the two calibration curves. However, it must be noted that K_b is for the conclusive binding constant of the drug (analyte) to the whole blood matrix, including blood cells and other components, such as proteins, although the method to obtain the binding constant can be universally applied to any pure matrix.

Compared to external calibration with equilibrium extraction, the kinetic calibration method improves the accuracy and simplifies the operation procedures; thus, it is suitable for on site and in vivo sampling.

Parallel Desorption Device. The 96-well plate-based parallel desorption device offered compactness and high efficiency during transportation as well as instrumentation. With its addressability, each probe used after in vivo sampling was positioned in a specific well of the plate, thus, the 90 probes used in this experiment were kept in one plate, providing better portability compared to the previously unwieldy field-sampling experiment using 3 bulky boxes. In addition, desorption can be conducted by simply transferring the cover of the desorption device with all of the probes to another 96-well plate filled with methanol, thus greatly simplifying the whole procedure and allowing for automation.

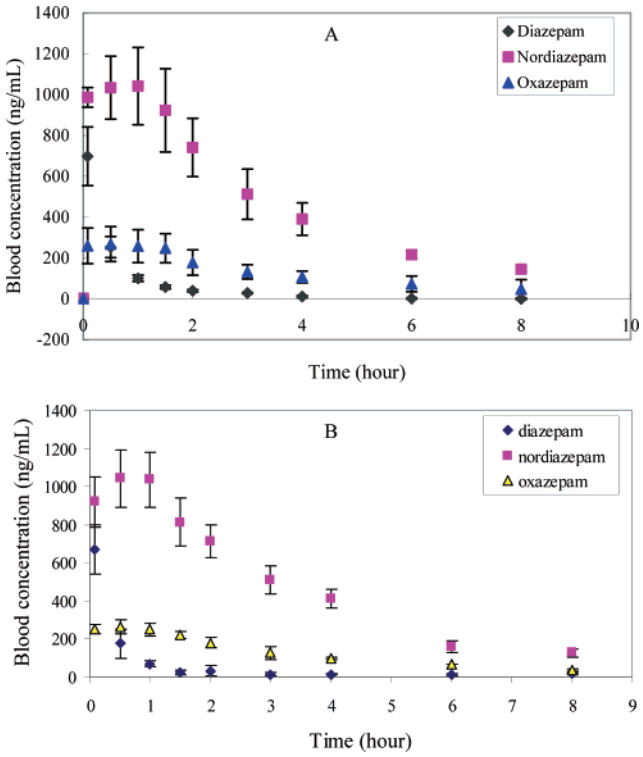

Figure 6. Averaged pharmacokinetic profiles of diazepam, nordiazepam, and oxazepam, which were monitored by in vivo SPME over 8 h on three dogs (n = 6 for the last point by duplicate trials, and n = 9 for all the other points by triplicate trials). (A) Pre-equilibrium extraction and kinetic calibration based on deuterated standards during the experimental course. (B) Equilibrium extraction and external calibration curve method.

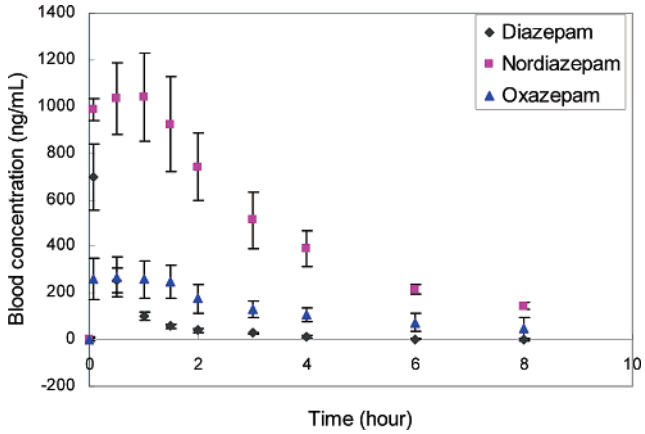

Figure 7. Averaged pharmacokinetic profiles of diazepam, nordiazepam, and oxazepam, which were monitored by conventional blood draws and an in vitro chemical assay over 8 h on three dogs ($n = 3$). Calibration was based on standard curves shown in Figure 4.

Validation of the in Vivo Pharmacokinetics by Conventional Drug Analysis. In previous studies, plasma concentrations were used to validate blood concentrations by SPME without considering the absorption of the drugs by blood cells.[6,7] However, it was found in this study that the percentages of the three analytes (diazepam, nordiazepam, oxazepam) that partitioned in the plasma were about 59, 63, and 68%, respectively, when the drug concentrations in whole blood were considered to be 100%. Consequently, the total concentrations obtained from conventional chemical analysis were used to validate the total concentrations from the SPME experiments. The calibration curves for the conventional blood assay are illustrated in Figure 5. The linear correlation coefficients (R^2) were better than 0.998, which demonstrate the validity of the chemical assay over the linear range (5−2000 μg/L).

For in vivo SPME experiments, diazepam pharmacokinetics in dogs was studied to evaluate the performance of the integrated microsampling system, including the C18-bonded silica/PEG probes, the pre-equilibrium extraction sampling strategy, and the kinetic calibration method. The pharmacokinetic profiles of diazepam and its two significant metabolites, nordiazepam and oxazepam, were monitored by in vivo SPME over 8 h, as shown in Figure 6A. For comparison, the equilibrium extraction and related calibration curve method were conducted, as shown in Figure 6B. Correspondingly, the results of conventional blood analysis are presented in Figure 7. All of the results from in vivo SPME (equilibrium extraction with the calibration curve method and pre-equilibrium extraction with the kinetic calibration method) were consistent with the results from the conventional assay; the correlation coefficients (r) were close to 1 (0.97−0.99). The results illustrate that the diazepam metabolism rate in beagles was rapid, as evidenced by the fact that nordiazepam was detected at higher concentrations, even quite early in the process, after drug administration. It was found that equilibrium extraction with the calibration curve method can provide higher sensitivity, which is more desirable for analysis of the late phase of the pharmacokinetic profile, but at the expense of sampling time and even temporal resolution. Conversely, the kinetic calibration method offers a fast sampling time and more accurate and convenient calibration and, thus is more applicable for full-process automation.

CONCLUSION

Accurate quantitative analysis is still the main concern for in vivo pharmacokinetic studies by SPME. This issue was addressed in both theoretical and experimental levels in this study. A number of issues, which inhibit the quantitative capability of SPME for in vivo sampling, were fully discussed and solved in this paper. First, the competitive effect from analogues of the analytes and other matrix components should be further evaluated before a fiber is used for in vivo sampling, especially for pharmacokinetic studies. Second, for a given probe, the appropriate strategies for extraction, equilibrium or pre-equilibrium extraction, and the related calibration method to obtain reliable results were thoroughly discussed. Finally, a simple method to obtain the product of the fiber volume and the partitioning constant and the binding constant of the drug metabolites to the blood matrix was proposed for calibration. Consequently, both total and free concentrations of the drug and its metabolites can be detected simultaneously. All the features were demonstrated by the in vivo sampling of the pharmacokinetic study.

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