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Blood sampling without blood draws for *in vivo* pharmacokinetic studies in rats

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ABSTRACT

Purpose: Pharmacokinetic (PK) studies in rats typically involve removal of serial blood samples following dosing. This research illustrates the development of a fast *in vivo* microextraction technique that has the potential to partly replace current sampling techniques based on blood draws, especially in the case of small animals.
Methods: The proposed sampling procedure is based on solid phase microextraction (SPME), an approach that has continued to revolutionize sampling and sample preparation ever since its discovery a decade ago. *In vivo* microextraction is faster than conventional methods, interferes minimally with the investigated system, minimizes errors associated with sample preparation and limits exposure of lab personnel to hazardous biological samples.
Results: Here we show the successful application of fast microextraction during a full PK study with diazepam in rats. The results were found to correlate very well with a standard analytical method. Three calibration strategies – external, standard on the fiber, and double extraction – were employed to correlate the amount extracted with the *in vivo* concentration.
Conclusions: Our results demonstrate the unique advantages of this technique and highlight its utility as a valuable new tool for fast bioanalysis in support of *in vivo* sampling and PK studies, particularly during the early drug discovery process. This approach can be used not only for drugs, but also for other exogenous or endogenous compounds.

1. Introduction

To study a drug's pharmacokinetic (PK) profile in an animal model, the drug is administered by the desired route of administration followed by sampling and analysis of a biological fluid such as whole blood or plasma. In order to obtain reproducible and good quality data, the sampling method is as important as the method of bioanalysis. For example, animals that experience stress during sampling can exhibit altered PK profiles [1,2].

Currently, many PK studies in rodents require either a large number of animals because they are performed by sacrificing several rodents for each data point [3–6], or a fewer number of catheterized animals which allows serial blood sampling. In the absence of blood volume replacement, the number of samples that can be taken is limited as serious hemorrhagic shock and tissue anoxia can occur if more than 20% of the total blood volume is drawn [7]. This can be ameliorated by replacing the sample volume with physiological saline or blood obtained from a donor animal. However, this introduces sample dilution and involves the sacrifice of more animals when sample volume is replaced with blood. Methods such as microdialysis and ultrafiltration do not require blood draws and are suitable for automation, but they are not appropriate for lipophilic drugs or drugs that are highly bound to plasma proteins, and have difficulty producing accurate quantitative results [8,9]. Sensors and sensor arrays are usually very small and have a short response time but they are very difficult to produce and may not be suitable for complex biological samples [10].

An ideal *in vivo* sampling technique should be highly specific, portable, solvent-free and offer integration of the sampling, sample preparation and analysis step. One of the most promising techniques for rapid sample preparation and subsequent analysis is solid phase microextraction (SPME), based on fibers coated with biocompatible materials that are exposed to the sample and then removed and analyzed. This technique causes minimal dis-

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turbances to the investigated system, as no liquid and only small fractions of analytes are removed [11–13].

The most widely used technique of sampling with SPME consists of exposing a small amount of extracting phase (coating) associated with a fiber to the sample for a predetermined amount of time. When the sample volume is large, the number of moles of analyte, n, extracted by the coating can be calculated as

$$n = K_{fs} V_f C_0 \quad (1)$$

where C_0 is the initial concentration of a given analyte in the sample, V_f is the fiber coating volume, and K_{fs} is the distribution coefficient of the analyte between the fiber coating and sample matrix [13]. Eq. (1) indicates that the amount of analyte extracted onto the coating at equilibrium (n) is linearly proportional to the analyte concentration in the sample (C_0), and points to the usefulness of the technique when the volume of the sample is unknown.

If sensitivity is not a major concern for analysis, shortening the extraction time is desirable. In these circumstances, the extraction is stopped and the fiber is analyzed before the equilibrium is reached. The kinetics of adsorption of analytes onto a fiber coating can be described as

$$n = n_0(1 - e^{-at}) \quad (2)$$

where n_0 is the amount of analyte adsorbed onto the fiber at equilibrium, t is the extraction time, and a is a time constant, representing how fast an equilibrium can be reached [14].

In contrast, when an SPME coating that is preloaded with a standard compound is exposed to an agitated sample matrix, desorption of the compound from the fiber occurs. The desorbed compound diffuses through the boundary layer into the bulk of sample matrix. The amount Q of standard remaining on the coating after a time t can be described as

$$Q = q_0 e^{-at} \quad (3)$$

where q_0 is the initial amount of standard present onto the fiber.

The constant a in Eq. (2) for adsorption has the same definition as constant a in Eq. (3) for desorption. The value of constant a, for the same analyte, should be the same for both adsorption and desorption of the analyte, under the same experimental conditions (*i.e.* sample bulk velocity and temperature).

Rearrangement of Eqs. (2) and (3) leads to

$$\frac{n}{n_0} + \frac{Q}{q_0} = 1 \quad (4)$$

Eq. (4) demonstrates that the sum of Q/q_0 (desorption) and n/n_0 (adsorption) should equal unity at any desorption/adsorption time. The isotropy of adsorption and desorption in SPME allows for the calibration of adsorption using desorption. This is especially important for the calibration of *on-site*, *in situ*, or *in vivo* analysis, because control of the agitation conditions of the matrix is sometimes difficult, and direct spiking of standards into the matrix is typically not possible in these cases [15].

When preloading the SPME coating with a standard for kinetic calibration is undesirable (or a suitable standard is not available), pre-equilibrium extraction can be calibrated by two successive extractions from the same sample. In this case, the sampling time for the two extractions must be different and the sample flow rate should be constant. For calculation of the amount of analyte extracted at equilibrium, Eq. (2) can be applied for both extractions:

$$n_1 = n_0(1 - e^{-at_1}) \quad (5)$$

$$n_2 = n_0(1 - e^{-at_2}) \quad (6)$$

where n_1 and n_2 represent the amount of analyte extracted at times t_1 and t_2.

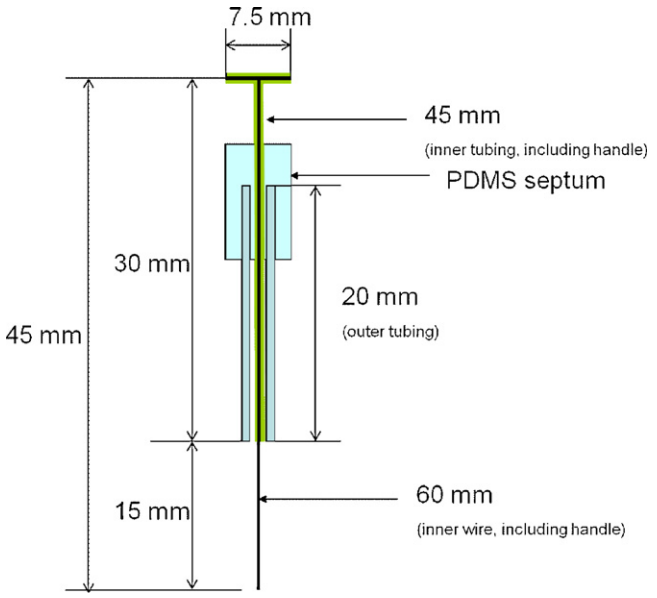

Fig. 1. Schematic representation of the SPME device for *in vivo* monitoring of drug concentrations in the blood of small animals (rats). Total length is 4.5 cm.

Rearrangement of Eqs. (5) and (6) leads to Eq. (7) which can be used to determine n_0, the amount of analyte extracted at equilibrium.

$$\left(1 - \frac{n_1}{n_0}\right)^{t_2/t_1} + \frac{n_2}{n_0} = 1 \quad (7)$$

In addition to convenient *in vivo* applications, SPME is also very useful for determining free concentrations [11,16,17]. Briefly, in the presence of an SPME fiber, the amount n (moles) of drug extracted by the fiber from the solution will be in equilibrium with the free concentration in solution. The free concentration of drug remaining in the sample is then given by

$$C_{free} = \frac{n}{f_c} \quad (8)$$

where f_c is the fiber constant and represents the product of the partition coefficient of the drug (between fiber and solution) and the volume of the fiber [16].

The present research is a continuation of our recent efforts to develop a blood draw-free, stress-free sampling technique for *in vivo* analysis [11]. With SPME, target compounds are quickly extracted directly from their *in vivo* surroundings, with the help of a special device (Fig. 1). The extraction time is short (40 s to 2 min), and the probes are desorbed in small volumes of solvents that can be analyzed with highly selective instruments.

Here we present the first application of fast *in vivo* microextraction for PK studies in rats. In addition, a kinetic calibration method that does not require any internal standards is introduced.

2. Materials and methods

The experimental work consisted of:

- Preparation of SPME probes, devices, and interfaces for *in vivo* application.
- Pharmacokinetic study in rats, by SPME and a standard approach (plasma sampling and analysis).

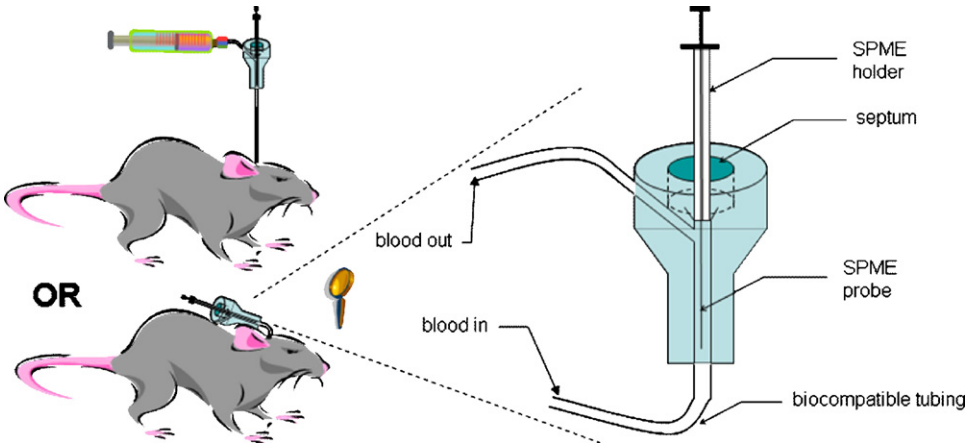

Fig. 2. *In vivo* SPME study on rats: placement of SPME devices and interface connection to the carotid artery.

2.1. Preparation of SPME probes

Biocompatible SPME fibers were prepared as previously described, by anodic oxidation of the pyrrole monomer (from Sigma/Aldrich, Mississauga, ON) [11].

2.2. Preparation of SPME devices

The custom-designed sampler for *in vivo* microextraction (Fig. 1) consisted of a flexible thin wire (0.005″ diameter) coated with biocompatible extraction phase and housed inside two concentric hypodermic tubes (medical grade stainless steel, Type 316 S/S, 30 gauge inner tube, 23 gauge outer tube, from Small Parts Inc., Miami Lakes, FL). The inner tube was used to reinforce the thin wire; its last 15 mm, including the wire, were made into a handle to facilitate the movement of the coated portion through the outer tubing. The outer tube was used to protect the coating and to pierce the sampling interface. The assembly was sealed with a piece of PDMS green septum (from Supelco, Bellefonte, PA).

2.3. Interfaces for in vivo application of SPME

Application of *in vivo* microextraction for pharmacokinetic studies with small animals required the construction of a special interface (Supplementary Fig. 1a–c—three different materials were investigated: Teflon, stainless steel and polyurethane).

2.4. In vivo microextraction

In vivo experiments were conducted with conscious male Sprague–Dawley rats implanted with jugular vein and carotid artery catheters. The catheters were plugged and exteriorized at the nape of the neck. Prior to dosing, the lower tube ("blood in", Fig. 2) of one of the interfaces in Supplementary Fig. 1 was connected to the carotid artery catheter. The upper tube of the interface ("blood out", Fig. 2) was either recirculated to the carotid artery catheter (interface a and b, Supplementary Fig. 1) or was connected to a syringe (interface c). Re-connection of the interface to the carotid artery catheter allowed for automatic return of the arterial blood, using the pumping action of the heart. Connection of the interface to a syringe pump allowed for precise control of the blood flow rate through the interface. The interface and the associated tubing were kept warm at 38 °C. Rats were administered either 1 or 0.5 mg/kg diazepam by bolus injection into the jugular vein catheter. At each sampling time point, a sterile SPME device was placed through the septum into the interface so that only the coated portion of the wire was exposed to the flowing blood (as shown in Fig. 2). Blood flow through the interface was either allowed to run freely *via* recirculation to the carotid artery or was induced by the push/pull action of a syringe attached to the upper tubing. When a syringe was used, 0.2 mL of blood was withdrawn at a flow rate of 0.6 mL/min and then pushed back at the same flow rate. This flow rate was well below the normal flow rate in the rat carotid artery, and produced minimal disturbance. Three push/pull cycles were completed during a 2-min interval of equilibrium SPME sampling. For kinetic calibration with the standard preloaded on the fiber, one push/pull cycle was performed (40 s). For kinetic calibration by double extraction, a short cycle (20 s) was followed by a regular cycle (40 s).

2.5. Standard compounds

Diazepam, nordiazepam, oxazepam, and diazepam-D5 standards (1 mg/mL in methanol) were purchased from Cerilliant (Austin, TX). The standards were diluted in methanol or phosphate-buffered saline (PBS), pH 7.4, to prepare mixtures of various concentrations for use in sample preparation and instrument calibration. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Fresh rat whole blood (sterile, with EDTA as anticoagulant) was purchased from Bioreclamation (Hicksville, NY), and maintained at 4 °C for maximum 2 weeks. Deionized water was obtained using a Barnstead/Thermodyne NANO-pure ultrapure water system (Dubuque, IA). The drugs were considered to be stable in whole blood over the course of the calibration procedure (less than 10 min at 38 °C).

2.6. Analysis of probes

All of the probes collected during a study were desorbed in parallel in plastic HPLC vial inserts with 20 μL desorption solvent consisting of acetonitrile and water (75:25) with 0.1% acetic acid. Lorazepam was included in the desorption solution as an internal standard (25 ng/mL), to control for variation in injection volume. The probes were removed after 1 min, when desorption was complete. The resultant solution was injected automatically in an LC–MS/MS (liquid chromatography coupled to tandem mass spectrometry) system.

2.7. LC–MS/MS assay

Chemical analysis was performed on an LC–MS/MS system consisting of a Shimadzu (Kyoto, Japan) 10AVP LC interfaced to a CTC-PAL autosampler and an MDS Sciex API 3000 tandem mass

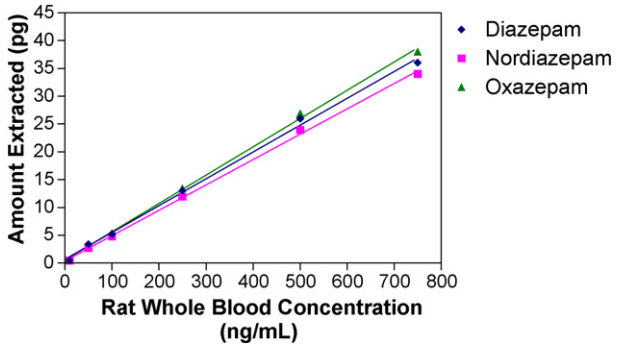

Fig. 3. Extraction calibration for diazepam, nordiazepam and oxazepam from rat whole blood. A six-point calibration ($n = 3$) from 5 to 750 ng/mL is shown.

spectrometer. The chromatographic analyses were performed as previously described [11,18]. The compounds were analyzed by electrospray ionization in positive ion mode with multiple reaction monitoring. The following transitions were monitored: diazepam, m/z 285.2/154.1; nordiazepam, m/z 271.1/140.0; oxazepam, m/z 287.1/241.1; diazepam-D5, m/z 290.4/198.4; lorazepam, m/z 321.1/275.1. Data were collected and analyzed using the Analyst 1.4.1 software from MDS Sciex.

2.8. Calibration methods

LC–MS/MS was used to quantify the analytes and internal standards present on the fiber. For equilibrium calibration, the amount of analyte extracted was correlated to the blood concentration using the calibration curve in Fig. 3. For kinetic calibration with standard preloaded on the fiber, both the extracted analyte and remaining internal standard were quantified and the value of n_0 (amount extracted at equilibrium) was calculated from Eq. (4); subsequently, the blood concentration was obtained using the calibration curve in Fig. 3. In the case of kinetic calibration by double extraction, n_0 was calculated from Eq. (7) and the blood concentration was obtained from the calibration curve.

2.9. Animal experiments

Male Sprague–Dawley rats (Charles River Labs, St. Constant, PQ) with an average weight of 300 g were acclimatized to their new environment for a minimum of 5 days prior to surgery. One day prior to dosing, rats were implanted with jugular vein and carotid artery catheters while under anesthesia with isoflurane (1.5% in oxygen delivered at a rate of 1 L/min). The catheters were plugged with removable metal plugs at the free end and then exteriorized by threading them under the skin and through a small incision at the nape of the neck. Animals were allowed to recover overnight prior to dosing. All rats were conscious and freely moving throughout the study. All procedures followed have been reviewed by the NoAb BioDiscoveries Inc. animal care committee and were performed in accordance with the principles of the Canadian Council on Animal Care (CCAC).

An interface was attached to the carotid artery catheter prior to dosing each rat. To insert the probes, the tip of the SPME device was used to pierce the interface septum. The length of the device was chosen so that the coated wire was completely exposed to the blood when the plunger was fully depressed. Before use, the SPME devices were sterilized in an autoclave at 121 °C and 15 psi for 30 min. For validation purposes, blood draws were taken from the same interface after each sampling with SPME. All of the probes were of single use.

Prior to dosing with diazepam, one blood draw (0.2 mL) was obtained from the interface and simultaneously a zero time probe analysis was performed. Rats were administered diazepam by bolus *i.v.* injection into the jugular vein catheter. Diazepam (purchased as 5 mg/mL injectable solution) was dosed at 0.5 mg/kg body weight for equilibrium extraction experiments and at 1 mg/kg body weight for kinetic calibration experiments. Diazepam-D5 was used as the standard on the fiber for kinetic calibrations. Drug concentrations were monitored for 6 h after dosing with diazepam. For each time point (5, 15, 30, 45, 60, 75, 120, 180, 240 and 360 min), the probes were in place for 2 min before the stated analysis time and blood draws were performed immediately after removing the probes. After the 2 min extraction time, the probes were removed, rinsed with water, and stored at −20 °C until the next day, when analysis was performed. All of the compounds are known to be stable on the fibers for at least 24 h [11]. For probe calibration, rat whole blood was spiked with appropriate amounts of the benzodiazepines and incubated with 10% CO_2 atmosphere. The probes were desorbed and analyzed as described in Section 2.6. The analytical range was 3–750 ng/mL.

2.10. Conventional plasma sampling and analysis

In addition to SPME sampling, 0.2 mL of blood was withdrawn from the interface at each time point. Plasma was isolated by centrifugation at 2500 rpm for 10 min and frozen at −20 °C in 2 mL cryovials (Wheaton Science Products, Millville, NJ) until analysis. For analysis, 0.05 mL of plasma was mixed with 0.25 mL acetonitrile containing 25 ng/mL lorazepam as internal standard in conical centrifuge vials. After vortex mixing (2400 rpm, 5 min) and centrifugation (14,000 rpm, 7 min), 0.2 mL of the supernatant was transferred to a 96-well plate and evaporated to dryness under flowing nitrogen. The residue was dissolved in 0.1 mL acetonitrile/water (75:25) on a shaking bed (150 rpm). Twenty μL was injected for analysis using the same chromatographic conditions as for the analysis of SPME probes. The linear range was 0.1–1000 ng/mL. Although this linear range may seem wide for a typical LC–MS method, triple quadrupole mass spectrometers can accurately quantify ionizable compounds over this concentration range.

2.11. Data analysis

For diazepam, the mean concentration *versus* time data were analyzed by a two-compartment model ($Y = Ae^{-\alpha t} + Be^{-\beta t}$) using WinNonlin Pro (Pharsight Corp., Mountainview, CA). Areas under the plasma concentration *versus* time curves (AUCs) represent the areas under the curve from the time of dosing and extrapolated to infinity. The distribution (α) and elimination (β) half-lives ($t_{1/2}$) were calculated as $\ln(2)/\alpha$ and $\ln(2)/\beta$, respectively. The total body clearance (CL) was estimated as Dose/AUC. The mean residence time (MRT) was calculated as AUMC/AUC, where AUMC denotes the area under the first moment curve. The steady-state volume of distribution (V_{ss}) was calculated as CL × MRT. Correlation coefficients between SPME and conventional analysis were calculated by least-square linear regression analysis using Microsoft Excel®.

For the two diazepam metabolites, nordiazepam and oxazepam, which were measured using equilibrium calibration for SPME, the mean concentration *versus* time data were analyzed by noncompartmental methods also using WinNonlin Pro. AUCs from 0 to the last time point were calculated by the linear/log-linear trapezoidal rule and then extrapolated to infinity by the addition of C_{last}/k, where k represents the apparent terminal rate constant. k was estimated by weighted ($1/Y^2$) regression analysis of at least four time points from the terminal (log-linear) portion of the concen-

tration *versus* time curve. Apparent half-lives ($t_{1/2}$) were calculated as ln(2)/k. AUMCs were also estimated and were used to calculate the corresponding MRTs. The time (t_{max}) at which maximum nordiazepam or oxazepam concentrations (C_{max}) were observed were determined from nominal values.

3. Results

The sensitivity, reproducibility and linear range of the assay were investigated by *in vitro* analysis of phosphate-buffered saline (PBS) spiked solutions (for determination of free concentrations) and whole rat blood spiked with a series of drug concentrations. All *in vitro* samples were incubated at 38 °C in 10% CO_2 atmosphere, in order to create experimental conditions similar to *in vivo* sampling and to generate accurate calibrations. The linear range for concentration of diazepam, nordiazepam, and oxazepam extracted from rat whole blood was from 3 to 750 ng/mL total concentration (Fig. 3), corresponding to 0.18–48 ng/mL free concentration. The free concentration was calculated using Eq. (8), and the fiber constant was determined by extractions from protein-free standard solutions (as previously described [16]).

For validation of the *in vivo* sampling approach, blood was also sampled after each probe extraction, and plasma was isolated by centrifugation. The short exposure time of the SPME devices allowed for the possibility to easily sample from three rats in parallel. Also, it was possible to draw the blood through the same interface that was used for the probes, resulting in less stress for the experimental animals.

The concentration *versus* time profiles for diazepam, nordiazepam, and oxazepam obtained with the probes, which measure concentrations in whole blood, are compared to the results of conventional sampling and analyses in plasma in Fig. 4, Supplementary Figs. 2 and 3. The total amount of time required to prepare a single sample ready for LC–MS/MS analysis was up to 3 min in the case of SPME and 90 min for conventional plasma analysis.

Due to the distribution of diazepam into blood cells, the ratio between plasma and whole blood concentration has been reported as variable and dependent on the hematocrit [19]. Since previous studies reported this ratio as being very close to one in the rat [20], the results of SPME and conventional sampling were compared directly. All three compounds show a good correlation between the concentration values obtained with SPME (in whole blood) and conventional sampling followed by plasma analysis, as shown in Fig. 4A (correlation coefficient 0.99), Supplementary Fig. 2 (correlation coefficient 0.95), and Supplementary Fig. 3 (correlation coefficient 0.99). The pharmacokinetic parameters determined for diazepam are also similar between methods (Table 1). The estimated values for CL, V_{ss} and the distribution and elimination half-lives ($t_{1/2}(\alpha)$ and $t_{1/2}(\beta)$, respectively), determined by the SPME method with equilibrium calibration, are close to those

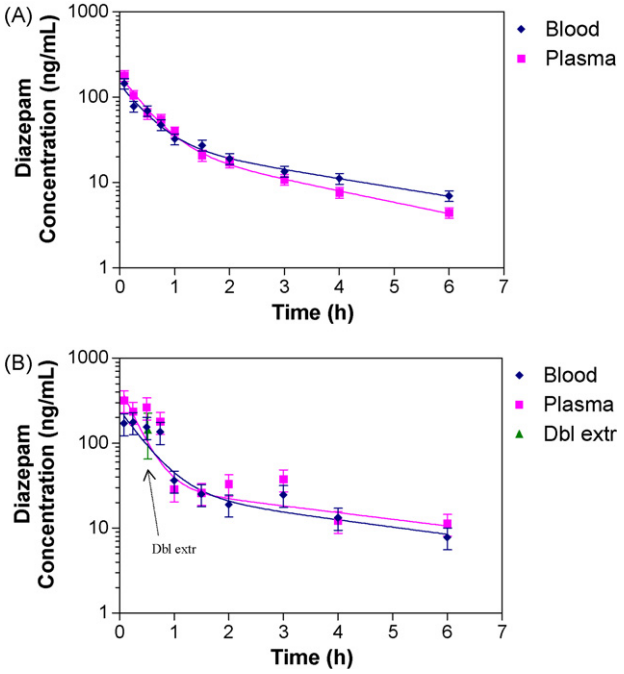

Fig. 4. Mean concentration *versus* time profiles for diazepam (n = 3 rats). "Blood": *in vivo* SPME from whole blood, "Plasma": conventional plasma sampling and analysis, "DblEx": calibration by double extraction method. (A) Profile obtained following 0.5 mg/kg *i.v.* diazepam; for SPME, equilibrium calibration was used. (B) Profile obtained following 1 mg/kg diazepam; for SPME, kinetic calibration was used. The lines fitted to the data were generated by nonlinear regression analysis using an equation for bi-exponential decay ($Y = Ae^{-\alpha t} + Be^{-\beta t}$).

estimated by conventional plasma sampling analysis. These parameters were also similar when kinetic calibration was performed for SPME, despite that greater overall variability was observed in both the blood (SPME) and plasma concentration *versus* time profiles (Fig. 4B, correlation coefficient 0.97). The data is also similar to previously published parameters for the pharmacokinetics of ^{14}C-diazepam in rats, especially with regard to total body clearance and distribution half-life [21]. The plasma concentration *versus* time profile in Fig. 4A fits very well to a two-compartment model, while Fig. 4B shows a more complex kinetics with evidence of enterohepatic recirculation (which is more evident in the plasma data but not in the blood data).

Mean blood and plasma concentrations of 2 of the metabolites of diazepam, nordiazepam and oxazepam, as determined by SPME and conventional plasma sampling and analysis, respectively, are also similar (Supplementary Figs. 2 and 3). Blood concentrations of the metabolites determined in the apparent

Table 1
Estimated pharmacokinetic parameters determined by two-compartment analysis of the mean diazepam concentration *versus* time curves following equilibrium and kinetic calibration methods

Parameter	Equilibrium calibration		Kinetic calibration		Published[a]
	Plasma	SPME/blood	Plasma	SPME/blood	Plasma
Dose (mg/kg)	0.5	0.5	1	1	1.2
AUC (h ng/mL)	157	172	303	236	267
$t_{1/2}(\alpha)$ (h)	0.301	0.310	0.198	0.304	0.20
$t_{1/2}(\beta)$ (h)	2.26	2.89	3.84	3.47	1.11
CL (mL/(h kg))	3193	2909	3304	4233	4530
AUMC (h^2 ng/mL)	319	527	1000	741	n/a
MRT (h)	2.04	3.06	3.31	3.14	n/a
V_{ss} (mL/kg)	6500	8917	10,921	13,294	5300

[a] Data was adapted from Ref. [21].

elimination phase by SPME appeared slightly higher than those determined in plasma, and therefore, the apparent elimination half-life and MRT determined by SPME are higher than those determined by conventional plasma analysis (Supplementary Table 1).

4. Discussion

The *in vivo* microextraction methodology was evaluated through studies of diazepam pharmacokinetics in rats. Quantification of extracted compounds (diazepam, nordiazepam, and oxazepam) was conveniently performed by a highly specific and sensitive liquid chromatography tandem mass spectrometric method.

The SPME probes (Fig. 1) were exposed to the blood flowing through a specially devised interface (Fig. 2 and Supplementary Fig. 1). The probes were placed in an interface since, unlike in previous experiments with larger animals, it was not possible to directly insert the probes into a blood vessel of a rat without significantly occluding it. Studies were conducted on three rats in parallel. Several materials were investigated for interface construction. The most suitable material that did not induce clotting in the interface, was found to be polyurethane. When blood was allowed to flow freely through the interface and was circulated back to the carotid artery, clotting occurred during many experiments, not only in the interface but in the connector tubes as well. It was found that sufficient pressure for keeping the blood flowing through the interface for extended periods of time could not be maintained. As an alternative, a syringe pump was used to draw and push blood through the interface. Clotting-related problems were not encountered with this approach. For fast equilibration, the blood should be flowing through the interface. However, if sensitivity or a longer extraction time is not an issue, extraction in static conditions can be employed. In this case, the interface is first filled with blood, then the fiber is introduced for extraction, and finally the blood is returned into the body. Damage to the catheters and interface by the animals is prevented by mounting the interface on top of the cage, out of the animal's reach. Because the interface (with or without the attached syringe) is free to rotate, the danger of twisting the tubing is very low; catheter twisting can also be prevented by using a movement-responsive cage [2].

For quantitative analysis, calibration was performed by both equilibrium extraction and kinetic calibration. For kinetic calibration, two methods were used: standard on the fiber and double extraction. Two of the major metabolites of diazepam, nordiazepam and oxazepam, were monitored as well. In order to relate the amount of analyte extracted to its total blood concentration, calibration curves in whole blood were prepared. While the results for total concentration are more accurate when the blood used for calibration has the same binding properties as *in vivo* blood, reliable measurements of the free concentration can be obtained even when the concentration of plasma proteins changes during the study, because the amount of analyte extracted by SPME is inherently related to the free concentration. This is an important advantage of SPME, as the free concentration is a valuable parameter in pharmacology. Supplementary Fig. 4 presents the free concentration profile of diazepam, oxazepam and nordiazepam in rat whole blood.

All concentration *versus* time profiles (Fig. 4, Supplementary Figs. 2 and 3) are in good agreement with literature values [5,21]. Kinetic calibration by double extraction was performed only for the data points collected at 30 min, as this was the first *in vivo* application, whereas equilibrium extraction and the standard on the fiber approach were applied for all data points.

5. Conclusions

The proposed sampling process does not require handling of the animal once the interface is installed. Because the animal is less stressed, the pharmacokinetic data is more relevant, and fewer animals are required to obtain reproducible data.

In this first application of fast microextraction and kinetic calibration for *in vivo* analysis in rats, sampling devices based on hypodermic tubes with SPME fibers were developed and successfully used for investigation of free and total concentration of diazepam and metabolites in whole blood.

There are important advantages in using this methodology: the exposure of lab personnel to blood is considerably decreased, the sample preparation process is much faster, fewer animals can be used for a full PK profile, and sampling can be simultaneously carried out at multiple sites in one animal without a reduction in blood volume. In addition, the technology can be miniaturized for applications in mice or soft tissues, and is suitable for automation. The technology has the potential to significantly decrease the turn-around time for acquiring pharmacokinetic profiles in small rodents, particularly during the early drug discovery process when *in vivo* PK studies are often considered a bottleneck for lead compound selection and drug candidate profiling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2008.03.028.

References

[1] H. Barton, J. Toxicol. Environ. Health A 68 (2005) 889–900.
[2] Y. Zhu, C.B. Kissinger, P.T. Kissinger, N. Bromet, Curr. Sep. Drug Dev. 21 (2005) 37–44.
[3] M.J. Anderton, M.M. Manson, R.D. Verschoyle, A. Gescher, J.H. Lamb, P.B. Farmer, W.P. Steward, M.L. Williams, Clin. Cancer Res. 10 (2004) 5233–5241.
[4] R.A. Corley, M.J. Bartels, E.W. Carney, K.K. Weitz, J.J. Soelberg, R.A. Gies, K.D. Thrall, Toxicol. Sci. 85 (2005) 476–490.
[5] I.I. Gueorguieva, I.A. Nestorov, M. Rowland, J. Pharmacokinet. Pharmacodyn. 31 (2004) 185–213.
[6] P. Scott-Stevens, J.R. Atack, B. Sohal, P. Worboys, Biopharm. Drug Dispos. 26 (2005) 13–20.
[7] K.J. Valenzano, L. Tafesse, G. Lee, J.E. Harrison, J.M. Boulet, S.L. Gottshall, L. Mark, M.S. Pearson, W. Miller, S. Shan, L. Rabadi, Y. Rotshteyn, S.M. Chaffer, P.I. Turchin, D.A. Elsemore, M. Toth, L. Koetzner, G.T. Whiteside, Neuropharmacology 48 (2005) 658–672.
[8] R.T. Kennedy, J.E. Thompson, T.W. Vickroy, J. Neurosci. Methods 114 (2002) 39–49.
[9] G. Leegsma-Vogt, E. Janle, S.R. Ash, K. Venema, J. Korf, Life Sci. 73 (2003) 2005–2018.
[10] D.R. Walt, Science (Washington, DC, U.S.) 308 (2005) 217–219.
[11] F.M. Musteata, M.L. Musteata, J. Pawliszyn, Clin. Chem. 52 (2006) 708–715.
[12] J. Pawliszyn, Aust. J. Chem. 56 (2003) 155–158.
[13] J. Pawliszyn (Ed.), Comprehensive Analytical Chemistry, vol. 37. Sampling and Sample Preparation for Field and Laboratory: Fundamentals and New Directions in Sample Preparation, 2002.
[14] J. Ai, Anal. Chem. 69 (1997) 1236–1239.
[15] Y. Chen, J. Pawliszyn, Anal. Chem. 76 (2004) 5807–5815.
[16] F.M. Musteata, J. Pawliszyn, J. Proteome Res. 4 (2005) 789–800.
[17] F.M. Musteata, J. Pawliszyn, J. Pharm. Biomed. Anal. 37 (2005) 1015–1024.
[18] A. Es-Haghi, X. Zhang, F.M. Musteata, H. Bagheri, J. Pawliszyn, Analyst 132 (2007) 672–678.
[19] A.W. Jones, H. Larsson, Ther. Drug Monit. 26 (2004) 380–385.
[20] Y. Igari, Y. Sugiyama, Y. Sawada, I. Tatsuji, M. Hanamo, J. Pharmacokinet. Biopharm. 11 (1983) 577–593.
[21] Y. Igari, Y. Sugiyama, Y. Sawada, T. Iga, M. Hanamo, Drug Metab. Dispos. 10 (1983) 676–679.