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In vivo solid-phase microextraction for single rodent pharmacokinetics studies of carbamazepine and carbamazepine-10,11-epoxide in mice

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ABSTRACT

The use of solid-phase microextraction (SPME) for *in vivo* sampling of drugs and metabolites in the bloodstream of freely moving animals eliminates the need for blood withdrawal in order to generate pharmacokinetics (PK) profiles in support of pharmaceutical drug discovery studies. In this study, SPME was applied for *in vivo* sampling in mice for the first time and enables the use of a single animal to construct the entire PK profile. *In vivo* SPME sampling procedure used commercial prototype single-use *in vivo* SPME probes with a biocompatible extractive coating and a polyurethane sampling interface designed to facilitate repeated sampling from the same animal. Pre-equilibrium *in vivo* SPME sampling, kinetic on-fibre standardization calibration and liquid chromatography–tandem mass spectrometry analysis (LC–MS/MS) were used to determine unbound and total circulating concentrations of carbamazepine (CBZ) and its active metabolite carbamazepine-10,11-epoxide (CBZEP) in mice ($n = 7$) after 2 mg/kg intravenous dosing. The method was linear in the range of 1–2000 ng/mL CBZ in whole blood with acceptable accuracy (93–97%) and precision (<17% RSD). The single dose PK results obtained using *in vivo* SPME sampling compare well to results obtained by serial automated blood sampling as well as by the more conventional method of terminal blood collection from multiple animals/time point. *In vivo* SPME offers the advantages of serial and repeated sampling from the same animal, speed, improved sample clean-up, decreased animal use and the ability to obtain both free and total drug concentrations from the same experiment.

1. Introduction

Solid-phase microextraction (SPME) is an equilibrium microextraction technique which combines sampling and sample preparation into one step [1]. The amount of an analyte extracted from a sample by SPME at equilibrium is given by Eq. (1),

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

where C_0 is the initial sample concentration of the analyte, n is the amount of analyte extracted, V_s is the sample volume, V_f is the fibre volume and K_{fs} is the analyte distribution constant between the fibre and sample matrix. However, under conditions of negligible depletion, when $V_s \gg V_f K_{fs}$, Eq. (1) reduces to Eq. (2), in which case the amount of analyte extracted by SPME is independent of the sample volume.

$$n = C_0 K_{fs} V_f \quad (2)$$

From a bioanalytical perspective, Eq. (2) permits the use of SPME to directly sample blood or tissue of animals *in vivo*, without having to withdraw an appropriate biofluid/tissue sample. Initially, *in vivo* SPME was applied to study the pharmacokinetics (PK) of diazepam and its primary metabolites in the circulating blood of Beagle dogs because of their large blood vessel size which permitted direct intravenous sampling using an in-dwelling catheter [2–5]. More recently, *in vivo* SPME was successfully applied to fish to study bioaccumulation of pharmaceuticals using direct muscle sampling [6,7], and to rats for PK studies of benzodiazepine drugs in the bloodstream [8].

However, although rats are extensively used for traditional PK studies in drug discovery, there is an increased interest in the use of mice due to the availability of various strains of gene knockout mice and indications that mice data may be more useful than

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rat data for allometric scaling of PK parameters from animals to humans [9]. Traditional PK studies in mice rely on the sacrifice of multiple animals at each time point of interest, which introduces inter-animal variation and dosing inaccuracies into the PK data. This disadvantage has been addressed by performing studies where all samples are acquired from a single animal by serial blood sample withdrawal, but to date such studies have been difficult to execute in mice due to a limited circulating blood volume (~1.85 mL/25 g mouse) and difficulties in the handling and sample preparation of the small blood sample volumes. To date, successful serial sampling and sample preparation approaches reported in the literature include the manual withdrawal of a small blood volume (4–10 μL) from the tail vein [10,11] or withdrawal of 30 μL blood using jugular vein cannulation [12] or lateral saphenous vein puncture [13] followed by plasma protein precipitation, withdrawal of 50–70 μL blood using jugular vein cannulation followed by turbulent flow chromatography on 2 μL aliquots [14] and sorbent sampling of 5 μL volumes of whole blood obtained from the tail vein in combination with protein precipitation and on-line solid-phase extraction [15]. In addition to reduced animal use and increased data accuracy, the advantages of such single rodent PK studies include: (1) a decrease in the amount of compound needed to perform the study which is particularly advantageous in early drug discovery where the quantity of test compound is limited, (2) elimination of the effects of anesthesia from the PK data, (3) the ability to study inter-animal variation, and (4) the opportunity to perform multiple and/or simultaneous pharmacokinetics/pharmacodynamics investigations in mice [10,11,13,16,17]. The major challenges with serial sampling approaches were that the withdrawal of higher blood volumes limited the number of time points that could be collected and sometimes exceeded recommended blood volume sampling guidelines (<20% of total blood volume). Furthermore, the tail vein bleeding was found to result in hemolysis in samples collected at later time points, which caused an overestimation of analyte concentrations [12]. The use of *in vivo* SPME could address both of these shortcomings, as no blood is withdrawn. SPME is also faster and offers the advantage of improved sample clean-up over the protein precipitation method because only a small amount of biocompatible sorbent is used and the partitioning of analytes in the sorbent is governed by their K_{fs} values. The main disadvantage of *in vivo* SPME is the lack of commercially available probes suitable for this type of application. To address this, Supelco recently developed prototypes of *in vivo* SPME probes and their suitability for single-use in biological fluids was successfully demonstrated *in vitro* for a selection of model drugs [18].

The primary objectives of the current study were to evaluate the performance of the new prototype SPME probes *in vivo* [18] and to modify *in vivo* SPME sampling procedures to make them applicable to serial sampling in mice for the first time because the blood-draw free nature of SPME sampling is particularly valuable for animals with such limited blood volume in the context of studies requiring repeated sampling of the same animal. This methodology was then applied to study the PK of carbamazepine (CBZ) and its active metabolite, carbamazepine-10,11-epoxide (CBZEP), in individual mice. The PK parameters obtained from total blood concentration *versus* time profiles using *in vivo* SPME were compared directly to the results obtained using automated serial blood sampling which collects small blood sample volumes and similarly permits the acquisition of an entire PK profile from a single animal. The data were also compared to the more traditional approach of manual blood withdrawal *via* terminal sampling from multiple animals at each time point followed by isolation of plasma. In order to facilitate the comparison between SPME and traditional methods, the blood to plasma concentration ratios of CBZ and CBZEP were determined by SPME.

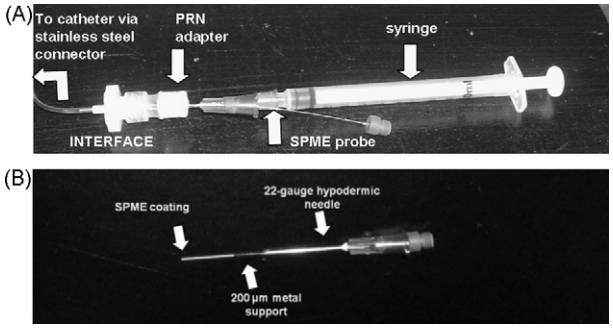

Fig. 1. (A) Photographic image of the mouse sampling interface shown with an SPME probe assembly inserted through the PRN adapter. (B) Close-up image of a prototype *in vivo* SPME probe assembly shown with the coating exposed.

2. Experimental

2.1. Chemicals and materials

CBZ, CBZEP and methyl carbamazepine, which was used as an internal standard for terminal blood collection experiments, were purchased from Sigma–Aldrich (Oakville, ON, Canada). Mianserin, which was used as an internal standard for serial blood collection experiments as methyl carbamazepine was no longer available commercially, was also purchased from Sigma–Aldrich. Diazepam was purchased from Cerilliant (Round Rock, TX, USA) as a 1 mg/mL methanolic solution and used as an internal standard for SPME experiments. Carbamazepine-d10 (CBZ-d10) was used for standard-in-fibre calibration and was purchased from Alltech (Deerfield, IL, USA) as a 100 μg/mL methanolic solution. Acetonitrile (HPLC grade), methanol (HPLC grade), and glacial acetic acid were purchased from Fisher Scientific (Ottawa, ON, Canada). Commercial prototype *in vivo* SPME assemblies were obtained as research samples from Supelco (Bellefonte, PA, USA). The two main components of the *in vivo* SPME assemblies were (i) a wire with immobilized SPME coating and (ii) a 22-gauge hypodermic needle which was used to protect and house the coated wire as well as pierce the septum of the sampling interface during sampling (Fig. 1). The SPME coating consisted of 5 μm C_{18} coated silica particles mixed with biocompatible binder and was immobilized on wire made of an inert, flexible metal alloy (200 μm diameter). The coating thickness was 45 μm and the length was 15 mm. Phosphate-buffered saline (PBS) solution, 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, when necessary.

2.2. Animals and intravenous (i.v.) administration of CBZ

Groups of male CD-1 mice (Charles River Labs, St. Constant, PQ, Canada) weighing 20–30 g were used. The mice were housed in the animal facility at NoAb BioDiscoveries Inc. and maintained on a 12 h light–dark cycle. Mice had access to water and to Lab Diet® 5015 Mouse Diet (Ren's Feed, Milton, ON, Canada) *ad libitum*. The animals were acclimatized to their environment for a minimum of 5 days prior to either surgery or dosing. Surgical placement of catheters was performed under isoflurane anesthesia and animals were allowed to recover and acclimatize individually to their cage for at least one day prior to dosing. For SPME sampling, a catheter was surgically implanted in the carotid artery and animals dosed *i.v. via* the tail vein. For automated serial blood draws, catheters were surgically implanted in the carotid artery (for sample withdrawal) as well as the jugular vein (for *i.v.* dosing). For terminal

blood draws, animals were dosed *i.v. via* the tail vein. Mice were administered single 2 mg/kg CBZ *i.v.* doses formulated in ethanol, propylene glycol and saline (1/1/3, v/v/v). All procedures followed were reviewed by NoAb BioDiscoveries' animal care committee and were performed in accordance with the principles of the Canadian Council on Animal Care (CCAC).

2.3. SPME experiments

2.3.1. In vivo SPME sampling procedure

In vivo SPME sampling experiments were conducted in 7 conscious mice. The animals were fully restricted during dosing and partially restricted during the 2-min SPME sampling periods in order to insert SPME device within the interface. Animals were allowed free unrestricted movement throughout the rest of the experimental period. A custom-made sampling interface, consisting of PRN adapter (BD, Franklin Lakes, NJ, USA) on one end was connected by a stainless steel tube connector (22 gauge, 2 cm in length) to the catheter implanted in the carotid artery of a mouse. For sampling, SPME probes were inserted into the interface via the septum of PRN adapter. The interface was similar to but smaller in size than that previously described for sampling from rats [8] in order to reduce total internal volume of the interface (including tubing from catheter) to 125 μL to permit sampling of mice. *In vivo* SPME sampling was performed prior to dosing and at 5, 15, 30, 60, 90, 120, 180 and 240 min post-dose. Approximately 70 s prior to each sampling time, the interface was filled with blood using a syringe inserted into the interface *via* a syringe needle. For each sampling period, a new *in vivo* SPME probe was inserted into the blood-filled sampling interface 1 min before the stated time and held in the interface 1 min after the stated time (total sampling time of 2.0 min). To prevent coagulation within the interface and to increase the amount extracted by SPME, agitation was applied within the interface by a manual pull–push action using a syringe. Within each 2 min-sampling period approximately 10 pull/push cycles were completed. After sampling was completed, the SPME probe was removed from interface and rinsed immediately for 30 s using purified water. Wash time should be kept as short as possible to avoid inadvertent desorption of the analytes from the coating. The probes were stored in the freezer (−20 °C) or on ice until analysis. After each sampling interval, blood remaining in the interface was returned to the animal and the interface and catheter flushed with heparin-containing saline.

2.3.2. In vivo SPME calibration procedures and in vitro validation

A kinetic on-fibre standardization calibration method was used for *in vivo* PK and *in vitro* validation experiments [4,8,19–21]. Simultaneous pre-conditioning and pre-loading of the *in vivo* probes were performed using 1000 ng/mL standard solution of CBZ-d10 calibrant prepared in methanol/water (1/1, v/v) for a minimum of 120 min with 1000 rpm vortex agitation, which was sufficient to reach equilibrium [18]. The amount of standard pre-loaded on the fibre was determined by immediate desorption of five probes (without performing *in vivo* sampling), followed by LC–MS/MS analysis.

Calibration standards and validation samples were prepared by spiking an appropriate volume of standard stock solution into mouse whole blood, plasma (1–2000 ng/mL) or phosphate-buffered saline (PBS, pH 7.4) in such a way as to keep the organic solvent concentration at exactly 1% methanol. Spiked blood and plasma samples were incubated overnight with refrigeration to permit protein binding to take place, as 1 h incubation was previously found to be insufficient [18]. Equilibrium calibration was performed in order to determine K_{fs} in Eq. (3) which is a necessary parameter in order to accurately calculate initial sample concentration of analytes. Calibration standards (0.3 mL sample volume in 0.3 mL capacity amber HPLC vial) were subjected to equilibrium SPME sampling procedure in parallel (16 h extraction, 200 rpm orbital agitation) using individual *in vivo* SPME probes for each standard. Fibres were rinsed for 30 s using purified water to eliminate any droplets from the surface of the fibres and desorbed as described in Section 2.3.3. The calibration curve was obtained by $1/y$ weighted linear regression analysis using SigmaPlot 2004 for Windows (version 9.0) software.

To assess the accuracy and reproducibility of the method, validation samples ($n = 4$ at three concentration levels) were extracted from 0.3 mL capacity polypropylene HPLC vials containing 0.3 mL of each spiked whole blood sample by placing the catheter end of the interface directly into the vial and performing SPME extraction exactly as described for *in vivo* samples in Section 2.3.1. Fibres were rinsed for 30 s using purified water to eliminate any droplets from the surface of the fibres and desorbed as described in Section 2.3.3.

2.3.3. Desorption of analytes from probes

The analytes on the probes were desorbed using 300 μL of desorption solvent (acetonitrile/water, 1/1, v/v) spiked with 50 ng/mL diazepam (internal standard used to correct for injection volume variability). The desorption was performed in 0.3 mL amber polypropylene HPLC vials (Labsphere, Brossard, PQ, Canada) for 60 min using vortex agitation at 1000 rpm on a multi-tube vortexer with a foam insert that can accommodate up to 50 HPLC vials at one time (model DVX-2500, VWR International, Mississauga, ON, Canada). These extracts were then analyzed directly using LC–MS/MS. High concentration samples were diluted, when necessary, by up to 10-fold using desorption solvent in order for the signal intensity to remain within the linear range of the analytical instrument.

2.3.4. Determination of blood to plasma concentration ratios

SPME was also used to investigate the blood to plasma concentration ratio of CBZ and its metabolite. This allowed a comparison of the blood concentration data determined by SPME and serial blood collection to plasma concentration data which had been obtained by the traditional method of terminal blood collection and isolation of plasma for subsequent bioanalysis. If the blood to plasma concentration ratio significantly differs from unity as a result of either exclusion of drug from red blood cells (ratio <1) or the partitioning into or binding of drug to red blood cells (ratio >1), a correction factor would need to be applied to the concentration data. The blood to plasma concentration ratio was determined from the same spiked blood sample, which was divided into two aliquots. One spiked whole blood aliquot was used to perform equilibrium SPME extraction exactly as described in Section 2.3.2 for calibration standards. The second aliquot was centrifuged (4 °C, 15,000 rpm, 15 min) to isolate plasma and equilibrium SPME extraction was carried out on 0.3 mL plasma aliquots exactly as described in Section 2.3.2 for calibration standards. The calibration curves from spiked whole blood and spiked plasma were used to determine the total concentration of analyte in each matrix.

2.4. Automated serial blood collection experiments

Serial blood sample collection experiments were performed in 3 conscious mice using an automated *in vivo* sampling system (Culex®, BASi, West Lafayette, IN, USA) equipped with a rotating cage (Raturn, BASi), which avoided the use of a liquid swivel. Blood samples (50 μL) obtained *via* the carotid artery catheter were collected serially from the same animal prior to dosing and at 5, 15, 30, 60, 90, 120, 180 and 270 min post-dose into a refrigerated fraction collector (4 °C). Following sampling, an equal volume of

heparin-containing saline (20 IU/mL) was administered to the animal to replace the sample volume. Detailed sample preparation procedures following sampling are described in Supplementary information.

2.5. Terminal blood collection experiments

The results of SPME sampling were compared to a more traditional sampling method, in which blood samples were collected by terminal cardiac puncture from three mice at each time point (total of 33 mice). Blood samples (0.6–0.8 mL) were obtained pre-dose and at 5, 15, 30, 60, 90, 120, 180, 240, 360 and 480 min post-dose by direct cardiac puncture while under CO_2/O_2 anesthesia. The collected blood was immediately transferred into tubes spray coated with heparin and then centrifuged (6800 rpm for 5 min at 4 °C) to isolate plasma. Plasma was stored at −70 °C until analysis. For preparation of calibration standards, blank blood was similarly collected from a group of undosed animals. Detailed sample preparation procedures following sampling are described in Supplementary information.

2.6. LC–MS/MS analysis

For SPME experiments, LC–MS/MS analyses were performed using a system consisting of Accela autosampler with cooled sample tray, Accela LC pumps and TSQ Vantage triple-quadrupole mass spectrometer equipped with HESI source (Thermo Fisher Scientific, San Jose, CA, USA). Xcalibur software (version 2.0.7. SP1) 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, Milford, MA, USA). Samples (10 μL) were injected in duplicate and kept at 5 °C on the autosampler while waiting for analysis. Mobile phases used were (A) acetonitrile/water/acetic acid (10/90/0.1, v/v/v) and (B) acetonitrile/water/acetic acid (90/10/0.1, v/v/v). Mobile phase gradient conditions were as follows: hold at 100% A for 0.5 min, linear increase to 90% B in 2.5 min, hold at 90% B for 0.5 min and re-equilibrate the column to initial conditions for 1.5 min. LC column effluent was diverted from the mass spectrometer (MS) for the first 1.5 min of run time. MS conditions used were: sheath gas = 50, auxiliary gas = 10, spray voltage 4000 V, and capillary temperature set to 275 °C. All of the compounds were analyzed in positive ion SRM mode using instrument settings described in Table S1 (Supplementary information). Baseline chromatographic separation of CBZ and CBZEP, such as that achieved by this method, was important for accurate analysis of CBZEP because approximately 0.3% of in-source oxidative conversion of CBZ to CBZEP was detected. In the absence of chromatographic separation of the two analytes, this could cause systematic bias when trying to simultaneously analyze low concentrations of CBZEP in presence of high concentrations of CBZ. For serial blood collection and terminal plasma collection experiments, detailed instrumental conditions are described in Supplementary information.

2.7. Pharmacokinetics data analysis

Data were analyzed by noncompartmental methods using WinNonlin Pro (Pharsight Corp., Mountainview, CA, USA). For SPME and serial blood collection experiments, blood concentration *versus* time data from each animal were analyzed individually. For the terminal plasma collection experiments, the mean plasma concentrations ($n = 3$ animals/time point) were analyzed. Areas under the concentration *versus* time curves (AUCs) were calculated by the linear/log-linear trapezoidal rule from the time of dosing to the time of the last measurable concentration and were extrapolated to infinity by the addition of C_{last}/k, where k represents the terminal rate constant. k was estimated by unweighted regression analysis of a minimum of 3 time points from the terminal (log-linear) portion of the concentration *versus* time curve. Terminal half-lives ($t_{1/2}$) were calculated as $\ln(2)/k$. Mean residence times (MRT) were calculated as AUMC/AUC, where AUMC denotes the area under the first moment curve. The total body clearance (CL) was estimated as dose/AUC. The steady-state volume of distribution (V_{ss}) was calculated as CL*MRT. The time, t_{max}, to reach the maximum concentration, C_{max}, was determined from the nominal values. The PK parameters estimated by SPME and automated serial blood sampling were compared by an unpaired t-test. A p value < 0.05 was considered statistically significant.

3. Results and discussion

To date, most *in vivo* SPME PK studies were performed using benzodiazepines as model analytes [2–5]. CBZ was selected as a model drug for current study because of its poor solubility and more variable plasma protein binding characteristics. CBZ is widely used for treatment of epilepsy, while its metabolite, CBZEP, formed through hepatic oxidative metabolism, is clinically important as it is pharmacologically active and may also contribute to the appearance of side effects [22,23]. Therapeutic drug monitoring of unbound (free) concentrations of CBZ is commonly performed because of significant inter-individual variations in free drug fractions while total CBZ concentrations remain within the therapeutic range [24,25]. This makes it particularly interesting for *in vivo* SPME sampling because the amount of drug extracted by SPME is proportional to the unbound fraction and thereby SPME is also useful for monitoring unbound drug concentrations.

3.1. SPME method development

The main disadvantage of *in vivo* SPME to date was the lack of commercially available probes suitable for this type of application. To address this, Supelco recently developed low-cost single-use *in vivo* SPME probes and their suitability for single-use in biological fluids was successfully demonstrated *in vitro* for a selection of model drugs including CBZ [18]. In this study, the performance of these prototype probes was tested *in vivo* for the first time. Research prototype *in vivo* SPME probes with three types of coating: (i) octadecyl silica (C_{18}), (ii) C_{16} with an embedded amide group and (iii) cyanopropyl, were previously evaluated *in vitro* as described in detail elsewhere [18]. Based on the latter results, probes coated with C_{18} were selected for use *in vivo* because of high extraction efficiency for CBZ. For optimum desorption of the analytes from the probes, acetonitrile/water (1/1, v/v) was selected as this solvent composition was compatible with direct LC–MS injection and produced a good chromatographic peak shape. The minimum volume of desorption solvent required for complete immersion of the coating in solution was 100 μL. However, increasing the volume to 300 μL reduced carryover to less than 0.5% while still providing adequate analytical sensitivity. As a consequence, 300 μL desorption solvent was used in the current study. All probes were used as single-use devices as recommended by the manufacturer.

Equilibrium sampling of hydrophobic drugs such as CBZ using these biocompatible prototype SPME probes is not suitable for *in vivo* applications due to the very long equilibration times which result in a loss of temporal resolution in the PK profile. The amount extracted *versus* time profiles for CBZ using probes coated with C_{18} (45 μm thickness), with and without agitation, are reported elsewhere [18]. When no agitation was employed, the time required to reach extraction equilibrium was extremely long (≥1200 min). However, quantitative analysis can be performed by using very

short pre-equilibrium sampling times and an appropriate kinetic calibration method, such as kinetic on-fibre standardization. The theory behind this calibration method has been described extensively elsewhere [19,20,26]. Briefly, the SPME probe is pre-loaded with an appropriate calibrant (such as the deuterated analogue of the analyte or CBZ-d10 in the current study). During sampling, a small portion of the pre-loaded calibrant is desorbed into the system under study. The process of desorption is then used to calibrate the simultaneous process of analyte extraction into the SPME coating provided that the kinetics (time constant a) of analyte and calibrant are similar. The initial concentration of the analyte in the sample, C_0, can then be determined using Eq. (3):

$$C_0 = \frac{nq_0}{q_0 - Q} \cdot \frac{1}{K_{fs}V_f} \quad (3)$$

where V_f and K_{fs} are the same as defined previously, n is the amount of analyte extracted into the SPME coating using a short pre-equilibrium sampling time, Q is the amount of calibrant remaining in the extraction phase after exposure to the sample matrix for the sampling time, and q_0 is the amount of calibrant that is pre-loaded in the extraction phase. The product of $K_{fs}V_f$, also referred to as the fibre constant (f_c), is determined by SPME calibration at equilibrium in the appropriate matrix (PBS for determination of unbound concentration and whole blood for total concentration). Under conditions of negligible depletion, f_c is approximately equal to the slope of the calibration curve, such as is the case for plasma and whole blood calibrations in the current study (Table S2 of Supplementary information). For PBS, where significant depletion of the calibrant was observed, f_c must be calculated by taking into account the sample volume employed for the analysis (Table S2). For *in vitro* sampling (such as calibration in blood or buffer), agitation can be used in order to significantly decrease the equilibration times for these fibres. For example, using 2400 rpm vortex agitation, equilibrium was reached in 60 min for CBZ using C_{18} coatings ($n = 3$ fibres) [18].

Reproducible calibrant loading is crucial for the success of this calibration procedure, and our previous studies with the three fibre prototypes from Supelco indicated that it is important to combine fibre pre-conditioning and standard loading into one step by using high concentrations of loading standard [18]. The reproducibility of the standard loading procedure was determined by pre-loading five additional fibres with CBZ-d10 and then desorbing the fibres directly in desorption solvent without sampling. Analysis of the desorption solvent indicated that the amount of CBZ-d10 pre-loaded onto the fibres (q_0) was ~1.1 ng and excellent precision was obtained as indicated by 7% RSD ($n = 5$).

3.2. Results of in vitro SPME method validation using mouse whole blood

The absolute matrix effect on the quantification of CBZ was evaluated by spiking 25 ng/mL CBZ into blank extract, obtained by performing the entire SPME sampling procedure on a blank whole blood sample, and comparing the intensity of the signal obtained for this preparation to a standard prepared at the same concentration in the desorption solvent. The intensity of the CBZ signal in the spiked extract relative to the standard solution was 102% indicating that there was no matrix suppression or enhancement at the retention time of the analyte.

Extraction efficiency (absolute recovery) was evaluated across the entire range of concentrations of interest ($n = 10$ for CBZ, $n = 6$ for CBZEP). Absolute recovery of CBZ was 68 ± 4%, 2.4 ± 0.3%, 2.1 ± 0.4% in PBS, plasma and whole blood, respectively, using one lot of SPME probes. Absolute recoveries of CBZEP were lower than for CBZ due to the slightly more polar nature of the compound, and were determined to be 39 ± 6%, 1.5 ± 0.1% 1.0 ± 0.2% in PBS, plasma and whole blood, respectively. Inter-probe reproducibility within the same lot was evaluated at pre-equilibrium and equilibrium and was typically <15% using short pre-equilibrium sampling times and <7% using equilibrium sampling times. Lot-to-lot SPME probe variability was higher with a mean absolute recovery for CBZ of 45 ± 11% (in PBS) for the 4 lots evaluated. This is probably caused by slight variations in the coating thickness between lots of fibres as well as slight variations in the ratio of the sorbent to biocompatible binder. Based on this data, it is recommended that probes from a single lot should be used for *in vivo* sampling and preparation of calibration standards, whenever possible.

A 30-s rinse step with purified water was added between the extraction and desorption steps in order to wash off salts and/or droplets remaining on the surface of the fibre following sampling. These rinse solutions were subsequently analyzed for the presence of CBZ. CBZ was not detected in any of the chromatograms indicating that no detectable loss of CBZ occurred during the rinsing step. The lower level of quantitation (LLOQ) for the SPME sampling procedure, which was determined using a 10× signal to noise ratio, was 1 ng/mL for CBZ and CBZEP in whole blood and exhibited acceptable precision (<20% RSD). The linear range evaluated in the current study was 1–2000 ng/mL for CBZ and 1–150 ng/mL for CBZEP in whole blood, covering the expected concentration ranges for the drug and metabolite. The linear concentration ranges were not limited by the probe extraction capacity and could be extended, if required. Table S2 (Supplementary information) summarizes the parameters calculated from representative calibration standards prepared in the 3 matrices (blood, plasma and PBS) and demonstrates that excellent linearity was obtained ($r^2 \geq 0.99$) in all cases even though each standard was extracted using a different *in vivo* SPME probe and internal standard was employed only to correct for differences in injection volume and not for inter-probe differences stemming from slight variations in the amount of immobilized SPME coating. The calibration was performed in this way because on-fibre standardization method utilized in this *in vivo* study does not compensate for inter-probe variability. This disadvantage of on-fibre standardization is not problematic when inter-probe variability is good as is the case with these commercial prototype probes. In addition, the amount of calibrant remaining on the probe can be used to identify any defective or improperly conditioned probes.

Accuracy and precision were assessed *in vitro* using pooled mouse blood spiked at three concentrations ($n = 4$ per concentration). Validation samples were extracted for 2 min by the same *in vivo* SPME sampling procedure, except that the catheter end of the sampling interface was placed in a vial containing 0.3 mL of spiked whole blood instead of being connected to the arterial catheter implanted in the mouse. The total concentrations of CBZ in these samples were then calculated from a CBZ calibration curve prepared in whole blood. Accuracy (% relative recovery) was calculated as the ratio of the experimentally determined amount of analyte to the true spiked amount × 100%. At 5,200 and 2000 ng/mL concentrations, mean % accuracy was determined as 96.8%, 109% and 93.1%, respectively, while precision (expressed as RSD) was 17%, 14% and 8%, respectively. This indicates an acceptable performance of the method and the on-fibre standardization approach.

On-fibre standardization approach introduces a small amount of calibrant into the animal blood stream. In current study, the total amount desorbed from the fibre into bloodstream was ~0.5 ng per sampling point. This small amount of calibrant is not expected to cause adverse reactions in the animal or affect the accuracy of *in vivo* SPME procedure in current application. Alternatively, recent study shows that any compound can be successfully used as calibrant for on-fibre standardization which opens up the possibility to use non-toxic, physiologically inactive calibrants [27].

3.3. Development of in vivo SPME sampling procedure for mice

In vivo SPME sampling in mice was conducted similarly to what was described previously for rats [8]. A sampling interface is required for SPME sampling from both rats and mice (unlike dogs) because the size of the blood vessels does not permit direct insertion of an SPME probe without occlusion of the blood vessel and interruption of blood flow. Compared to that used for rats, the sampling interface designed for the mice was miniaturized to keep the internal volume of the interface to a minimum (total volume of 125 μL including tubing leading from catheter). The interface used for mice is shown in Fig. 1A. The SPME probe was inserted into the interface by piercing the septum of PRN luer-lock adapter with the hypodermic needle of *in vivo* SPME probe (Fig. 1B). For sampling, the plunger of the probe is depressed to expose the coating to blood within the catheter tubing. To draw and push blood over the exposed SPME coating, manual pull and push using the syringe inserted in the interface was used. This provided the sample agitation required to increase the amount of analyte extracted (and calibrant desorbed) by the coating within the short 2 min-sampling time.

The ability of the SPME sampling method to respond to rapidly changing concentrations *in vivo* was evaluated using an *in vitro* experiment. The analyte peak area determined from a probe which was initially exposed to a blank PBS solution for 30 s, followed by 90 s sampling from a 50 ng/mL CBZ standard solution in PBS, was compared to that of the same probe which was exposed to the 50 ng/mL CBZ standard solution for the entire 2 min of sampling. To determine the effect of the agitation rate, the probe was exposed to the 50 ng/mL CBZ solution following a 30 s delay, but the sampling rate was decreased from 10 to 4 pull/push cycles. As shown in Fig. 2, no difference was observed between the two conditions as long as the sampling speed of 10 pull/push cycles over 2 min was used (the response was decreased at the lower sampling rate). This indicates that SPME can respond reasonably well to rapidly changing concentrations using the sampling parameters employed in this study. The use of speed faster than 10 pull/push cycles was not considered in order to keep the sampling flow rate well below the animals' blood flow rate.

3.4. Determination of blood to plasma concentration ratio

In order to enable a direct comparison of the whole blood concentrations obtained by SPME sampling to the plasma concentrations that had been obtained by the traditional approach of terminal blood sampling from multiple animals at each time point, it is important to determine the whole blood to plasma concentration ratio, in case the analyte partitions into or binds to red blood cells (RBC) [28]. The distribution of CBZ and CBZEP between whole blood and plasma was investigated *in vitro* by spiking drug at various concentrations into whole blood and then dividing each blood sample into two aliquots. Equilibrium SPME sampling was performed directly in one aliquot of spiked whole blood and in plasma obtained after centrifugation of the second aliquot of spiked whole blood as described in Section 2.3.4. Table 1 summarizes the concentrations of CBZ that were determined in the blood and plasma samples, using the appropriate matrix-matched calibration curves. The experimental blood to plasma concentration ratio for CBZ (1.1 ± 0.2) is not statistically different from unity as determined by a paired *t*-test ($p = 0.38$). This allowed the concentrations measured by *in vivo* SPME sampling of whole blood and conventional plasma sampling to be compared directly without application of a correction factor. A similar experiment was performed for CBZEP (1–150 ng/mL) and the whole blood to plasma concentration ratio was determined as 1.3 ± 0.4. A paired *t*-test indicated that the difference between whole blood and plasma concentrations is not statistically significant ($p = 0.43$). The results obtained compare well to literature values of RBC to total plasma concentration ratio in humans of 1.06 ± 0.21 and 1.53 ± 0.45 for CBZ and CBZEP [29], respectively, and to blood to plasma concentration ratios of 1.37 ± 0.125 and 1.32 ± 0.134 for CBZ and CBZEP, respectively, in rats [30].

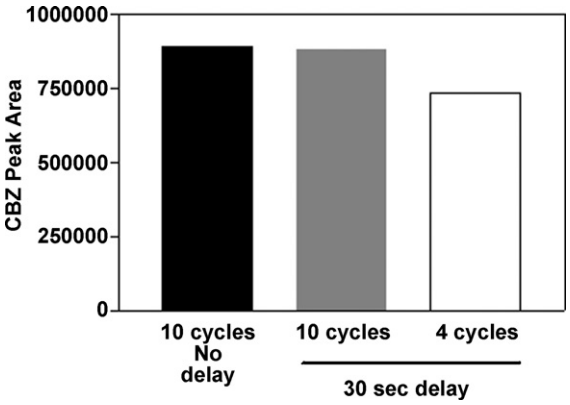

Fig. 2. Evaluation of the response (chromatographic peak area of CBZ) of SPME sampling to a rapid change in concentration *in vitro*. A fibre was placed in a 50 ng/mL CBZ solution in PBS for 2 min with 10 pull/push cycles over the sampling period. The same fibre was also placed in blank PBS for 30 s, then in 50 ng/mL CBZ in PBS for the remaining 90 s sampling time using 10 pull/push cycles and in blank PBS for 30 s, then in 50 ng/mL CBZ in PBS for the remaining 90 s sampling time using 4 pull/push cycles. RSD of $n = 3$ determinations was <3%.

Table 1
Determination of whole blood to plasma concentration ratio as determined by equilibrium *in vitro* SPME sampling procedure described in Section 2.3.4.

Total CBZ concentration (ng/mL)		Whole blood/Plasma concentration ratio
Whole blood	Plasma	
1.19	1.19	1.00
11.7	14.8	0.79
83.4	72.1	1.16
439	323	1.36
769	643	1.20
1370	1440	0.95
Mean ($n = 6$)		1.1
SD ($n = 6$)		0.2
% RSD ($n = 6$)		19

3.5. In vivo PK after single 2 mg/kg i.v. dose of CBZ

The blood concentration *versus* time profiles of CBZ and CBZEP for individual mice that were obtained by *in vivo* SPME sampling and by automated serial blood collection after an *i.v.* dose of 2 mg/kg CBZ are depicted in Figs. 3 and 4, respectively. Blood concentrations of CBZ, determined by both methods, decreased rapidly over time. The formed metabolite, CBZEP, appeared rapidly and differences in the concentrations of CBZEP between individual mice were apparent. The concentrations and AUC of the metabolite reflect the formation, distribution and metabolism of the metabolite and not solely the amount converted. In previously published studies on rats, the inherent variability in the clearance of CBZ among animals was estimated as 20–40% [30]. However, the variability between animals appeared to be more pronounced for the SPME sampling technique. The reason(s) for this is not clear but may be due to a variety of experimental factors. One possible factor is the site of administration. Mice sampled by SPME were restrained during administration of CBZ *via* the tail vein whereas those sampled by the automated sampler were administered CBZ *via* the jugu-

Table 2
Mean (±SD) estimated PK parameters for CBZ following 2 mg/kg *i.v.* administration of CBZ to mice. Blood was sampled by SPME sampling ($n = 7$ mice) and by serial automated blood draws ($n = 3$ mice) and plasma was sampled by terminal blood draws ($n = 3$ mice/time point, 2 experiments).

Parameter	Units	SPME sampling[a]	Serial blood draws[a]	Terminal blood draws[b]
$C_{initial}$[c]	ng/mL	600 ± 150	820 ± 350	680
Terminal $t_{1/2}$	min	32 ± 12	50 ± 23	45
$AUC_{0\text{-inf}}$	min ng/mL	27,000 ± 10,000	43,000 ± 8000	37,000
CL	mL/min/kg	87 ± 35	48 ± 10	55
$MRT_{0\text{-inf}}$	min	40 ± 11	50 ± 7	47
V_{ss}	mL/kg	3200 ± 800	2400 ± 800	2600

[a] Parameters are estimated from individual whole blood concentration *versus* time profiles for each animal.
[b] Parameters are estimated from the mean plasma concentration *versus* time profiles.
[c] $C_{initial}$ is initial CBZ concentration extrapolated to time 0.

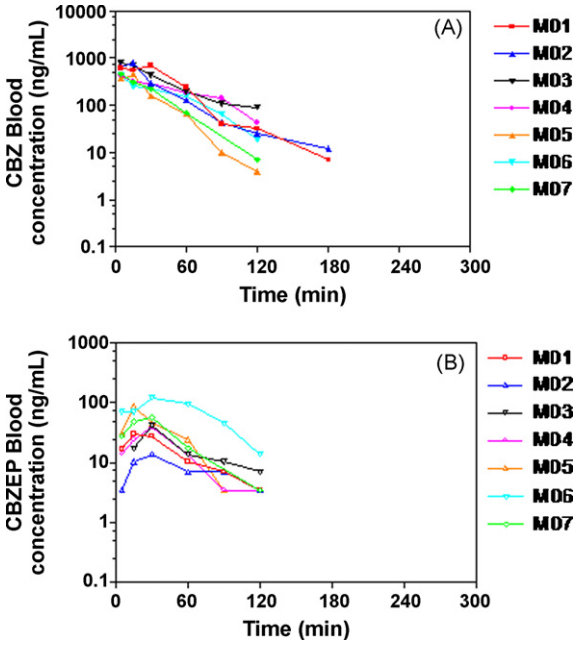

Fig. 3. Whole blood concentration *versus* time profiles obtained by *in vivo* SPME sampling for CBZ (A) and its formed metabolite, CBZEP (B), in 7 individual mice (M01 to M07) following administration of a single *i.v.* dose of 2 mg/kg CBZ. Samples were collected up to 240 min, but no CBZ and CBZEP was detected in some of the late time point samples as shown in the figure.

lar vein catheter since the instrument is designed to eliminate handling of the animals during the dosing and sampling periods. Another possible factor is that greater variations in the extraction and desorption of the analyte and calibrant under *in vivo* conditions existed as compared to *in vitro* conditions. The variability in the concentrations determined by SPME is also determined by SPME-method specific factors which differ from those involved in traditional analyses, as discussed below. Despite the variability observed between individual mice, the mean blood concentrations

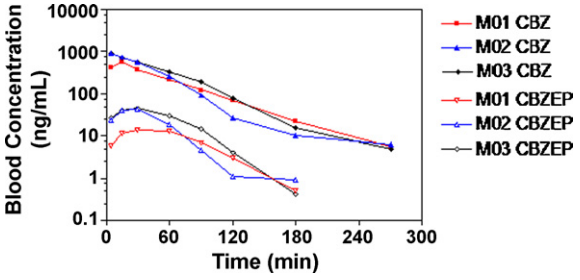

Fig. 4. Whole blood concentration *versus* time profiles obtained by automated serial sampling for CBZ and its formed metabolite, CBZEP, in 3 individual mice (M01 to M03) following administration of a single *i.v.* dose of 2 mg/kg of CBZ.

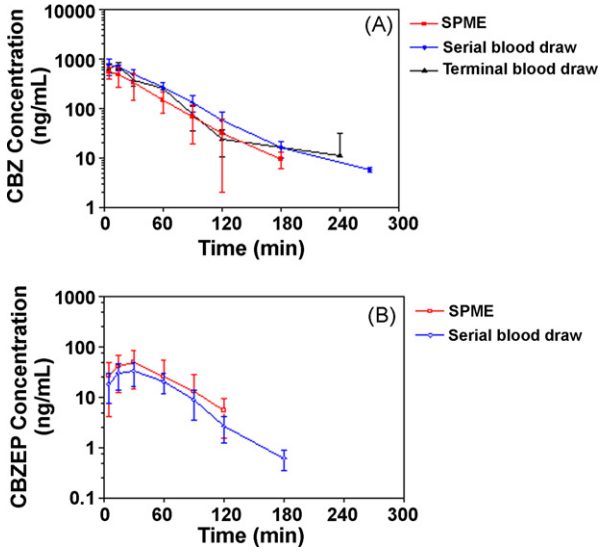

Fig. 5. Mean (±SD) concentration *versus* time profiles of CBZ (A) and the formed metabolite, CBZEP (B), following 2 mg/kg *i.v.* administration of CBZ to mice. Samples were taken by serial SPME sampling ($n = 7$ mice), by serial automated blood draws ($n = 3$ mice) or by terminal blood draws (3 mice/time point). SPME and serial blood sampling measured whole blood concentrations whereas terminal sampling measured plasma concentrations.

of CBZ and CBZEP are similar between the two methods and exhibit parallel profiles (Fig. 5). The PK parameters estimated by the two methods for CBZ and CBZEP are summarized in Tables 2 and 3, respectively. While the systemic clearance (CL) estimated by the SPME method was generally greater than that estimated by serial automated sampling, the difference is not statistically significant ($p > 0.05$). Similarly, no statistically significant differences were observed for the steady-state volume of distribution (V_{ss}) or terminal half-life ($t_{1/2}$) estimated by the two methods. Given that the PK results obtained by two methods are equivalent, SPME sampling has a number of advantages over serial blood sample withdrawal as mentioned previously. The most obvious advantage is that no blood is withdrawn, thus maintaining a constant circulating blood volume and composition, and removing the limitation in the number of samples that can be taken. For serial sampling, 50 μL of

Table 3
Mean (±SD) estimated PK parameters in blood for the formed metabolite, CBZEP, following 2 mg/kg *i.v.* bolus administration of CBZ to mice. Blood was sampled by SPME sampling ($n = 7$ mice) and by serial automated blood draws ($n = 3$ mice).

Parameter	Units	SPME sampling	Serial blood draws
t_{max}	min	34 ± 26	30 ± 0
C_{max}	ng/mL	57 ± 37	33 ± 17
Apparent $t_{1/2}$	min	36 ± 17	23 ± 5
$AUC_{0\text{-inf}}$	min ng/mL	3400 ± 2500	2200 ± 1000
$MRT_{0\text{-inf}}$	min	62 ± 20	52 ± 9

blood was withdrawn at each sampling point (total 450 μL of blood withdrawn over entire experiment), which does exceed recommended 20% of total blood volume and can be expected to cause some adverse effects. To compensate for blood withdrawn, fluid replacement was performed using heparin saline solution, as is common practice in such studies. Theoretically, Culex unit can be programmed to take samples as low as 5 μL, so this method of sampling can be improved to have less impact on the animal in future. However, the success of such approach then rests heavily on the development of appropriate technology that can handle such small sample volumes with high accuracy and precision as coventional sample preparation workflows are not suitable. The unbound drug concentration in blood, in addition to the total concentration, can also be determined by SPME (whereas blood withdrawal typically determines total blood or plasma concentrations after disruption of protein binding). Since extraction by SPME is proportional to the unbound concentration in the sample, the unbound concentration can be determined from calibration standards prepared in plasma water or buffer while the total concentration is determined from calibration standards prepared in blood. As well, the difficulties in handling the small blood and plasma volumes associated with serial blood withdrawal are avoided, as sampling and sample clean-up are essentially combined into one step. *In vivo* SPME also does not require the use of automated blood samplers, reducing costs and increasing the number of animals that can be sampled per day.

The mean CBZ plasma concentration *versus* time profile obtained following a terminal blood sampling experiment with 3 mice/time point is also depicted in Fig. 5A and the PK parameters estimated from the mean data are summarized in Table 2 (CBZEP was not measured in the latter experiment). As mentioned in Section 3.4, the blood to plasma concentration ratio for CBZ is unity and, therefore, plasma concentrations could be compared directly to blood concentrations. Mean plasma concentrations of CBZ obtained following terminal blood sampling decreased rapidly over time and were similar in magnitude to blood concentrations determined by SPME and serial blood collection experiments. The PK parameters estimated from the terminal collection experiment are also in good agreement with those estimated by SPME and serial blood collection. The major difference in these PK parameters is that, unlike serial sampling methods, terminal blood sampling does not allow one to assess the inter-animal variability in the PK parameters (as they are estimated from the mean concentration *versus* time profile).

When comparing the differences in results obtained between serial and discrete terminal sampling in mice, Peng et al. reported better precision of drug concentrations estimated from serial sampling (<35% RSD, range 12–33%) than those from discrete sampling (<50% RSD, range 16–50%) [13]. These results are similar to those obtained in the current study with the serial sampling (mean 29% RSD, range 11–47%) and discrete terminal sampling (mean 50% RSD, range 4–173%). The overall precision at each time point in CBZ blood concentrations determined by SPME ranged from 39–109% RSD with mean RSD of 60% which makes it comparable to the results obtained by discrete terminal sampling approaches. The overall variability reported for SPME differs from that of traditional analyses as it includes inter-probe variability which was estimated using *in vitro* experiments to be 5–10%, the experimental error inherent in the determination of the amount of on-probe calibrant and inter-animal variability in the extent of plasma protein binding, since only the unbound drug is extracted by SPME. In humans, the variability of unbound fractions was reported as 0.15–0.3 for CBZ and from 0.33 to 0.67 for CBZEP following oral CBZ administration, and no significant relationship was established between unbound and total CBZ concentrations indicating that degree of binding can vary significantly *in vivo* [31]. The variability in plasma protein binding which can result in significant differences in the unbound fraction, does not affect total blood or plasma concentration data obtained by conventional methods which include the disruption of drug–protein binding prior to analysis using organic solvent as employed in current study. However, this variability in bound fraction does affect the total concentration determined by SPME unless the blood of each individual animal is used for calibration (within-animal calibration) which is not feasible for mice due to limited blood volume. Therefore, this is one of the major contributing factors to higher RSD values observed for *in vivo* SPME versus traditional methods. In contrast, free (unbound) concentrations determined by SPME are very accurate and provide important information about the biological availability of a given drug and can also be used to estimate the inter-animal variability of drug–protein binding *in vivo*.

4. Conclusions

In vivo SPME sampling was applied for the first time to PK studies in mice using a miniaturized sampling interface, although the approach presented here can be easily extended to other types of studies such as metabolomics. The developed sampling methodology for mice was validated against both traditional serial and terminal sampling approaches. The use of *in vivo* SPME in small rodents such as mice permits the construction of an entire blood concentration *versus* time profile without a change in blood volume or composition. It combines sampling and sample preparation into one step thereby increasing the throughput of sample analysis. The methodology allows the determination of both total and unbound drug concentrations, thus presenting an important advantage over traditional analyses which determine only total drug concentrations, especially for highly protein bound drugs. The results from the present study indicate that *in vivo* SPME holds promise as a valuable method for blood-draw free sampling from rodents. SPME may be particularly useful for performing PK studies in 'precious' animals, such as transgenic mice and murine disease models, as well as being useful in multiple crossover studies. In order to expand the usefulness of the methodology for early drug discovery and drug candidate screening *in vivo*, other pre-equilibrium calibration methods which do not require the use of any calibrant, are currently being evaluated. In addition, automation of the pull/push sampling cycle is currently under development to allow the sampling to be performed without handling the animal and without restricting animal movement. Most importantly, this new technology could play an important role in the fields of metabolomics, drug metabolism and toxicology by capturing unstable and/or short-lived metabolites that may be degraded/converted during procedures based on blood withdrawal.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.07.060.

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