CARBAMAZEPINE PHARMACOKINETICS IN MICE USING *IN VIVO* SOLID-PHASE MICROEXTRACTION SAMPLING

University of Waterloo

Dajana Vuckovic[(1)], Inés de Lannoy[(2)], Brad Gien[(2)], Victor Saldivia[(2)], Florin Marcel Musteata[(3)], Janusz Pawliszyn[(1)]

(1) Department of Chemistry, University of Waterloo, Waterloo, Canada, dvuckovi@sciborg.uwaterloo.ca, janusz@uwaterloo.ca
(2) NoAb BioDiscoveries, Mississauga, Canada
(3) School of Pharmacy, Memorial University of Newfoundland, St. John's, Canada

ABSTRACT

The use of solid-phase microextraction (SPME) for *in vivo* sampling of drugs and metabolites in the bloodstream of freely moving animals such as dogs, pigs, and rats eliminates the need for blood withdrawal in order to generate pharmacokinetic profiles in support of pharmaceutical drug discovery studies (1-4). The fact that *in vivo* SPME does not require removal of blood samples is particularly important for small rodents such as mice because it enables the use of a single animal to construct the entire pharmacokinetic profile. In contrast, when rodents are used in conventional pharmacokinetic studies, one animal per each time point is typically needed due to the availability of limited blood volume from a single animal. This necessitates the use of a large number of animals in order to generate profiles with a sufficient number of data points. The current efforts to further develop, miniaturize and commercialize *in vivo* SPME probes, in order to enable their use in small rodents for the first time, are presented. A new polyurethane sampling interface was also designed to facilitate repeated sampling from the same animal. The performance of commercial prototype *in vivo* SPME assemblies produced by Supelco was evaluated as part of this study. The extractive coating (45 µm thickness) of these assemblies consists of a mixture of biocompatible binding agent with 5 µm porous silica particles coated with C18. *In vivo* SPME sampling and liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) was then used to determine free and total circulating concentrations of carbamazepine in mice and to construct a pharmacokinetic profile. Standard-in-fibre calibration method was used for quantitative analysis. The obtained results compare well to the traditional methods relying on blood withdrawal. In addition, *in vivo* SPME offers the advantages of speed, decreased animal use, improved accuracy of data due to the elimination of inter-animal variation from the profile, and the ability to obtain both free and total drug concentration from the same experiment.

1.0 INTRODUCTION

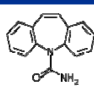


Figure 1 Structure of carbamazepine (CBZ)

2.0 EXPERIMENTAL

2.1 IN VIVO SPME SAMPLING PROCEDURE

In vivo SPME experiments were conducted using conscious male CD-1 mice (20-25 g) implanted with a custom made sampling interface connected by a stainless steel connector to a catheter implanted in the common carotid artery. The catheter is implanted surgically the day before the experiment begins. The interface is connected to the catheter on the day of the experiment. Mice were administered 4 mg/kg carbamazepine by intravenous injection into the tail vein. The dosing solution consisted of ethanol, propylene glycol, and saline (1:1:3, v/v/v). In vivo SPME sampling was performed prior to dosing and at 5, 15, 30, 60, 90, 120, and 240 min post-dose. During each sampling, a new in vivo SPME probe was inserted into the sampling interface 1.0 minute before the stated time and held in the interface until 2.0 minute sampling time elapsed. To prevent coagulation within the interface and increase the amount extracted by SPME, additional agitation was applied within the interface using manual push-pull action using a syringe. Within each 2 minute sampling period about 10 push/pull cycles were completed. After sampling was completed, the SPME probe was removed from interface and rinsed for 10 seconds using purified water. The probes were stored in freezer or dry ice until analysis.

2.2 PROBE DESORPTION

The probes were desorbed using 100 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 0.15mL polypropylene HPLC inserts (Supelco, Bellefonte, USA) for 5 min using vortex agitation of 2400 rpm. These extracts were then analysed directly using LC-MS/MS.

2.3 IN VIVO SPME CALIBRATION PROCEDURES

Kinetic (standard-in-fibre) calibration method was used for in vivo pharmacokinetic and in vitro validation experiments. All in vivo probes were pre-loaded with carbamazepine D10 internal standard using 500 ng/mL standard solution prepared in purified water for 30 minutes (static conditions). Standards and validation samples were prepared by spiking an appropriate amount of each drug standard ranging from 1 ng/mL to 1000 ng/mL in mouse whole blood or phosphate-buffered saline buffer (PBS, pH 7.4) in such a way as to keep the organic solvent concentration at exactly 1% methanol. Calibration standards were then subjected to equilibrium SPME procedure in parallel (120 min extraction, 2400 rpm vortex agitation) using individual in vivo SPME probes for each standard. Fibres were rinsed for 30 seconds using purified water to eliminate any droplets from the surface of the fibres and desorbed as described in section 3.2. The calibration was performed using 1/x2 weighted linear regression in SigmaPlot 2004 for Windows (version 9.0) software. Validation samples were extracted from HPLC vials containing 0.5mL of each spiked whole blood sample by placing the interface directly into the vial and performing SPME extraction exactly as described for in vivo samples in section 3.1. Fibres were rinsed for 30 seconds using purified water to eliminate any droplets from the surface of the fibres and desorbed as described in section 3.2.

2.4 LC-MS/MS ANALYSIS

All LC-MS/MS analyses were performed using a system consisting of CTC-HTS PAL autosampler with cooled sample tray, Shimadzu 10AVP LC with dual pumps (Shimadzu LC10ADvp) and system controller (SCL10Avp) and Applied Biosystems API3000 tandem mass spectrometer equipped with TurboIonSpray source. Analyst software (version 1.4.1) was used for data acquisition and processing. The column used for the separation of the analytes was Symmetry Shield RP18 with dimensions of 2.1 x 50 mm and 5 µm particles (Waters, Millford, MA, United States). Samples (20 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 2.0 min, hold at 90%B for 1.0 min and re-equilibrate the column to initial conditions for 1.5 min. A bypass pump and a Waters switching valve were used to divert the flow of column effluent for the first 1.0 min of run time. MS conditions used were: nebulizer gas = 9, curtain gas = 7, CAD gas = 9, ionspray voltage 4500 V, and source temperature set to 300 °C. All of the compounds were analysed in positive ion MRM mode using instrument settings described in Table 1.

Table 1 Summary of MS Parameters

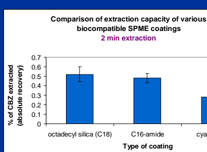
Analyte	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
Carbamazepine	236.9	194.2	20	80	10	35	18
Carbamazepine D10	247.3	204.2	17	70	9.8	34	14
Diazepam	285.0	153.9	92	120	7.5	39	10

4.0 RESULTS AND DISCUSSION

4.1 SPME method development and evaluation of prototype in vivo SPME probes from Supelco

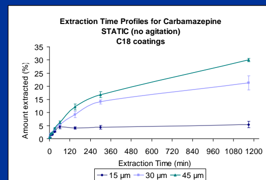
4.1.1 SELECTION OF SPME COATING

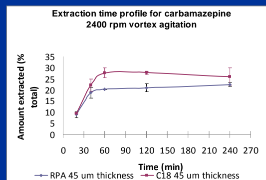
- The extraction capacity of three different types of biocompatible SPME coatings (coating thickness = 45 µm) was evaluated for carbamazepine (CBZ).
- Extraction was performed from 100 ng/mL CBZ standard solution dissolved in PBS buffer for 2 min without agitation in order to mimic in vivo sampling conditions.
- C18 and C16-amide coatings were found to have similar extraction capacity as shown in Figure 2, while more polar cyano-propyl coating had significantly lower extraction capacity.



4.1.2 EXTRACTION TIME PROFILES

- Extraction time profiles for CBZ using C18 coatings of varying thickness (n=3 fibres) were constructed in order to determine the time required to reach equilibrium. As shown in Figure 3, using static conditions the equilibrium was reached in 60 minutes (15 µm coating) and 300 minutes (30 µm coating). These times are too long to perform equilibrium in in vivo SPME sampling thus necessitating the use of kinetic calibration method. For in vitro testing, agitation can be used in order to significantly decrease the equilibration times for these fibres as shown in Figure 4. Using 2400 rpm vortex agitation equilibrium was reached in 60 minutes for C18 and C16-amide (45 µm) coatings (n=3 fibres).

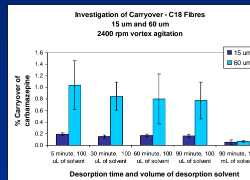




3.0 EXPERIMENTAL/RESULTS/DISCUSSION – GENERIC SPME COATINGS

EVALUATION OF CARRYOVER

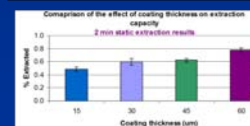
Figure 5 shows the results of carryover test for 15 µm and 60 µm coatings. Minimum amount of solvent required for desorption is 100 µL in order to achieve complete immersion of the entire length of coating with desorption solution. As shown in Figure 5, increasing desorption time from 5 to 90 minutes did not result in further reduction of carryover. Increasing desorption solvent volume to 1.5 mL resulted in carryover of 0.1% for both coatings. To achieve adequate sensitivity during pharmacokinetic studies, sample dilution is undesirable. Therefore, 100 µL desorption solvent and 5 min desorption time were chosen as optimum desorption conditions for current study.



EVALUATION OF INTER-FIBRE REPRODUCIBILITY

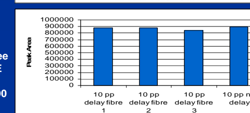
The inter-fibre reproducibility of RPA coatings of varying thickness was evaluated using both 2 min and 60 min extraction times (static agitation). The results for 2 min extraction are shown in Figure 6.

The inter-fibre reproducibility of 45 µm RPA coating was better than of 15 and 30 µm coatings, so they were selected for all subsequent experiments.



EVALUATION OF SAMPLING SPEED

The ability of SPME to respond to rapidly changing concentrations in vivo was evaluated. In figure 6, the first three bars represent three individual samplings using in vivo SPME where probes I switched during sampling from solution containing no analyte (sampled for 30 seconds) followed by 90 seconds exposure to 50 ppb CBZ standard.



2. In vitro method validation using mouse whole blood

In order to assess accuracy and precision, in vitro mini-validation was performed using mouse whole blood at three concentrations (5 replicates at each concentration). In vitro validation samples were extracted for 2 minutes using the same in vivo SPME sampling procedure used for the mice. The total concentrations of CBZ in these samples were then calculated using CBZ calibration curve in whole blood. Accuracy (% relative recovery) was calculated as the ratio of experimentally determined amount over true spiked amount x100%. Very good accuracy was found at 5 ng/mL (96.8% mean accuracy) and slightly lower accuracy at 50-1000 ng/mL levels (~70% mean accuracy). Precision was found to be good as shown by 17-23% RSD at all levels. The dilution of 1000 ng/mL samples resulted in better accuracy (~90%, 2 samples re-analyzed) potentially indicating that matrix suppression of the signal by co-extracted compounds may have played a role.

	Linear range	# of stds	Slope	Std. Error - slope	Y-intercept	Std. Error - Y-intercept	R^2
Whole blood	1-2000 ng/mL	10	0.027	0.0012	-0.003	0.004	0.992
PBS buffer	1-100 ng/mL	5	0.464	0.031	0.147	0.078	0.995

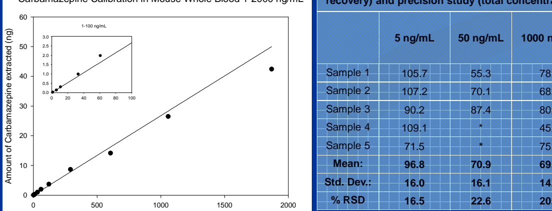
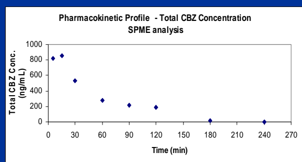


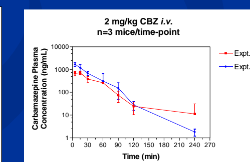
Table 5 Results of in vitro accuracy (% relative recovery) and precision study (total concentration)

	5 ng/mL	50 ng/mL	1000 ng/mL
Sample 1	105.7	55.3	78.2
Sample 2	107.2	70.1	68.9
Sample 3	90.2	87.4	80.7
Sample 4	109.1	*	45.3
Sample 5	71.5	*	75.6
Mean:	96.8	70.9	69.8
Std. Dev.:	16.0	16.1	14.4
% RSD	16.5	22.6	20.6

3. Application of optimized method to the study of carbamazepine pharmacokinetics in mice

Pharmacokinetic profile for a single mouse was obtained using in vivo SPME after intravenous dosing at 4 mg/kg. The results obtained are shown in Figure 9. The results for traditional plasma analysis are shown in Figure 10. SPME results follow the same general profile as obtained for traditional analysis, but the concentrations appear to be somewhat under-estimated considering dosing for SPME study was performed at 4 mg/kg rather than 2 mg/kg. In vitro validation results indicate good performance of the developed SPME method. In vivo results may have somewhat poorer accuracy due to the unavailability of whole blood from the same mouse to perform SPME calibration. Instead, SPME calibration was performed using pooled blood obtained from several mice. The exact amount of binding proteins varies from individual-to-individual, and may have caused a small systematic error in in vivo results. In fact, Dewe et al. report up to 20% bias in pharmacokinetic profiles when calibration was performed using pooled lot of blood. The found that with-animal calibration strategy improved both precision and accuracy by up to 20%. However, such an approach is not feasible with mice due to limited blood volume. Furthermore, it is possible that the results obtained represent true animal variation as a study performed by Watanabe relying on serial miniscule bleeding in mice found inter-animal variation as high as 200% at any given time point.





5.0 SUMMARY AND CONCLUSIONS

In vivo SPME was applied for the first time for pharmacokinetic sampling of mice. The use of in vivo SPME in such studies permits the construction of an entire pharmacokinetic profile from a single mouse, and combines sampling and sample preparation in one-step thus increasing sample throughput. A single rodent is sufficient to obtain the entire pharmacokinetic profile, thus reducing animal use by up to 10-15 times. The data generated using in vivo SPME is improved since inter-animal variation is eliminated from the profile. In future with additional miniaturization of SPME probes, this methodology can be extended to tissue and cellular sampling or sampling of multiple sites on the same animal in order to evaluate compound distribution or bioaccumulation within the animal. Additional studies on multiple mice and further validation against traditional techniques are currently under way.

5.0 ACKNOWLEDGMENTS

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6.0 REFERENCES

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