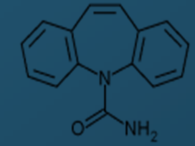


USE OF *IN VIVO* SOLID-PHASE MICROEXTRACTION FOR SINGLE RODENT PHARMACOKINETIC STUDIES



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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 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. The aim of the current research was to apply SPME for *in vivo* sampling of mice for the first time. This was achieved using new commercial *in vivo* SPME probes with biocompatible extractive coating (a mixture of biocompatible binding agent with 5 µm porous octadecyl silica particles) and a new polyurethane sampling interface designed to facilitate repeated sampling from the same animal and insertion of the SPME probes. Pre-equilibrium *in vivo* SPME sampling and liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) were used to determine free and total circulating concentrations of carbamazepine in mice after 2 mg/kg intravenous dosing. Standard-in-fibre calibration method was used for quantitative analysis. The method was linear in the range of 1-2000 ng/mL in whole blood with acceptable accuracy (>70%) and precision (<23% RSD). The pharmacokinetic results compare well to the traditional methods relying on blood withdrawal but *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.

INTRODUCTION

$$n_e = \frac{C_0 K_f V_s V_f}{K_f V_f + V_s} \quad V_s \gg V_f K_f \Rightarrow n_e = C_0 K_f V_s$$

Standard-on-fibre (kinetic) calibration method uses the desorption of preloaded standard from extraction phase to calibrate the process of extraction of analyte into the extraction phase [5].

$$\frac{n}{n_e} + \frac{q}{q_0} = 1$$

n – amount of analyte extracted by SPME at time t
 n_e – amount of analyte extracted by SPME at equilibrium
 q – amount of standard remaining in the extraction phase
 q_0 – amount of standard preloaded on the extraction phase

RESEARCH OBJECTIVES

- To apply *in vivo* SPME sampling to mice for the first time
- To acquire all points of pharmacokinetic profile using a single animal
- To compare pharmacokinetic profiles of carbamazepine (CBZ) in mice acquired using *in vivo* SPME and compare to profiles obtained using traditional method based on blood withdrawal

EXPERIMENTAL

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 was implanted surgically the day before the experiment began. The interface was connected to the catheter on the day of the experiment. Mice were administered 2 mg/kg CBZ 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, 180 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 by manual push-pull action using a syringe. After each 2 minute sampling period about 10 push/pull cycles were completed. The probes were stored in freezer or dry ice until analysis.

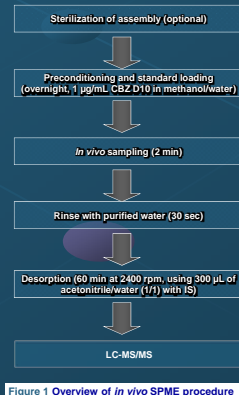


Figure 2 Commercial *in vivo* SPME probe from Supelco Inc.

Figure 1 Overview of *in vivo* SPME procedure

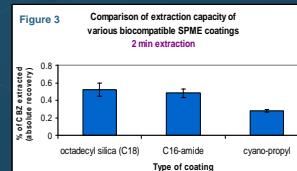
EXPERIMENTAL-CONTINUED

Table 1 LC-MS Parameters								
Instrument:	CTC-HTS PAL autosampler, Shimadzu 10AVP LC, API 3000 (triple quadrupole), Analyst software version 1.4.1							
Ionization:	Turbospray, Positive ESI, nebulizer gas = 9, curtain gas = 7, CAD gas = 9, ionspray voltage 4500 V, and source temperature set to 300 °C							
SRM Transitions:	Analyte	Q1 Mass (amu)	Q3 Mass (amu)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
	CBZ	236.9	194.2	20	80	10	35	18
	CBZ D10	247.3	204.2	17	70	9.8	34	14
	Diazepam (IS)	285.0	153.9	92	120	7.5	39	10
Analysis time:	5.0 min							
Flow rate:	500 µL/min							
Column:	Waters Symmetry Shield 5 µm RP18, 50x2.1 mm							
Mobile phase:	A: Acetonitrile/water/acetic acid (10/90/0.1) B: Acetonitrile/water/acetic acid (90/10/0.1) Gradient: 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							

RESULTS: OVERVIEW OF *IN VIVO* SPME METHOD DEVELOPMENT

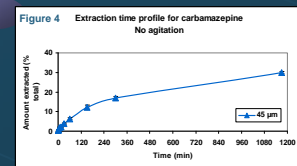
SELECTION OF SPME COATING

The extraction capacity of three different types of biocompatible SPME coatings (coating thickness = 45 µm) was evaluated using 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 3, and C18 coating was selected for all subsequent experiments.



SPME EXTRACTION TIME AND SELECTION OF CALIBRATION PROCEDURE

Extraction time profiles for CBZ using C18 fibres of 45 µm thickness (n=3) were constructed in order to determine the time required to reach equilibrium. As shown in Figure 4, using static conditions the equilibrium was reached in 1200 min which is too long to perform equilibrium *in vivo* SPME sampling thus necessitating the use of kinetic calibration method. Using 2400 rpm vortex agitation equilibrium was reached in 60 minutes.



Kinetic calibration requires preloading of appropriate standard into extraction phase, prior to performing *in vivo* sampling. When preloading step was performed using CBZ D10 standard prepared in purified water, extraction efficiency of C18 coating was reduced drastically as shown in Figure 5. To avoid this problem, coating preconditioning and standard preloading were combined in one step by performing the preloading from a high concentration CBZ D10 standard solution with high organic content (methanol/water, 1/1). Table 2 shows that sufficient amount of CBZ D10 can be loaded using 0.25 mL of 1 µg/mL CBZ D10 standard. The precision of selected loading procedure (n=5 fibres) was subsequently found to be 7% RSD.

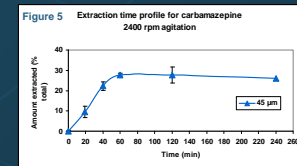
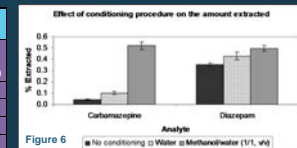
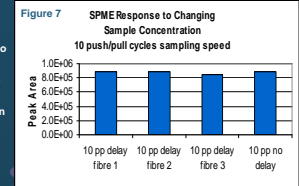


Table 2 Optimization of standard preloading procedure			
Concentration (ng mL ⁻¹)	Volume used for loading (mL)	Amount loaded - CBZ D10 (ng)	% loaded - CBZ D10
100	0.25	0.17	0.67
100	1.7	0.17	0.10
1000	0.25	1.68	0.67
1000	1.7	1.65	0.10



RESULTS: METHOD VALIDATION

The ability of SPME to respond to rapidly changing concentrations *in vivo* was evaluated. In Figure 7, three individual samplings using *in vivo* SPME where probes were switched during sampling from solution containing no analyte (sampled for 30 seconds) followed by 90 seconds exposure to 50 ppb CBZ standard were compared to sampling where the probe was exposed to 50 ppb CBZ standard for 120 seconds. No difference in the amount extracted was found as long as sufficient assisted agitation was applied (10 push/pull cycles).



In order to assess accuracy and precision, *in vitro* mini-validation was performed using pooled mouse whole blood at three concentrations (n=5). *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 kinetic calibration. Accuracy (% relative recovery) was calculated as the ratio of experimentally determined amount over true spiked amount x100% (Table 3). Excellent 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.

Table 3 *In vitro* assessment of accuracy and precision of kinetic calibration method

CBZ Conc.	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

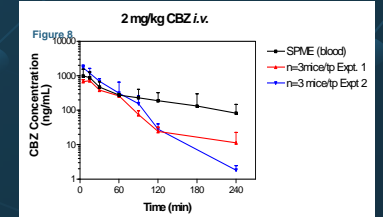
Linearity of SPME-LC-MS/MS method was established in whole blood in 1-2000 ng/mL concentration range (Table 4). Excellent linearity was obtained even though each point of the calibration curve was obtained using individual single-use fibres.

Table 4 <i>In vitro</i> assessment of linearity of SPME-LC-MS/MS method							
	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	0.6-300 ng/mL	8	0.306	0.012	0.267	0.195	0.992

RESULTS: PHARMACOKINETIC STUDY

Pharmacokinetic profile for a single mouse (n=3) was obtained using *in vivo* SPME after intravenous dosing at 2 mg/kg. The results are shown in Figure 8 and Table 5. The results for traditional plasma analysis (n=3 micetime point) are also shown in Figure 8. SPME results show good agreement with traditional analysis for early time points (up to 90 min), but show slower elimination of CBZ than predicted by traditional analysis. Further *in vivo* SPME studies are currently underway to further investigate this observation. It is possible that the results obtained represent true animal variation as a recent study relying on serial miniscule bleeding in mice found inter-animal variation as high as 200% at any given time point [6].

Table 5 C _{Free} CBZ in ng/mL (n=3 mice)			
t	AVG	% RSD	
5	58	57	
15	51	45	
30	27	42	
60	16	10	
90	13	76	
120	11	71	
180	7.7	127	
240	4.8	79	



CONCLUSIONS

- In vivo* SPME sampling was successfully applied to mice for the first time and entire pharmacokinetic profile was collected using a single animal.
- In vivo* SPME is a potentially useful tool to reduce animal use and to study inter-animal variation.

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