In Vivo Calibration Methods of SPME and Application to Pharmacokinetic Studies

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Solid phase microextraction (SPME) has gained much popularity for *in vivo* applications recently. Thus far, there are two types of pre-equilibrium kinetic calibration that have been applied to *in vivo* SPME: on-fibre standardization and dominant pre-equilibrium desorption. Both of these techniques have their own advantages and disadvantages. To address the limitations presented by these two techniques, a third pre-equilibrium kinetic calibration method, the diffusion-based interface model, was investigated. The diffusion-based interface model had been successfully applied to air and water samples but was never utilized for *in vivo* SPME studies. For the first part of the research, on-fibre standardization, dominant pre-equilibrium desorption, and diffusion-based interface model were compared in terms of accuracy, precision, and experimental procedures, by using a flow-through system. These three kinetic calibrations were further validated by equilibrium SPME extraction and protein-plasma precipitation, a current state-of-the-art sampling method.

The potential of diffusion-based interface model was yet again demonstrated in the second part of the research project. This calibration method was applied to comparative pharmacokinetic studies of two drugs, fenoterol and methoxyfenoterol, on 5 rats. To provide a constant sampling rate as required for diffusion-based interface model, a SPME animal sampling autosampler, AccuSampler®, was utilized. It custom-written program allowed the entire SPME sampling procedure excluding insertion and removal of SPME probes to be automated. Furthermore, to validate the results obtained by SPME, the AccuSampler® was programmed to withdraw blood after each SPME sampling time point for conventional method analysis using protein-plasma precipitation. The well correlated data obtained by SPME sampling and the

conventional method illustrated the potential of diffusion-based interface model as an excellent choice for future *in vivo* SPME applications.

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DEDICATION

To my parents, John Yeung and Catherine Kwan, for their loving caring, understanding, and encouragement

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACN	Acetonitrile
β_2-AR	β_2-adrenoceptor
C18	Octadecyl-silica
CAD	Collision activated dissociation
CCAC	Canadian council on animal care
CN	Cyano
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IV	Intravenous
LC	Liquid chromatography
LOQ	Limits of quantification
m/z	Mass to charge ratio
MeOH	Methanol
MR	Matrix reservoir
MS	Mass spectrometry
PBS	Phosphate buffered saline
PP	Peristaltic pump
RBC	Red blood cell
RPA	Reversed phase amide
RSD	Relative standard deviation
SP	Syringe pump
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected Reaction Monitoring

Chapter 1: Introduction

In vivo studies have always had importance in biological and chemical processes for drug development. Since *in vivo* studies are conducted within a living organism, results obtained for therapeutic and toxic effects of a drug are more relevant in comparison with *in vitro* studies. There are many factors to consider when selecting an *in vivo* sampling technique. These include sensitivity, accuracy, and precision of the overall method.

Solid phase microextraction (SPME), which was invented by Dr. Janusz Pawliszyn in the late 1980s, has recently gained much popularity for *in vivo* studies due to its advantage of integrating the sampling and sample preparation procedures. Owing to the minimal number of experimental steps required that prevent significant error such as sample loss and operation error, high sensitivity, accuracy, and precision are achievable using SPME.

1.1 Principles of Solid Phase Microextraction (SPME)

There are three main experimental steps for analysis with SPME: extraction, desorption, and instrumental analysis [1]. During extraction, analyte in the sample partitions into the extraction phase of the SPME fibre, and further partitions into the desorption solvent during desorption. Subsequently, the desorption solvent is injected into the analytical instrument for separation and quantitation [1].

At the initial stage of extraction, the amount extracted increases linearly with time. However, later on, extraction reaches equilibrium. In other words, equilibrium is reached between the amount extracted on the fibre and what is left in the sample matrix.

The time at which equilibrium occurs is termed the equilibration time. In biological studies with SPME, sampling often occurs with matrices such as plasma or whole blood, which contain biomolecules such as red blood cells and proteins [1]. At the equilibration time, the free concentration of the analyte and the concentration of the bound analyte on the biomolecules are also at equilibrium with each other (Figure 1). In other words, the amount extracted is at its maximum during equilibrium and a longer extraction time has no effect on this amount [2].

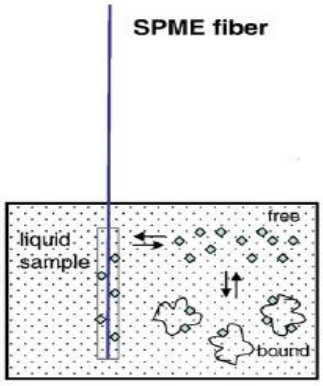

Figure 1: Schematic representation of direct fibre extraction [1]. Equilibrium of the free concentration of the analyte occurs between the extraction phase on the fiber and in the sample matrix as well as between free concentration of the analyte in the sample matrix and the bound analyte on the proteins.

The amount extracted by SPME at equilibrium can be calculated as follows:

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

where n_e is the amount extracted, K_{fs} is the distribution constant of the extraction phase and sample, V_f is the volume of the extraction phase, V_s is the sample volume, and C_0 is the initial concentration of the target analyte [1]. However, when the volume of the sample matrix is very large, so that $K_{fs} \cdot V_f << V_s$, the amount extracted n can be calculated as follows:

$$n_e = C_0 K_{fs} V_f \tag{2}$$

The simplification from equation 1 to equation 2 illustrates the advantage of SPME for on site or *in vivo* analysis; a defined volume is unnecessary for direct exposure of the probe to the sample matrix [1].

1.2 *In Vivo* SPME

Traditional *in vivo* studies on small animals may require sacrificing animals due to the removal of blood samples. For such *in vivo* analysis, SPME offers two main advantages. First, since blood withdrawal is not necessary and the circulating blood volume does not change, the number of animals used is minimized. Second, only a minimal disturbance to the chemical balance of the system occurs since substance depletion is negligible as only a small amount of the free analyte is extracted [3]. Therefore, since only a small amount of analyte is extracted and the circulating blood volume is preserved, more data points can be obtained per animal and thus the inter-animal variances are decreased and accurate results are achieved.

Early *in vivo* studies with SPME measuring venous blood concentrations of a substance involved sampling in the vein directly where a catheter was placed in a

peripheral vein and sealed with a PRN adapter [4]. The SPME probe was loaded into a needle that was used to pierce the PRN adapter. Subsequently, the probe slid into the catheter, exposing the extraction phase, and the needle was withdrawn [4]. However, this technique was rather cumbersome. Blood exposure and blood leaking from the catheter during the insertion and removal of the probes were inevitable. To overcome these obstacles, a new type of SPME device was invented that prevents any leaking of blood and minimized exposure to blood (Figure 2) [5].

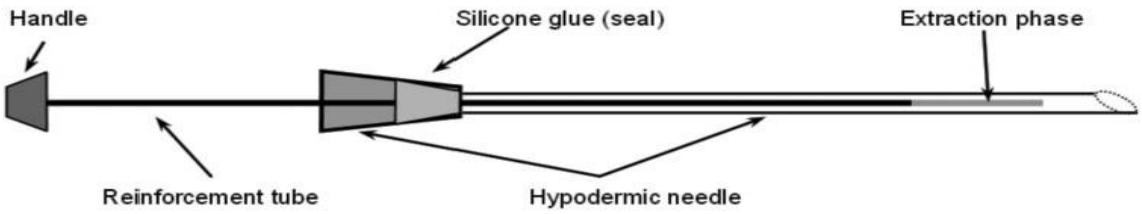

Figure 2: A special SPME device for *in vivo* extraction [5]

The SPME device consists of a thin flexible wire coated with an extraction phase housed in a hypodermic needle [5]. The assembly including the wire and needle is sealed with silicone glue. During extraction, the reinforcement tube is depressed to allow the exposure of the extraction phase into the flowing blood of the vein [5].

This new SPME device simplifies extraction procedures and sampling can be done much more efficiently. Its usefulness has been demonstrated in applications such as monitoring intravenous concentrations of drugs and metabolites in beagles and rats [6, 7], and field sampling of pharmaceuticals in fish [8].

1.2.1 Applications Of I*n Vivo* SPME

In the study of the pharmacokinetics of diazepam by Lord et al. [4], the venous concentration of diazepam and its metabolites, oxazepam and nordiazepam, were monitored in three beagles following intravenous administration of diazepam and were compared with the profiles obtained with SPME by conventional analysis for validation. The pharmacokinetic profiles of diazepam, oxazepam, and nordiazepam are shown in figure 3.

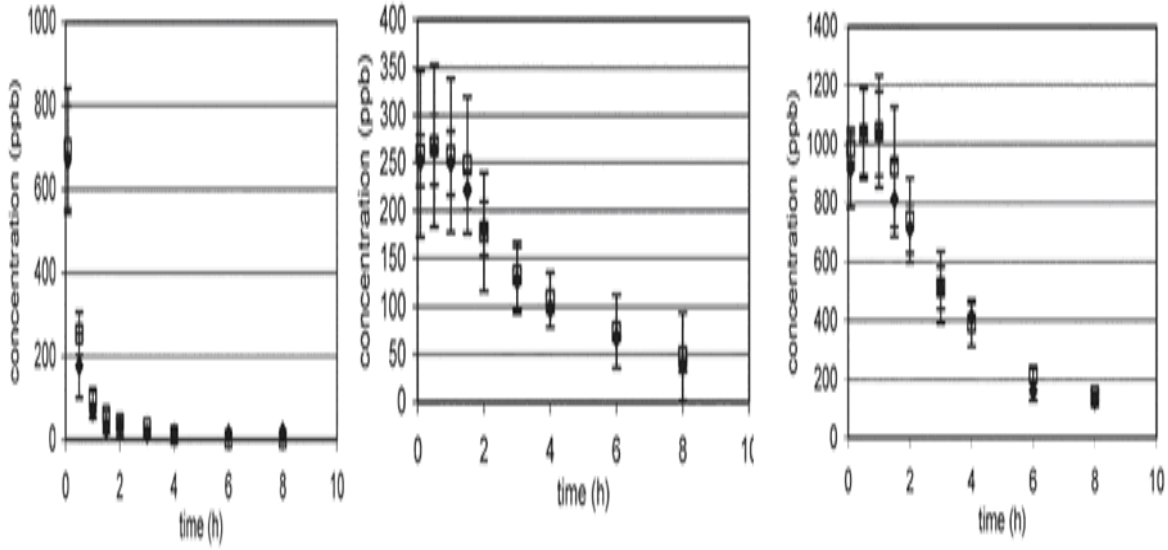

Figure 3: Pharmacokinetic profiles of A) diazepam B) oxazepam and C) nordiazepam from the average of three studies in three beagles after dosage with diazepam. The venous concentration was monitored over the course of 8 hours for the 3 drugs. (n=6 for the last point and n=9 for all the other points). Diamonds represent SPME probe and squares represent conventional method [4].

In the pharmacokinetic profile of diazepam, a rapid distribution phase followed by a less rapid decay in concentration occurred over the period of study. The rate of metabolism from diazepam to nordiazepam was fast since a high concentration of nordiazepam was

detected at early time points (Figure 3C) [4]. For oxazepam, the rate of formation and the rate of elimination was the same up to 1.5 hours of post dosage of diazepam.

The advantage of SPME is seen more prominent when pharmacokinetic studies are performed in smaller animals such as rodents (Figure 4) [7].

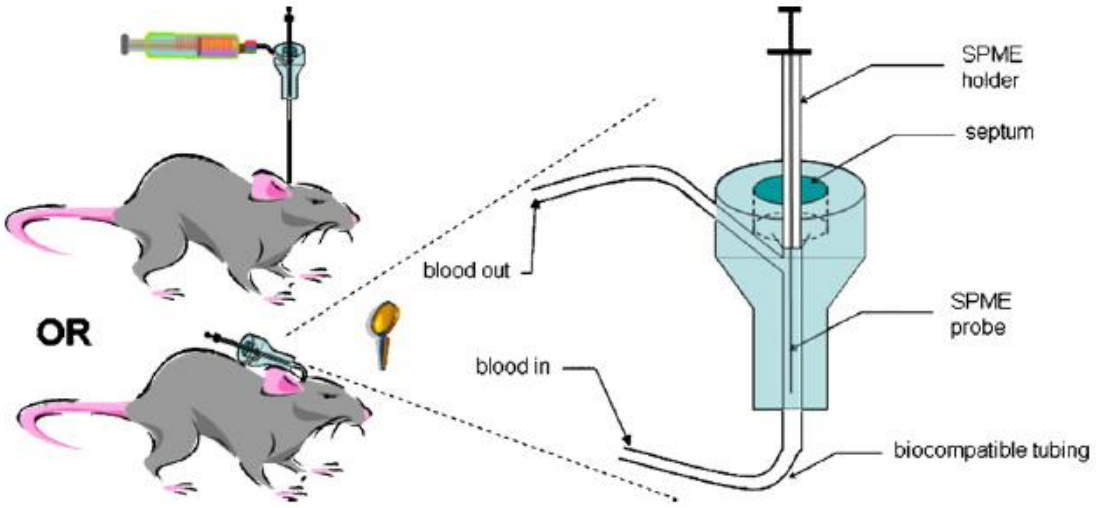

Figure 4: Schematic diagram of SPME sampling with rodents. The lower tube was connected to the carotid artery catheter and the upper tube was either recirculated to the carotid artery catheter or was connected to a syringe [7]

The limited blood volume in small animals will result in sacrificing animals when *in vivo* sampling requires withdrawal of large volumes of blood. A recent article published by Musteata et al. had demonstrated the success of pharmacokinetic studies of diazepam and its metabolites in rats using SPME [7]. The results were found to correlate well with the conventional method of blood draw, plasma separation, and detection.

The feasibility of *in vivo* SPME was demonstrated by Zhou et al. [8] in the field sampling of pharmaceuticals in fish muscles. In the study, wild fishes were collected from different river locations with variability in municipal wastewater effluent. The fishes were sampled by SPME and the amount extracted was compared linearly with the waterborne concentrations of pharmaceuticals understudy [8].

1.3 Calibration Procedures in SPME

Successful usage of SPME depends on the selection of calibration method. Equilibrium extraction remains an attractive option because during *in vivo* SPME testing in a conscious animal, the blood flow rate within the catheterized blood vessel, which is analogous to the speed of agitation, is uncontrollable. However, the amount of analyte extracted at equilibrium is independent of flow rate, so accurate and reliable quantitation can be achieved using equilibrium sampling [9].

Lord et al. performed the first *in vivo* SPME pharmacokinetic studies to measure the circulating concentration of diazepam and its metabolites in Beagle dogs using an extraction time of 30 minutes which was sufficient to establish equilibrium between the fibre and blood [4]. However, such a long sampling time limits temporal resolution because the determined concentration would be an average of the overall sampling time period and is feasible only when very thin coatings such as polypyrrole [4] are used. For some SPME fibres, however, equilibrium extraction is not practical, since equilibration takes too long [10]. In this case, the use of pre-equilibrium sampling strategy in combination with kinetic calibration is a better method of sampling [11-19].

1.3.1 On-fibre Standardization

Ai et al. has proposed a theoretical model based on a diffusion-controlled mass-transfer process to describe the kinetic process of SPME both in the linear and equilibrium regime [11-12]. Using this model, Chen et al. demonstrated the isotropy of extraction and desorption processes and therefore proposed that the extraction process could be calibrated using the process of desorption of standard loaded on the fibre [13]. The isotropic relationship between extraction and desorption exists when the addition of the rate of extraction and rate of desorption is close to or equal to 1 and therefore can be described using equation 3 (Figure 5).

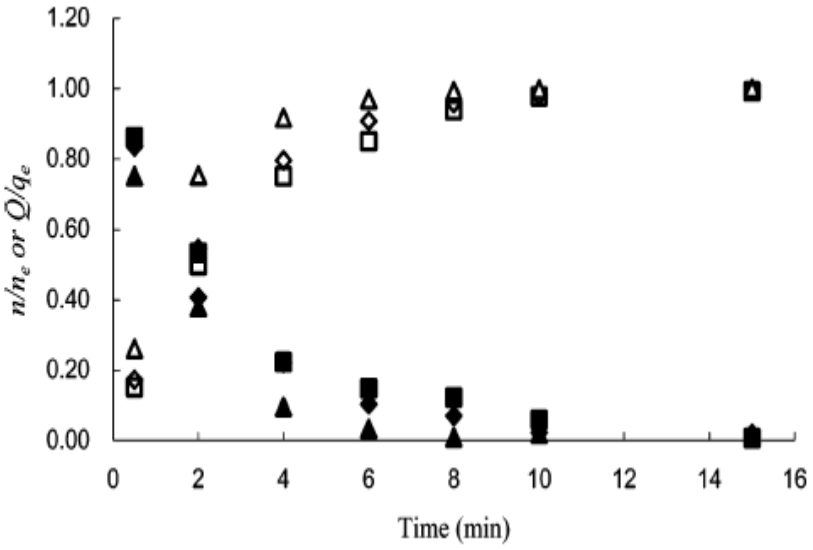

Figure 5: Absorption time profiles for diazepam (◊), nordiazepam (□), and oxazepam (Δ), and desorption time profiles for their deuterated analogues, diazepam-d_5 (♦), nordiazepam-d_5(■), and oxazepam-d_5(▲).

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

where n is the amount of analyte extracted at time t, n_e is the amount of analyte extracted at equilibrium or maximum extraction, q_0 is the amount of standard pre-loaded on the extraction phase, and Q is the amount of standard left on the extraction phase after sampling time t. Therefore, by sampling with a pre-loaded fibre, the amount desorbed into the sample matrix can calibrate the extracted analyte [13].

Rate of desorption of the standard can be described by the mathematical equation as follows:

$$\frac{Q}{q_0} = \exp(-at) \tag{4}$$

where a is a time constant that is dependent on the volume of fibre coating and sample matrix, mass transfer coefficients, distribution coefficients and the fibre's surface area [13]. Extraction of analytes onto the extraction phase can be described as follows [13]:

$$\frac{n}{n_e} = 1 - \exp(-at) \tag{5}$$

When isotropy exists between extraction and desorption, that is, when the two processes have the same time constant, equation 4 and 5 can be combined to equation 3 [14]. The calculated value for n_e can subsequently be used to calculate the free concentration of the sample matrix from equation 1 or 2 depending on sample size. Overall, on-fibre

standardization method for *in vivo* studies compensates for matrix effects and the effects of agitation or blood flow rate when sampling from an animal [7, 15].

1.3.2 Dominant Pre-equilibrium Desorption

On-fibre standardization has been successfully applied to *in vivo* studies [7, 15] and other areas such as environmental monitoring [16] and food [14]. However, for this standardization technique, a standard is required for pre-loading onto the fibre. These standards, usually radioactive compounds or deuterated analogues of the analyte, are relatively expensive and the availability is limited. Moreover, addition of standard into the sample matrix can possibly have a negative impact on the system under investigation. Therefore, a standard-free calibration method was introduced by Zhou et al [18]. Similar to on-fibre standardization, dominant pre-equilibrium desorption, uses the isotropic relationship between the amount extracted and amount desorbed for calibration.

For dominant pre-equilibrium desorption, it was found that the rate of desorption is constant when the pre-loaded amount is four-fold higher than the potential extracted amount from the sample matrix. When this requirement is fulfilled, desorption becomes dominant and extraction by the same fibre is negligible [18]. Another prominent factor to consider in this calibration method is the distance between the desorption fibre and the extraction fibre. Because the preloaded standard is the same as the extracted analyte, these fibres must be kept apart from each other in order to prevent them from affecting each other but not too far so that the matrices between desorption and extraction are different [18].

Pre-equilibrium desorption was applied to a jade plant (*Crassula ovata*) for pesticide sampling. Figure 6 illustrates the locations where the desorption and extraction

fibres were inserted. The desorption fibres were previously pre-loaded with the pesticides of interest [18].

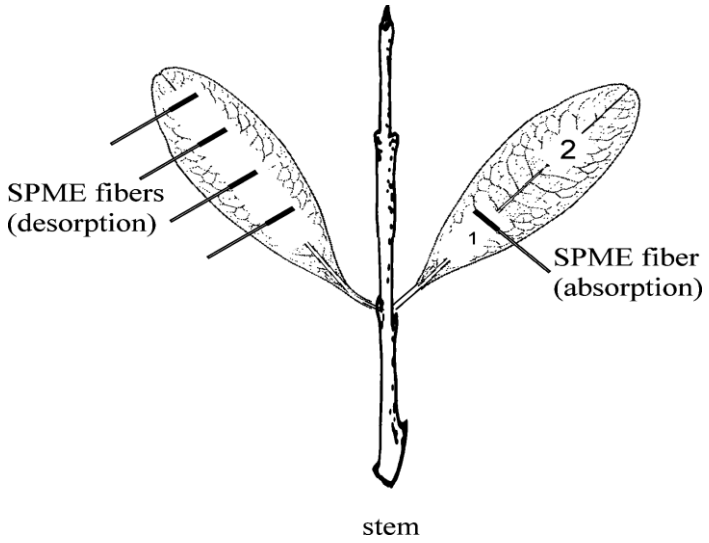

Figure 6: Application of pre-dominant desorption on jade plants. The locations for SPME absorption and desorption are illustrated [18]

In addition to jade plants, this technique has been applied to study pharmaceutical accumulation in wild fish using separate animals for desorption and extraction [8].

1.3.3 Diffusion-based interface Model

Although dominant pre-equilibrium desorption does not require other standards, this calibration method may be too costly because different fibres are needed for both extraction and desorption. In addition, a different animal may be used for calibration if the sampling area of the animal is too small to fit both an extraction and a desorption fibre as was the case when sampling muscle of wild fish where one fish was used for extraction and another for desorption [8].

To address the limitations of dominant pre-equilibrium desorption, another standard-free pre-equilibrium calibration method, the diffusion-based-interface calibration model was investigated for *in vivo* SPME studies [19-22]. Similar to dominant pre-equilibrium desorption, the diffusion-based-interface model uses the analyte itself to calibrate the amount extracted. However, unlike dominant pre-equilibrium desorption, this method requires fewer SPME fibres and the size of sampling area is not of great concern.

Diffusion-based interface model calibration is based primarily on the principle of analyte diffusion from the sample matrix to the fibre [19]. First, the analyte travels from the sample matrix to the surface of the fibre. Depending on the types of fibre used and thus the mode of extraction, the analyte will partition into the bulk of the polymer, in the case of absorption extraction, or adsorb onto the binding sites available on the surface of the coating, in the case of adsorption extraction. In the mass transfer process, the analyte must travel through the boundary layer, the layer between the bulk of the sample and the fibre surface (Figure 7), to the surface of the fibre [19]. When a fibre is submerged into the sample matrix for extraction, the fluid that is touching the surface of the fibre is stationary. As the distance from the fibre surface increases, the fluid movement increases as well, corresponding to the bulk flow in the sample [1].

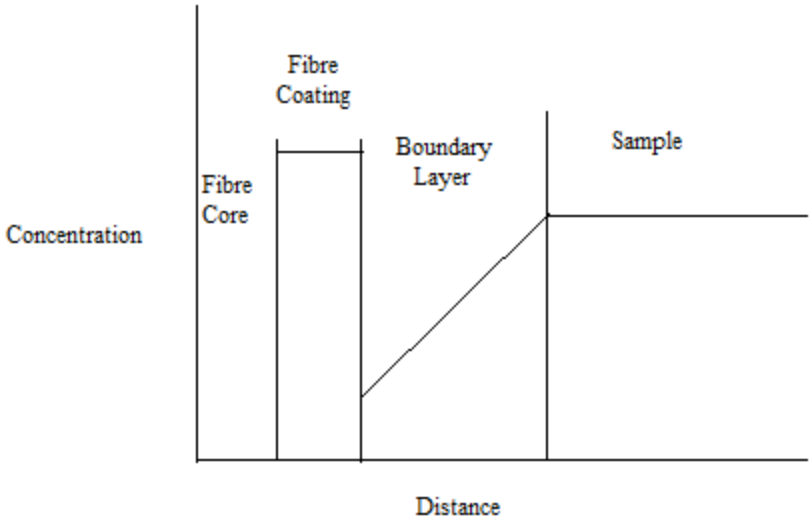

Figure 7: Diagram illustrating the concentration gradient in the boundary layer and sample

During pre-equilibrium extraction, the concentration gradient of the analyte across the boundary layer is high since the concentration of analyte in the sorbent ($C_{sorbent}$) is practically zero [19]. Therefore, diffusion of the analyte occurs from high concentration, the bulk sample, to low concentration, the sorbent. This allows the rate of extraction to be correlated linearly to the concentration of the analyte in the sample and calibration can be performed according to the extraction rate [19]. The concentration of the bulk sample can be determined as follows:

$$C = \frac{n \ln((b+\delta)/b)}{2\pi L t D_L} \tag{6}$$

where b is the outside radius of the fibre coating, δ is thickness of boundary layer, D_L is the diffusion coefficient of the analyte in the sample matrix, and L is the length of the fibre [19]. The thickness of boundary layer, δ, can be calculated as follows:

$$\delta = 9.52(b / \mathrm{Re}^{0.62}\, Sc^{0.38}) \tag{7}$$

where Re is the Reynolds number and Sc is the Schmidt number.

Using the amount extracted and the known concentration in the sample, the calibration constant can be determined by rearranging equation (6) and defining the calibration constant as follows:

$$\text{Calibration Constant} = \frac{\ln((b+\delta)/b)}{2\pi L t D_L}$$

Once the calibration constant is determined, it can be applied to samples with unknown concentration.

For diffusion-based interface model calibration, it is important to note that with a prolonged extraction time or extraction at equilibrium, $C_{sorbent}$ cannot be assumed as zero and therefore diffusion-based calibration cannot be applied. In addition, since extraction is based on the concentration gradient across the boundary layer, the size of the boundary layer must be kept constant during sampling for an accurate calibration. Since speed of agitation has a direct effect on the thickness of the boundary layer, a control of the sampling speed during extraction is required.

Diffusion-based interface calibration is convenient as it uses the analyte of interest for calibration. In some cases where the target analyte is well researched, the calibration constant can be calculated by knowing the radius and the length of the

coating, the extraction time, and diffusion coefficient for the analyte [20]. This calibration method, therefore, offers an attractive alternative to other pre-equilibrium calibration methods.

1.4 Research Proposal

The utility of *in vivo* SPME sampling has been demonstrated using animals of different sizes with different calibration methods. On-fibre standardization has been applied successfully on beagles and rats in studying the pharmacokinetics of diazepam and its metabolites. However, this method requires radioactive or deuterated standards which may be difficult to find or harmful to the investigated system. To overcome this problem, a novel standard-free calibration technique, dominant pre-equilibrium desorption, was introduced. Nevertheless, this method has several shortcomings; first, it requires more fibres than other calibration methods since different sets of fibre for extraction and pre-loading for desorption are needed. This not only increases the cost of the experiment, but also decreases the precision of the data due to fibre variability. Second, when *in vivo* studies are performed on animals, two different animals may be required if the sampling area is too small to fit both an extraction fibre and a pre-loaded fibre.

Due to these reasons, the diffusion-based interface model, another standard-free calibration method, was investigated. In this method, the bulk concentration is determined by the calibration constant, which can be determined experimentally or

theoretically. In this research, three calibration methods were compared, using a flow-through system, and validated by SPME equilibrium extraction and plasma-protein precipitation. All methods were compared in terms of accuracy, precision, and ease of operation. The best calibration method, the diffusion-based interface model, was selected and applied in pharmacokinetic studies of fenoterol and methoxyfenoterol in rats. AccuSampler®, an automated *in vivo* sampling system, was used to provide a constant sampling rate as required for the diffusion-based interface method. All SPME sampling procedures except for the insertion and removal of the SPME probe were automated with the custom-written program installed in AccuSampler®. All SPME data obtained were validated with plasma-protein precipitation.

Chapter 2: *In vivo* SPME calibration method comparison: on-fibre standardization, dominant pre-equilibrium desorption, and diffusion-based interface model on flow-through system

2.1 Overview

SPME has recently gained ground for *in vivo* studies relying on direct extraction of analytes of interest from a living system. However, success of applying SPME is greatly dependent on the selection of the calibration method. In this research, three *in vivo* SPME calibration methods, on-fibre standardization, dominant pre-equilibrium desorption, and diffusion-based interface model, were compared in terms of precision, accuracy, and ease of experimental procedures using a flow-through system. In addition, these calibration methods were further validated using SPME equilibrium extraction and a conventional method that involved protein precipitation. The comparison was performed using fenoterol as the analyte of interest and liquid chromatography (LC)-tandem mass spectrometry (MS) was used for the analysis. All three methods compared well with both the equilibrium extraction and the conventional method in terms of accuracy. In terms of precision, diffusion-based interface model had the best precision of 9-14% RSD in whole blood, and RSD of 9-15% in phosphate buffered saline (PBS). Dominant pre-equilibrium desorption had the poorest precision of 20-28% RSD in whole blood, and a RSD of 26-30% in PBS. The poorer precision observed for the dominant pre-equilibrium desorption method can be explained by the need to use more fibres in comparison to the other two calibration methods. In terms of ease of experimental procedures, the diffusion-based interface model was the simplest, as it did not require procedures such as fibre loading. This research suggests the potential use of diffusion-based interface model as the best calibration method for future *in vivo* SPME studies.

2.2 Materials and methods

2.2.1 Materials

(R,R)-Fenoterol and (R,R)-methoxyfenoterol were obtained from National Institute of Health (Baltimore, MD, US). Pseudoephedrine, ammonium acetate and silicone oil were purchased from Sigma-Aldrich (St. Louis, MO, US). High performance liquid chromatography (HPLC) grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ, US). Fresh rat whole blood (sterile, with sodium heparin as anticoagulant) and plasma were purchased from Lampire Biological Laboratories Inc. (Pipersville, PA, US). Rat whole blood was maintained at 4^0C for a maximum of 1 week and plasma was kept frozen at -20^0C until use. Deionized water was obtained from a Barnstead/Thermodyne Nano-pure ultrapure water system (Dubuque, IA, US).

2.2.2 LC-MS/MS Assay

For analysis of fenoterol and methoxyfenoterol, a Shimadzu (Kyoto, Japan) 10AVP LC consisting of a system controller and dual binary pumps, a CTC-PAL autosampler, and MDS Sciex API 3000 tandem MS were used. The MS system was operated in the electrospray ionization positive ion mode with selected reaction monitoring (SRM). Methoxyfenoterol was monitored using SRM transition of m/z 318.2→121.1, fenoterol was monitored at m/z 304.3→107.1, and pseudoephedrine, the internal standard, was monitored at m/z 165.8→148.2. The optimum settings for MS parameters were obtained using direct infusion of 1 μg/mL standard solution. The source temperature was set to 500^0C, ionspray voltage 4500 V, collision

activated dissociation (CAD) gas was 10, the nebulizer gas was 15, and the curtain gas was 10. The remaining parameters can be found in Table 1:

	Declustering Potential (V)	Focusing Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
Methoxyfenoterol	50	70	7	35	15
Fenoterol	17	60	13	33	10
Pseudoephedrine	15	70	10	15	10

Table 1: MS parameters for methoxyfenoterol, fenoterol and pseudoephedrine

The LC-MS method was modified from Kim et al. [1]. Atlantis HILIC Silica 3µm (2.1 mm X 50 mm) (Waters, Milford, MA, US) column was used. Before the first injection, the column was pre-conditioned for 1 hour with mobile phase B, which consisted of acetonitrile:ammonium acetate (pH unadjusted, 10 mM) (95:5, v/v). Mobile phase A was acetonitrile:ammonium acetate (pH unadjusted, 10 mM) (50:50, v/v). The flow rate was 0.5 mL/min throughout the entire run time of 7 minutes. To avoid any contamination in the MS detector, the first minute of effluent was diverted to waste using Waters switching valve. The gradient is shown in the following table:

Time (mins)	Events	Parameter
0.01	Event	2
1.00	%B	100
1.01	Event	0
2.50	%B	50
3.00	%B	50
3.01	%B	100
5.99	%B	100
7.00	STOP	100

Table 2: The LC gradient for fenoterol and methoxyfenoterol analysis

The data collected were processed using the Analyst 1.4.1 software from MDS Sciex.

2.2.3 *In Vitro* SPME Method Development

For SPME method development, both fenoterol and methoxyfenoterol were studied as methoxyfenoterol, although used only as an isotropic standard for on-fibre standardization in this chapter, pharmacokinetics study of methoxyfenoterol was performed in the following chapter..

Selection of a commercially prototype fibre was based on highest extraction efficiency for the compounds of interest and lowest inter-fibre variations. Commercial prototype reverse phased amide (RPA) fibre, C18, and cyano (CN) type fibre were used for extraction under static conditions for 2 minutes in 100 ng/mL standard PBS buffer solution of fenoterol and methoxyfenoterol. RPA fibres were selected for subsequent experiments. A preconditioning method was selected based on the highest extraction efficiency. Fibres were either not preconditioned or preconditioned for various lengths of time in either desorption solvent of

ACN:MeOH (80:20, v/v) or in 100% MeOH followed by 2 minutes extraction in static conditions in 100 ng/mL fenoterol in PBS buffer solution.

Desorption time was determined based on the lowest percentage of carryover. To determine the extraction time for subsequent *in vivo* studies, extractions were performed in 100 ng/mL standard whole blood solution for either 2 or 4 minutes at 2 different pump flow rates of 0.6 mL/min and 1.4 mL/min using a syringe pump (Kloehn Co. LTD, Las Vegas, Nevada, US). Extraction for 4 minutes at 1.4 mL/min was selected based on the highest sensitivity in comparison with other combinations. An extraction time profile was constructed using the flow rate of 1.4 mL/min and extraction in 100 ng/mL standard whole blood solution.

The stability of fenoterol and methoxyfenoterol was investigated. Fibres were desorbed after extraction times of 24, 36 and 72 hours. The extracted fibres were kept in a freezer and away from light until desorption. The linear ranges for SPME and plasma protein precipitation were determined from 10, 250, 500, 1000, 2000, 10000, to 20000 ng/mL. Each fibre was of single use. For extraction at higher concentrations, serial dilutions were made to the desorption solvent for SPME and supernatant for plasma protein precipitation method until the peak area was within the linear range of the instrument response. In addition, prior to desorption, the fibres were rinsed with nano-pure water for approximately 1 second to dissolve any impurities. The residual water on the fibre was blotted on Kimwipe tissue.

2.2.4 Flow-through System

For the comparisons of the three kinetic calibration methods, a flow- through system was used (Figure 8).

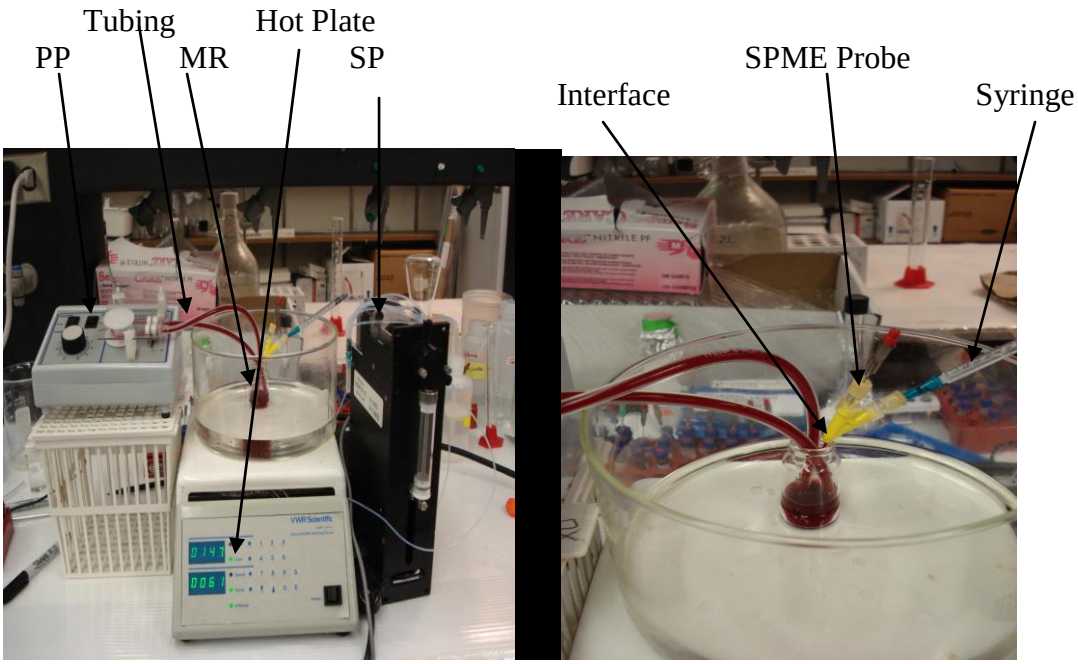

Figure 8: A) Snapshot of the flow-through system with a peristaltic pump (PP) for providing a controlled linear velocity of fluid from the matrix reservoir (MR) to the tubing. A hole was drilled into the tubing where an interface was placed. On one side of the interface, a syringe or syringe pump (SP) was pierced into the septum of the interface where it provided the power for blood to flow through the interface. The SPME probe was pierced on the other side of the interface. A hotplate (HR) was used to control the temperature of the sample at 37-38^{0}C. B) Close up of the interface, SPME fibre and syringe

The peristaltic pump (VWR, cat # 57951-016, 4-600 mL/min) functioned as an artificial heart. The tubing functioned as an artificial vein and was selected based on three criteria: suitability for peristaltic pump usage, biocompatibility, and low or minimal extraction. Two types of tubing, Tygon S-50-HL and Tygon LFL were selected as they fulfilled the first two requirements. Subsequently, Tygon S-50-HL of I.D. X O.D.: 1/8 X 1/4 inch and wall thickness of 1/16 inch (VWR, cat # 63010-231) was selected as it extracted smaller amount of analytes compared to Tygon LFL. The peristaltic pump was set at a flow rate of 75 mL/min according to a previous experiment [2] but with an addition of a hot plate and a crystallizing dish on top with silicone oil

to maintain temperature at 38^0C. Extractions were performed when the tubing was equilibrated at 37-38^0C. The setup was optimized using Beagle dog parameters as the pressure range in the circulation loop was within the same range as that expected in Beagle dog vein and incorporation of any external flow resistance was not necessary [2-3].

2.2.5 Preparation of Calibration Standards

PBS buffer of pH 7.4 was prepared by dissolving 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, and 0.24g of KH_2PO_4 in water. To evaluate the accuracy of the calibration methods, extractions were performed in whole blood and PBS buffer with spiked fenoterol concentrations at 10, 5000, and 20,000 ng/mL which fall in the dynamic linear range determined previously. Prior to extraction, the blood was incubated with the spiked fenoterol for 24 hours. This allowed the drug to distribute evenly in the sample. The calibrated amount was compared with the true concentration in the sample. Three determinations were performed at each concentration level and using each kinetic calibration method in order to evaluate method precision. Equilibrium extraction and conventional method of plasma protein precipitation were performed for comparison with the three kinetic calibrations.

2.2.6 On-fibre Standardization

For on-fibre standardization, methoxyfenoterol was used as the standard. To determine whether isotropic exists between fenoterol and methoxyfenoterol, the ratios of n/n_e for both drugs were determined. For the determination of n, extraction was performed in 100 ng/mL whole blood using a flow rate of 1.4 mL/min. For n_e, extraction was performed for 1 hour, the time it takes to reach equilibrium between whole blood and fibre according to the extraction time profile. Methoxyfenoterol, either 10 ng/mL or 1000 ng/mL in PBS buffer, was loaded overnight as the

standard on fibre. To obtain the value for q_0, the fibres that were not used in subsequent extractions were desorbed in desorption solvent consisting of ACN and MeOH (80:20, v/v).

For extraction, the fibre loaded with methoxyfenoterol was pierced through one of the septa of the interface that was drilled through the tubing of the flow-through system. Meanwhile, a syringe was pierced through the other septa and used to manually push and pull the blood up to the interface at 1.4 mL/min. Extraction was performed for 4 minutes followed by desorption for 1 hour in desorption solvent. For this calibration method, both Q and n were determined simultaneously using a single fibre.

2.2.7 Dominant Pre-equilibrium Desorption

For dominant pre-equilibrium desorption, the preloaded amount must be at least 4-fold higher than the potential extracted amount from the sample matrix [4], so an experiment to determine the pre-loading concentration was first performed. Fibres were loaded with varying amounts of fenoterol using a series of different concentrations (5000, 10,000, 20,000, 50,000 ng/mL) and were desorbed in desorption solvent to determine how much was loaded to obtain q_0. Subsequently, 4 minute extractions were performed on the flow-through system using a syringe and non-loaded fibre to determine n. The concentrations of fenoterol were 10, 5000, and 20,000 ng/mL. The amount extracted was compared with the amount loaded to decide on the concentration required for loading.

Due to the small area of the interface, extraction and desorption of the preloaded fibre were not performed simultaneously. The pre-loaded fibre was exposed to the flowing blood sample or PBS buffer of 10, 5000, and 20,000ng/mL of fenoterol for a 4 minutes desorption via a syringe again using the flow-through system. Subsequently, the desorbed pre-loaded fibre were desorbed in the desorption solvent for 1 hour to obtain Q.

2.2.8 Diffusion-based Interface Model

For diffusion-based calibration, the calibration constant was determined experimentally by performing five extractions in the flow-through system using 100 ng/mL fenoterol for 4 minutes in both PBS buffer and whole blood. All extractions, both for the actual experiment and calibration constant, were performed with a syringe pump. To calculate the concentration of the sample, the experimentally determined calibration constant was applied according to equation 6. The calibration constant was also determined theoretically by calculations using equation 6 for comparison with the experimentally determined calibration constant.

2.2.9 Equilibrium Extraction

For equilibrium extraction, each extraction was performed for 1 hour to reach equilibrium using the syringe pump and the flow-through system at 10, 5000, and 20,000 ng/mL in PBS buffer and whole blood followed by 1 hour desorption. A calibration curve was constructed from 10 ng/mL – 20,000 ng/mL at the same extraction time to calculate the initial concentration in the sample.

2.2.10 Conventional Blood Analysis

For validation purposes, 0.3mL blood was withdrawn using a syringe from the same interface where SPME probe was inserted. The blood sample was subsequently centrifuged (14,000 rpm, 5 min, 4^0C) and plasma was isolated. A 0.08 mL portion of plasma was mixed with 0.30 mL of HPLC grade ACN in conical centrifuge vials for protein precipitation. After vortex mixing (2400 rpm, 5 min) and centrifugation (14,000 rpm, 10 min, 4^0C), 0.01 mL of the supernatant was transferred to a 0.1 mL insert (Supelco, Bellefonte, PA, US). 0.09 mL of desorption solvent for dilution and 0.01 mL of pseudoephedrine (50 ng/mL) as internal standard

to control for injection volume variation were added. The resulting solution was vortexed manually to ensure thorough mixing. External calibration was performed to determine the amount of fenoterol in the plasma sample. For this, blank plasma was spiked with different concentrations of fenoterol. Following the same procedures as above, the spiked plasma was mixed with ACN for protein precipitation followed by vortex mixing and centrifugation.

2.3 Results & Discussions

2.3.1 SPME Method Development

Three commercial prototype coatings, C18, RPA, and CN were selected for evaluation [5]. Extractions were performed for 2 minutes using 100 ng/mL at static conditions to determine the extraction efficiency. Ten fibres were used for each type of coating for extraction. RPA displayed the highest extraction capacity and lowest inter-fibre variability (Figure 9). Therefore, it was selected for subsequent *in vivo* studies.

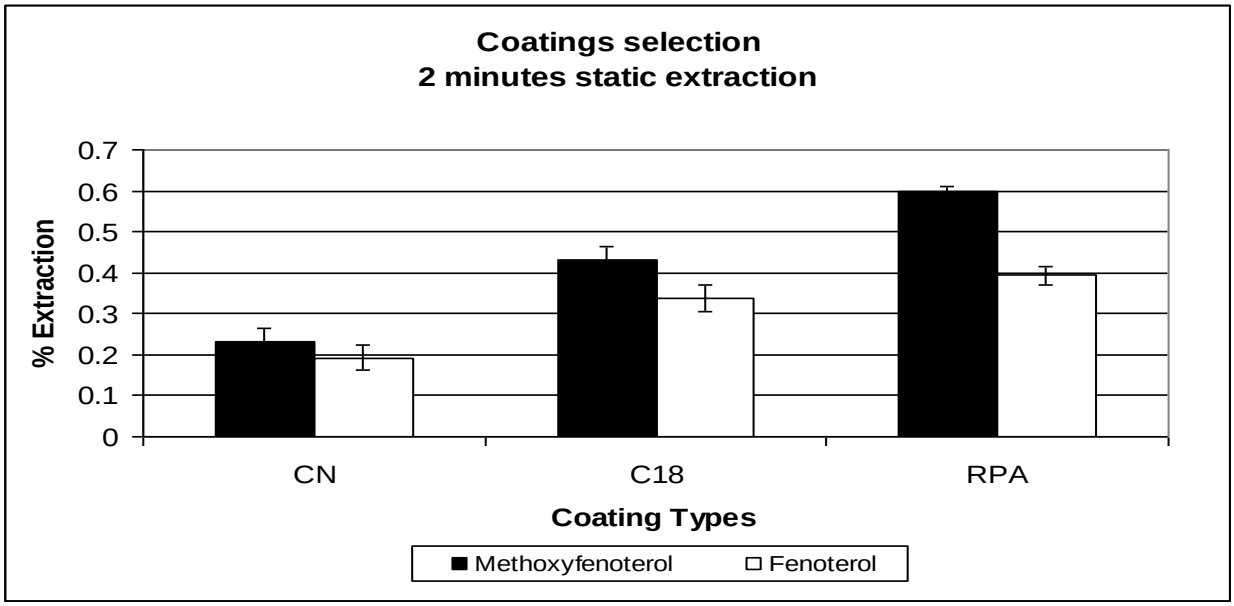

Figure 9: Extraction capacity and inter-fibre variability of three types of commercially available coating: cyano, C18, and RPA (n=10). Error bars represent one standard deviation from the mean. The extraction time was 2 minutes in static condition in 100 ng/mL standard PBS buffer solution

From previous experiments, it was known that pre-wetting or pre-conditioning the coating by soaking the fibres into organic solvent greatly enhanced the extraction efficiency. Therefore, two organic solvents, consisting of ACN and MeOH (80:20, v/v) and 100% methanol were selected. Extractions were performed in 100 ng/mL after 0, 15, 30, and 60 minutes of pre-conditioning in desorption solvent. This was compared with extraction performed after 60 minutes of pre-conditioning in MeOH (Figure 10). It was observed that amount extracted was below the limits of quantification (LOQ) when no pre-conditioning was performed on the fibres. No difference in extraction efficiency was found after 30 and 60 minutes of pre-conditioning with desorption solvent or MeOH. Therefore, on the basis of these results, each RPA probe was pre-conditioned for at least 30 minutes using desorption solvent before any extraction was performed.

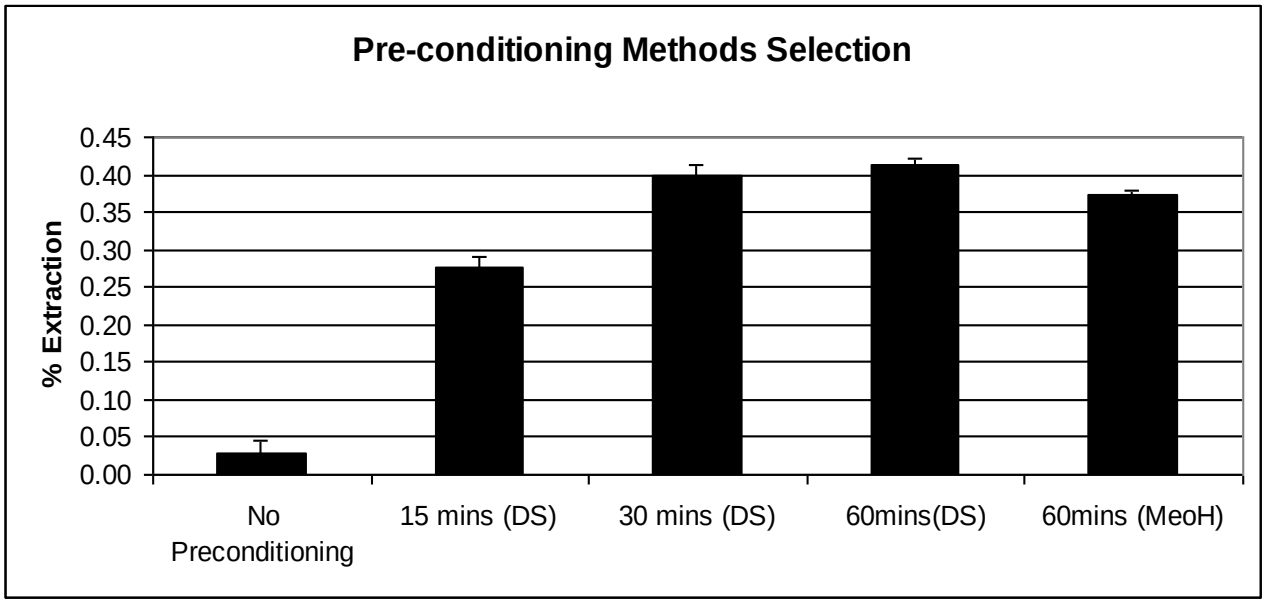

Figure 10: Effect of pre-conditioning method on amount extracted after the indicated time and solvent type (n=3). Error bars represent one standard deviation from the mean. Two solvents were selected for pre-conditioning: desorption solvent (DS) and methanol (MeOH). The extraction time was 2 minutes in static condition in 100 ng/mL PBS buffer standard solution of fenoterol

For SPME method development, it is important to determine the desorption time. The selected time should be long enough to allow almost all of the analyte to be desorbed from the fibre. In this experiment, extractions were performed in 100 ng/mL fenoterol in PBS buffer for 60 minutes with vortex (2400 rpm) followed by desorption for 15, 30, 45, and 60 minutes. Carryover was determined by subsequent desorption for another 60 minutes in fresh desorption solvent (Figure 11).

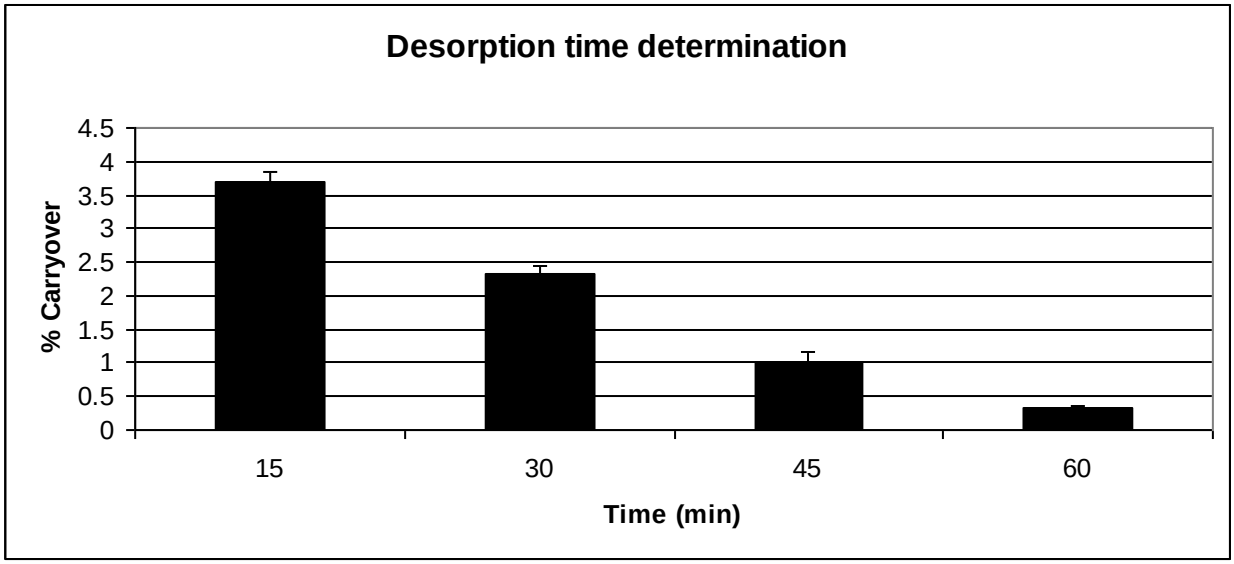

Figure 11: Desorption time determination by extraction with 100 ng/mL fenoterol in PBS buffer for 60 minutes with vortex followed by desorption at various time in desorption solvent (n=3). Error bars represent one standard deviation from the mean.

A 60 minute desorption time was selected for subsequent experiments as it gave less than 0.5% carryover.

For pre-equilibrium extraction using the three kinetic calibrations, on-fibre standardization, dominant pre-equilibrium desorption, and diffusion-based interface model, the amount extracted must be far less than the amount extracted at equilibrium. There are two factors that affect the amount extracted: sampling speed and sampling time. Since a syringe or syringe pump would be used to push and pull the flowing sample through the interface, this agitation would increase the amount extracted by the fibre in comparison with extractions performed at static conditions. Therefore, it is important to consider the pump flow rate or the manual push-pull rate by the syringe. Because the kinetic calibration methods, specifically the diffusion-based interface model, would be applied to sampling real animals such as rats, the sampling rate should be well below the normal flow rate in the animal in order to give minimal disturbance. 0.6 mL/min was selected based on previous pharmacokinetic studies [6].

To determine the optimal sampling time to use for *in vivo* study, one must consider both the overall sensitivity of the SPME method and temporal resolution when performing pharmacokinetic studies. This is because as the extraction time lengthened the sensitivity of the SPME method increases because of the increase in the amount extracted by SPME. However, the temporal resolution of the pharmacokinetic profile decreases since the concentration measured is an average of the extraction time. This factor is especially detrimental to the early time points because the concentration changes rapidly immediately after drug administration by i.v. dosing. Based on this factor, 2 and 4 minutes were selected for extraction. However, it was discovered that the amount extracted at 2 and 4 minutes at the flow rate of 0.6 mL/min was below the sufficient amount extracted to observe fenoterol according to previous pharmacokinetic study of fenoterol [7]. When the sampling rate was increased to 1.4 mL/min with 4 minutes extraction, the sensitivity was sufficient for subsequent pharmacokinetic study based on the amount extracted (Figure 12).

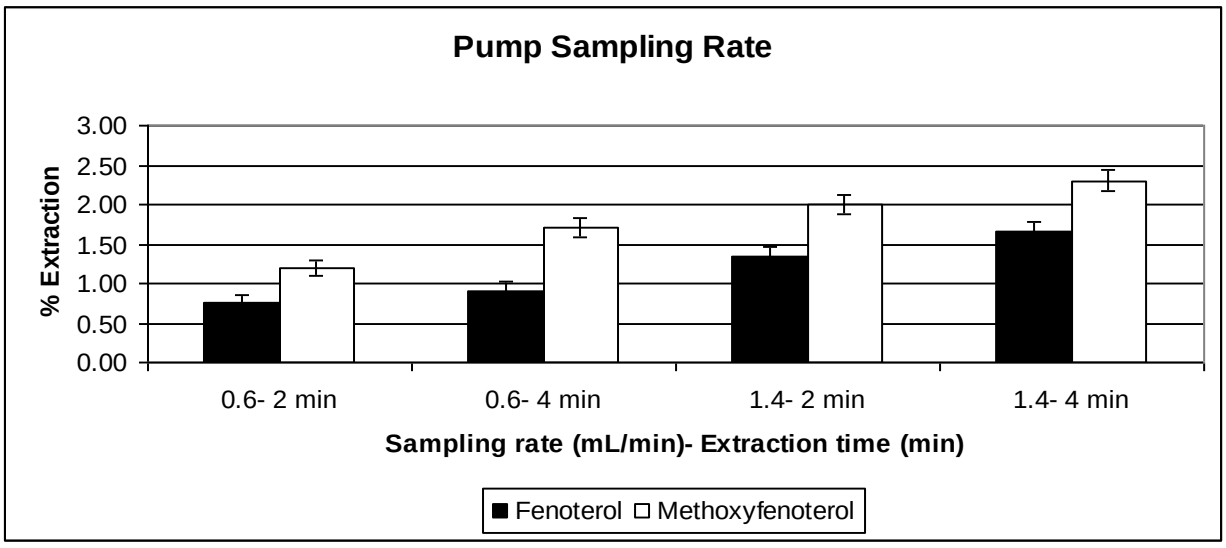

Figure 12: Pump sampling rate versus percent extraction for fenoterol and methoxyfenoterol (n=3) and error bars were constructed based on standard deviations. Extractions were performed in 100 ng/mL standard whole blood solution.

In order to confirm that the amount extracted with flow rate of 1.4 mL/min for 4 minutes was below what would be at equilibrium, an extraction time profile in 100 ng/mL standard rat whole blood was constructed for both fenoterol and methoxyfenoterol. The extraction was performed at 4 minutes with 1.4 mL/min flow rate (Figure 13).

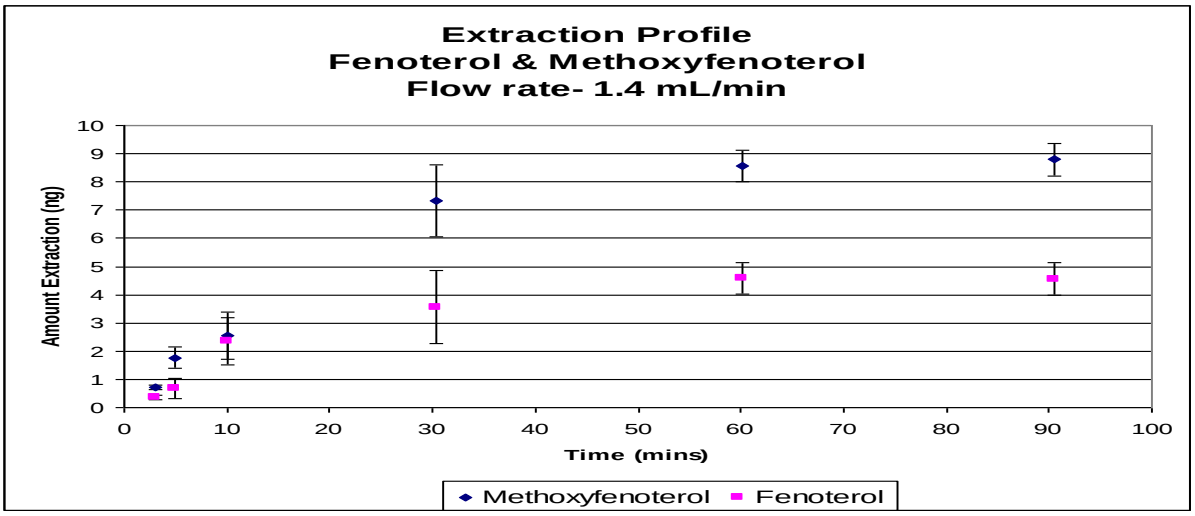

Figure 13: Extraction time profile in rat whole blood for fenoterol and methoxyfenoterol. For each time point (n=3), the error bars were constructed based on standard deviations.

Based on the extraction time profile, it was confirmed that the amount extracted with 4 minutes sampling time was far from the amount extracted at equilibrium thus meeting the requirement for kinetics. For *in vitro* studies, fibres were desorbed right away after extraction. However, for *in vivo* studies using animals, sampling would be performed at NoAb Biodiscoveries Inc. and fibres would not be desorbed until hours after extraction. Therefore, stability of the analytes on the fibre was important to be determined. From figure 14, both fenoterol and methoxyfenoterol were stable on fibres with less than 10% lost after 48 hours of extraction.

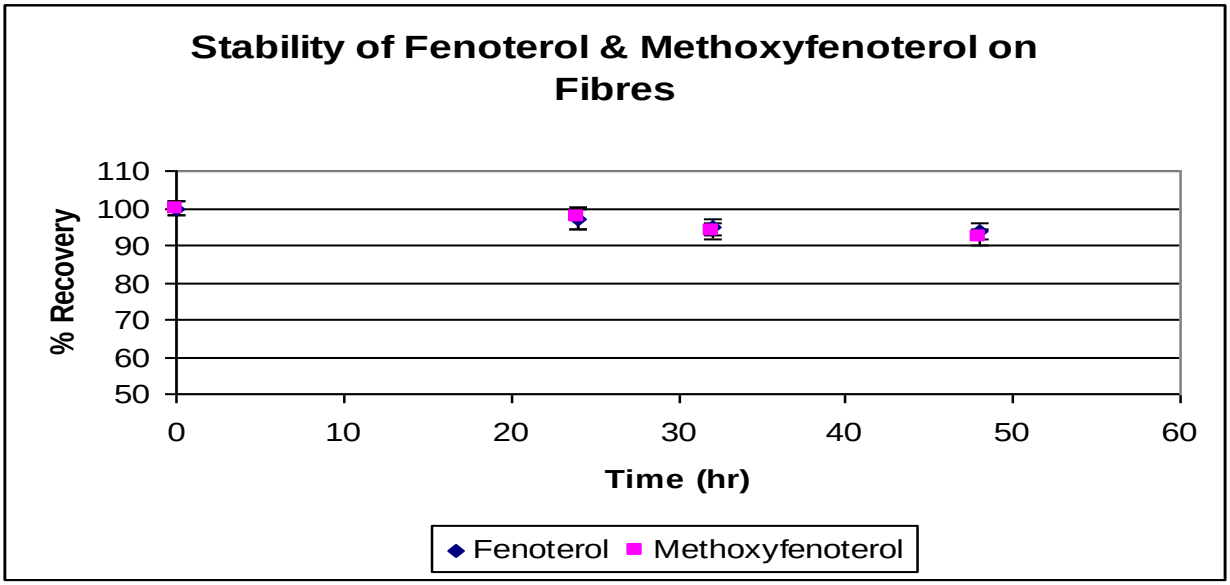

Figure 14: Stability of fenoterol and methoxyfenoterol on fibres. Extractions were performed with vortex (2400 rpm, 60 minutes) at 100 ng/mL standard PBS buffer solution followed by desorption after 24, 32, and 48 hours.

The dynamic range for fenoterol and methoxyfenoterol using kinetic SPME and plasma-protein precipitation was studied. The dynamic range for methoxyfenoterol was determined for experiments in chapter 3. Kinetic SPME was performed with the syringe pump at 1.4 mL/min with sampling time of 4 minutes and 1 hour for equilibrium extraction. The dynamic range using equilibrium extraction was determined only for fenoterol as it was needed for the experiments in chapter 2. The regression slope, intercepts, and errors for equilibrium extraction can be found in Table 15.

	Linear Range (ng/mL)	Number Of Standards	Slope	Standard Error Slope	+/- Intercept	Standard Error Intercept	R^2
Whole Blood SPME							
Fenoterol	10-20,000	7	7.1E-3	2.0E-4	2.2E0	1.5E0	0.9986
Methoxyfenoterol	8-20,000	7	2.1E-2	3.0E-4	9.2E-1	2.9E0	0.9972
Conventional Method: Protein Precipitation							
Fenoterol	10-20,000	7	4.2E-2	1.2E-3	5.1E-1	1.3E0	0.9958
Methoxyfenoterol	10-20,000	7	4.6E-2	6.0E-4	-1.5E0	5.8E-1	0.9993

Table 3: A seven point extraction of fenoterol and methoxyfenoterol (n=3) using SPME extraction in rat whole blood and plasma protein precipitation. The regression slope was obtained from graph of concentration (ng/mL) versus amount extracted (ng)

SPME and conventional plasma protein precipitation methods were compared in terms of absolute matrix effects using the same LC-MS method. For plasma protein precipitation method, the relative signal intensity of standard spiked post extraction versus standard prepared directly in solvent was 56% and 59% for fenoterol and methoxyfenoterol, respectively. No ionization suppression was observed using SPME, as indicated by the relative signal intensities of 95% and 102% for fenoterol and methoxyfenoterol, respectively. This illustrates that SPME can provide significantly improved sample clean up where it was not achievable with the conventional method. For plasma protein precipitation, the slope for fenoterol and methoxyfenoterol was greater in comparison with the SPME method. This is because plasma protein precipitation is an exhaustive extraction method where SPME only extracts the free analyte.

2.3.2 On-Fibre Standardization

For on-fibre standardization, the selection of standard is dependent on the isotropic character to the analyte of interest [9-13]. Methoxyfenoterol was initially selected due to its

similarity in structure to fenoterol. Previously, Zhou et al. had demonstrated that if the analyte and standard were isotropic, the time constant, a, for desorption of the standard, should be similar to the time constant of the extraction process of the analyte [14, 15]. Based on this conclusion and equations (3) and (4), isotropism can be confirmed either using the ratio of n/n_e or Q/q_0 for both the analyte and standard. If the ratios for analyte and potential standard have similar values, their isotropic relationship is positive. This can save much time from determining the extraction and desorption time profile for the analyte of interest and standard respectively.

	Fenoterol	Methoxyfenoterol
n (ng)	8.2E-2	1.5E0
n_e (ng)	4.9E0	9.1E0
n/n_e	0.17	0.17

Table 4: Ratio of n/n_e for fenoterol and methoxyfenoterol (n=3)

To obtain q_0, the loaded amount of methoxyfenoterol, excess fibres were loaded overnight. It is important to load fibres with enough time to achieve equilibrium between the loading solution and fibre since the amount loaded would be more consistent. q_0 can be determined by preloading several extra probes and immediately desorbing them. Two concentrations, 10 and 1000 ng/mL were selected for loading (Table 5). For lower concentration sample such as 10 ng/mL, fibres were loaded with 10 ng/mL of methoxyfenoterol and for higher concentration, 1000 ng/mL of methoxyfenoterol were loaded. This was due to the consideration of serial dilution which would be required for higher concentrations before injecting the sample into the instrument. If a low concentration was used for loading, the instrument might not be sensitive enough to pick up methoxyfenoterol after dilution.

Concentration of methoxyfenoterol loading solution (ng/mL)	Loaded amount of methoxyfenoterol on fibre (ng)	RSD (%) (n = 3)
10	0.23	5
1000	23	4

Table 5: The amount of methoxyfenoterol loaded on fibres after overnight extraction with agitation (2400 rpm). The loading solution consisted of methoxyfenoterol in 1.5 mL PBS buffer

Table 6 displays the results for n, Q, q_0 (either 10 or 1000 ng/mL) and the calculated n_e by using equation 5. Both n and Q were obtained with the same fibre sampling in whole blood and PBS buffer with concentration of 10, 5000, and 20,000 ng/mL fenoterol. That is, both extraction and desorption were performed simultaneously with 1 fibre. A syringe was used to push and pull the sample at a flow rate of 1.4 mL/min through the interface. A syringe pump was not used since a control of sampling rate was not required for this calibration method. For actual *in vivo* application of on-fibre standardization, a syringe was used instead of syringe pump.

	n (ng) (RSD)		Q(ng) (RSD)		q_0(ng) (RSD)		n_e(ng) (RSD)	
Concentrations	Buffer	Blood	Buffer	Blood	Buffer	Blood	Buffer	Blood
10 (ng/mL)	1.0E-01 (13)	4.9E-03 (13)	1.3E-01 (5)	2.2E-01 (6.2)	2.3E-01 (5)	2.3E-01 (5)	2.6E-01 (19)	1.4E-01 (17)
5000 (ng/mL)	8.1E+01 (24)	2.3E+00 (22)	1.0E+01 (8)	2.2E-01 (6)	2.3E+01 (4)	2.3E-01 (4)	1.4E+02 (19)	7.6E+01 (15)
20000 (ng/mL)	2.8E+02 (19)	9.3E+01 (26)	1.1E+01 (3)	1.6E+01 (6)	2.3E+01 (4)	2.3E+01 (4)	5.2E+02 (20)	2.9E+02 (16)

Table 6: On-fibre standardization- Results for n, Q, q_0 which was obtained with fibres (n=3) that were not used for extraction, and n_e which was calculated with equation 5

To calculate the bulk concentration of fenoterol in the sample using n_e determined with the aid of equation 3, the product of K_{fs} and V_f was obtained from the slope of calibration plot obtained by finding the amount of fenoterol extracted under equilibrium conditions from standard solutions containing different amounts of fenoterol (Figure 15).

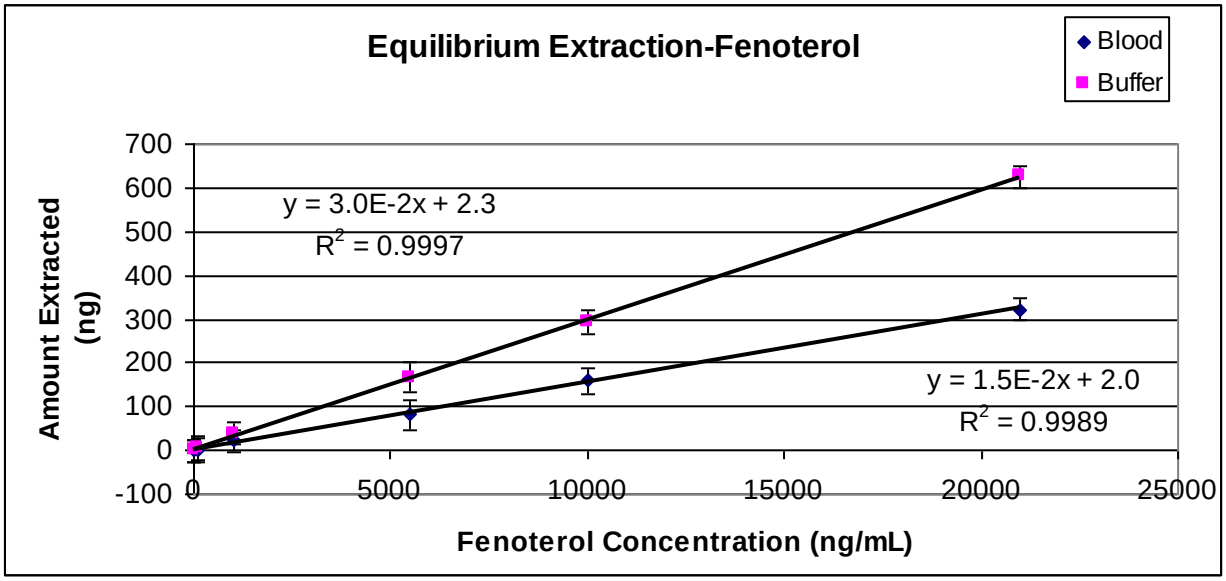

Figure 15: Equilibrium extraction of fenoterol in PBS buffer and blood. The slope of the line was used to determine the product of K_{fs} and $V_{f.}$

The differences observed in Figure 15 between extraction from whole blood and PBS buffer are due to the differences in free concentration of fenoterol in the two matrices. In whole blood, fenoterol can bind to biomolecules present, thus lowering the free concentration of analyte in solution. The concentration calculated using the slope of the line in figure 15 and equation 2 can be found in Table 7:

		C_0 (ng/mL)	
Concentrations	Fibre	Buffer	Blood
10 (ng/mL)	1	7.7E00	9.0E00
	2	1.1E00	8.1E00
	3	9.4E00	1.1E00
	Mean	9.4E00	9.4E00
	RSD (%)	19	17
5000 (ng/mL)	1	4.1E03	6.1E03
	2	5.6E03	4.9E03
	3	5.9E03	4.6E03
	Mean	5.2E03	5.2E03
	RSD (%)	19	15
20000 (ng/mL)	1	1.5E04	2.2E04
	2	2.1E04	2.1E04
	3	2.2E04	1.6E04
	Mean	1.9E04	2.0E04
	RSD (%)	19	16

Table 7: Concentrations calculated using on-fibre standardization

2.3.3 Dominant Pre-equilibrium Desorption

For isotropic desorption, the amount pre-loaded on the fibre must be 4-fold higher than the potential extracted amount [14,16]. Therefore, to determine the loading concentration, four concentrations of fenoterol, 5000, 10,000, 20,000 and 50,000 ng/mL were selected for loading. Similar to on-fibre standardization, fibres were loaded from PBS buffer using equilibrium overnight extraction.

After deciding the appropriate loading concentration (Figure 16) by comparing amount extracted and amount loaded, the loaded fibre was desorbed into the respective sample in order to determine n_e (5) using a syringe.

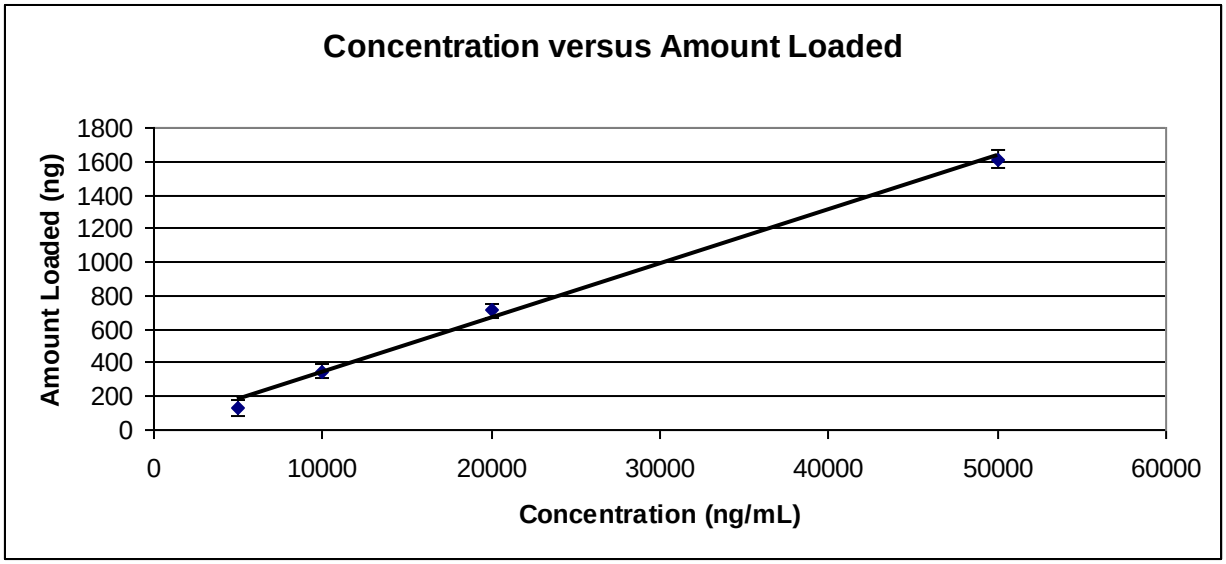

Figure 16: Amount loaded versus loading concentration of fenoterol (n=3)

Unlike on-fibre standardization, the desorption of the loaded fibre in dominant pre-equilibrium desorption was performed separately from extraction. Due to the small surface area of the interface, both an extraction and desorption fibre were not able to fit together. Results of n, Q, q_0, and the calculated n_e from equation 3 are displayed in Table 8.

	n (ng) (RSD)		Q(ng) (RSD)		q_0(ng) (RSD)		n_e(ng) (RSD)	
Concentrations	Buffer	Blood	Buffer	Blood	Buffer	Blood	Buffer	Blood
10 (ng/mL)	2.3E-01 (30)	7.7E-02 (21)	3.8E+01 (6)	6.8E+01 (9)	1.3E+02 (5)	1.3E+02 (7)	3.2E-01 (30)	1.6E-01 (20)
5000 (ng/mL)	6.5E+01 (29)	6.9E+00 (21)	3.9E+02 (7)	6.5E+02 (6)	7.1E+02 (6)	7.1E+02 (7)	1.4E+02 (27)	7.7E+01 (21)
20000 (ng/mL)	2.5E+02 (26)	8.9E+01 (24)	8.8E+02 (9)	1.1E+03 (5)	1.6E+03 (4)	1.6E+03 (5)	5.4E+02 (23)	3.0E+02 (23)

Table 8: Dominant pre-equilibrium desorption- results for n, Q, q_0 which was obtained with fibres (n=3) that were not used for extraction, and n_e which was calculated with equation 5

The calculated concentration using dominant pre-equilibrium desorption can be found in the following table.

Concentrations	Fibre	Buffer	Blood
10 (ng/mL)	1	1.5E+01	1.0E+01
	2	1.3E+01	1.3E+01
	3	7.9E+00	9.4E+00
	Mean	1.2E+01	1.1E+01
	RSD (%)	30	20
5000 (ng/mL)	1	3.7E+03	4.5E+03
	2	5.1E+03	4.9E+03
	3	6.5E+03	6.5E+03
	Mean	5.1E+03	5.3E+03
	RSD (%)	27	28
20000 (ng/mL)	1	2.3E+04	2.4E+04
	2	2.2E+04	1.6E+04
	3	1.5E+04	2.2E+04
	Mean	2.0E+04	2.1E+04
	RSD (%)	26	23

Table 9: Concentrations calculated using dominant pre-equilibrium desorption

2.3.4 Diffusion-based Interface Model

For diffusion-based interface model calibration [17-21], the calibration constant was obtained by extraction of 100 ng/mL of fenoterol standard solution prepared in whole blood and PBS buffer, respectively. Using the amount extracted and the known concentration in the sample, the calibration constant can be determined using equation (6). In this experiment, five extractions were performed to improve the accuracy of the experimental calibration constant with an acceptable RSD. However, the number of extractions can vary depending on the overall method precision.

	n (ng)		Calibration Constant	
Fibres	Buffer	Blood	Buffer	Blood
1	4.3E-01	3.5E-01	2.3E+02	2.9E+02
2	5.0E-01	3.6E-01	2.0E+02	2.8E+02
3	6.4E-01	2.9E-01	1.6E+02	3.4E+02
4	5.1E-01	2.8E-01	2.0E+02	3.5E+02
5	5.5E-01	2.9E-01	1.8E+02	3.4E+02
		Mean	1.9E+02	3.2E+02
		RSD (%)	14	11

Table 10: Determination of experimental calibration constant for buffer and blood using 100 ng/mL fenoterol (n=5)

The calibrated concentration using the experimental calibration can be found in the following table:

	n (ng)		Calibrated Concentration (ng/mL)	
Concentrations	Buffer	Blood	Buffer	Blood
10 (ng/mL)	4.6E-02	2.8E-02	8.8E+00	9.0E+00
	4.9E-02	3.0E-02	9.5E+00	9.6E+00
	5.9E-02	3.6E-02	1.1E+01	1.2E+01
		Mean	9.1E+00	9.3E+00
		RSD (%)	15	14
5000 (ng/mL)	2.3E+01	1.4E+01	4.5E+03	4.6E+03
	2.5E+01	1.7E+01	4.9E+03	5.5E+03
	2.9E+01	1.6E+01	5.6E+03	5.1E+03
		Mean	5.0E+03	5.1E+03
		RSD (%)	11	9
20000 (ng/mL)	1.2E+02	6.3E+01	2.3E+04	2.0E+04
	1.1E+02	6.9E+01	2.0E+04	2.2E+04
	1.0E+02	5.7E+01	2.0E+04	1.8E+04
		Mean	2.1E+04	2.0E+04
		RSD (%)	9	9

Table 11: Calibrated concentration using the experimental calibration constant for buffer and blood

The calibration constant can also be determined or verified theoretically by knowing the radius and length of the coating, the extraction time, the diffusion coefficient and calculating the thickness of boundary layer (7) (Table 12). The calibration constant can be calculated as follows:

$$\text{Calibration Constant} = \frac{\ln((b+\delta)/b)}{2\pi LtD_L} \quad (8)$$

For b, the radius of the fibre was 0.145 mm. The δ was calculated from equation 7. The Reynolds number was calculated using Re = ud/ ν, where u, is the linear velocity of the sample, d is the diameter of the fibre used, and ν is the kinematic viscosity of the sample matrix. The kinematic viscosity of blood is 198 mm^2/min at 20^0C [22] and of PBS buffer, which was estimated as water, is 39.5 mm^2/min at 40^0C [23]. These literature values were used to estimate the calibration constant as the kinematic viscosity for blood and PBS buffer at 38^0C are unavailable to the best of author's knowledge. The Schmidt number was calculated by Sc = ν/ D_L, where D_L is the diffusion coefficient of fenoterol in whole blood and buffer. The diffusion coefficient in both media was estimated using parameters from water due to the lack of references available for fenoterol in whole blood and PBS buffer. The diffusion coefficient in liquid can be calculated as follows:

$$D_L = 7.4X10^{-10}\frac{(\psi_2 M_2)^{0.5}T}{\eta_2 v_1^{0.6}} \quad (9)$$

where ψ_2 is a constant and has the value of 2.6 for water [17], M_2 is the molecular weight of water, η_2 is the viscosity of water and is 0.0423 g/mm•min [17], T is the temperature of the experiment, 311.5 K, and v_1 is the molar volume of fenoterol and is 309800 mm^3/mol, calculated by summing the size of the atoms making up the chemical's structure [25].

Fenoterol	b (mm)	δ (K•min^2/mm^3)	Re	Sc (mm^4/min^2•K)	D_L (K•min/mm^2)	L (mm)	t (min)
Blood	0.145	3.15E-4	0.540	1.05E10	1.89E-8	15	4
PBS buffer	0.145	2.14E-4	2.704	2.09E9	1.89E-8	15	4

Table 12: Parameters used to calculate calibration constant for fenoterol in blood and PBS buffer

Fenoterol	Whole Blood	PBS Buffer
Experimental (n=5)	320.4 ± 11	192.6 ± 14
Theoretical	304.2*	207.1*

Table 13: A comparison between experimental and theoretical calibration constant for fenoterol in whole blood and PBS buffer *(The kinematic viscosity of water was taken to be at 40^0C and blood at 20^0C when the actual experimental temperature was 38^0C)

From Table 13, the theoretical calibration constant for fenoterol is very close to the range of the determined experimental constant for whole blood and PBS. Sample calculation to obtain the theoretical constant for fenoterol in blood is illustrated:

$$\text{Calibration Constant} = \frac{\ln((b+\delta)/b)}{2\pi LtD_L}$$

b = Radius of fibre coating) = Diameter (mm)/ 2
= 290 μm / 2
= **0.145 mm**

$$\delta = \text{boundary layer} = 9.52(b / \mathrm{Re}^{0.62}\, Sc^{0.38})$$

Re = Reynolds number = $\frac{ud}{\nu}$

u = linear velocity = r/A

r = sampling rate = 1.4 mL/min = 1400 mm^3/min

A = area of interface = $\pi r^2 = \pi(1.1\text{mm})^2$

$$u = \frac{1400\ \text{mm}^3/\text{min}}{\pi\,(1.1\text{mm})^2} = 368.479\ \text{mm/min}$$

d = diameter of fibre = 290 mm

ν = kinematic viscosity = 198 mm^2/min

Re = $\frac{368.29 \text{ mm/min (0.290mm)}}{198 \text{ mm}^2\text{/min}}$

= 0.540

Sc = Schmidt number = $\frac{\nu}{D_L}$

ν = kinematic viscosity = 198 mm^2/min

D_L = Diffusion coefficient $= 7.4X10^{-10}\frac{(\psi_2 M_2)^{0.5}T}{\eta_2 v_1^{0.6}}$

Ψ_2 = constant =2.6 for water

M_2 = Molecular weight = 18.0 g/mol for water

T = 311.5 K

v_1 = molar volume of fenoterol

= 17(C) + 21(H) + 1(N) + 4(O) –2 rings

= 18(16.5) + 23(2) + 1(5.7) + 4(5.5) – 2(20.2)

= 309.8 cm^3/mol = 309800 mm^3/mol

η_2 = 0.0423 g/mm•min for water

$$D_L = 7.4X10^{-10}\frac{(2.6 \bullet 18.0 g/mol)^{0.5} 311.5K}{(0.0423 g/mm \bullet \text{min})(309800 mm^3/mol)^{0.6}}$$

= **$\underline{1.892 \text{ E -8 K}\cdot \text{ min/mm}^2}$**

Sc = $\frac{198 \text{ mm}^2\text{/min}}{1.892 \text{ E -8 K}\cdot \text{ min/mm}^2}$

= 1.047E10 mm^4/ K·min^2

$$\delta = 9.52\left(\frac{0.145mm}{(0.54)^{0.62}(1.047E10 mm^4/K\min^2)^{0.38}}\right)$$

= **$\underline{3.15\text{E-4 K}\cdot\text{min}^2\text{/mm}^3}$**

$$\text{Calibration Constant} = \frac{\ln((0.145mm + 3.15E-4K\min^2/mm^3)/0.145mm)}{2\pi \bullet 15mm \bullet 4\min \bullet (1.892E-8K\min/mm^2)}$$

= 304.2 mm^{-3}

	Calibrated Concentration experimental calibration constant(ng/mL)		Calibrated Concentration theoretical calibration constant(ng/mL)	
Concentrations	Buffer	Blood	Buffer	Blood
10 (ng/mL)	8.8E+00	9.0E+00	9.4E+00	8.6E+00
	9.5E+00	9.6E+00	1.0E+01	9.2E+00
	1.1E+01	1.2E+01	1.2E+01	1.1E+01
Mean	9.1E+00	9.3E+00	1.1E+01	9.6E+00
RSD (%)	15	14	14	13
5000 (ng/mL)	4.5E+03	4.6E+03	4.8E+03	4.4E+03
	4.9E+03	5.5E+03	5.2E+03	5.2E+03
	5.6E+03	5.1E+03	6.0E+03	4.9E+03
Mean	5.0E+03	5.1E+03	5.4E+03	4.9E+03
RSD (%)	11	9	11	9
20000 (ng/mL)	2.3E+04	2.0E+04	2.5E+04	1.9E+04
	2.0E+04	2.2E+04	2.2E+04	2.1E+04
	2.0E+04	1.8E+04	2.1E+04	1.8E+04
Mean	2.1E+04	2.0E+04	2.3E+04	1.9E+04
RSD (%)	9	9	9	9

Table 14: Comparison of calibrated concentrations obtained by experimental calibration constant and theoretical calibration constant

From Table 14, results obtained by using the experimental calibration constant were more accurate since the theoretical calibration constants were only estimated. However, the accuracy of results predicted using theoretical calibration constant was also very good, indicating the usefulness of this method.

In addition, for successful application of diffusion-based interface model calibration, two requirements must be fulfilled: the concentration of the analyte in sorbent must be close to zero in order for a concentration gradient to be constructed, and a constant agitation or sampling speed is required to keep the boundary layer constant. For the first requirement, a prolonged extraction time or extraction at equilibrium is detrimental since the sorbent concentration cannot be assumed as zero. For the second requirement, because extraction is based on the concentration gradient

across the boundary layer, the size of the boundary layer must be kept constant during sampling for an accurate calibration. In order to achieve a constant size of boundary layer, the speed of agitation must be constant.

2.3.5 SPME Equilibrium Extraction

Equilibrium extraction was performed to compare with the three kinetic calibration methods. Extractions were performed in whole blood and PBS buffer of 10, 5000, and 20000 ng/mL fenoterol using the flow-through system The extraction time was 1 hour using a syringe pump with flow rate at 1.4 mL/min as this was the extraction time and flow rate to reach equilibrium according to figure 13. To determine the initial concentration in the sample, external calibration was used. The amount extracted at equilibrium versus concentration was plotted in figure 15. The amount extracted n_e was calculated using the graph in figure 15 and the following weighted equation (reciprocal y square) (Table 15):

	Linear Range (ng/mL)	Number Of Standards	Slope	Standard Error Slope	+/- Intercept	Standard Error Intercept	R^2
Whole Blood	10-21,000	6	1.4E-2	1.8E-3	-2.7E-2	1.5E-2	0.9424
PBS buffer	10-21,000	6	3.2E-2	2.0E-4	9.7E-2	3.7E-2	0.9841

Table 15: Weighted equation using reciprocal y square for figure 15

The weighted equation was used since the y-intercept of the non-weighted equation was larger than the amount extracted at 10 ng/mL. The calculated concentration using equilibrium SPME are shown in the following table:

	n_e (ng)			C_0 (ng/mL)	
Concentrations	Buffer	Blood		Buffer	Blood
10 (ng/mL)	4.4E-01	1.2E-01		1.1E+01	1.1E+01
	4.2E-01	1.1E-01		9.0E+00	9.7E+00
	4.4E-01	1.2E-01		1.1E+01	1.0E+01
			Mean	1.0E+01	1.1E+01
			RSD(%)	9	9
5000 (ng/mL)	1.6E+02	7.6E+01		5.5E+03	5.3E+03
	1.6E+02	6.4E+01		4.9E+03	4.5E+03
	1.5E+02	6.9E+01		4.3E+03	4.9E+03
			Mean	4.9E+03	4.9E+03
			RSD(%)	12	8
20000 (ng/mL)	7.3E+02	2.9E+02		2.3E+04	2.2E+04
	6.1E+02	2.8E+02		1.9E+04	2.0E+04
	6.3E+02	2.6E+02		2.0E+04	1.8E+04
			Mean	2.0E+04	2.0E+04
			RSD(%)	10	9

Table 16: Amount extracted n and calculated concentration using equilibrium extraction at 3 concentrations (n=3)

2.3.6 Conventional Method-Plasma Protein Precipitation

Conventional method of plasma protein precipitation was performed to validate the data obtained with SPME. To calculate the concentration in the sample using plasma protein precipitation, a calibration curve of concentration from 10 – 20,000 ng/mL versus the peak area ratio of fenoterol and the internal standard pseudoephedrine was constructed (Figure 17). The area ratio obtained from samples of 10, 5,000, and 20,000 ng/mL was substituted into the weighted equation from figure 17 to calculate the concentration (Table 17).

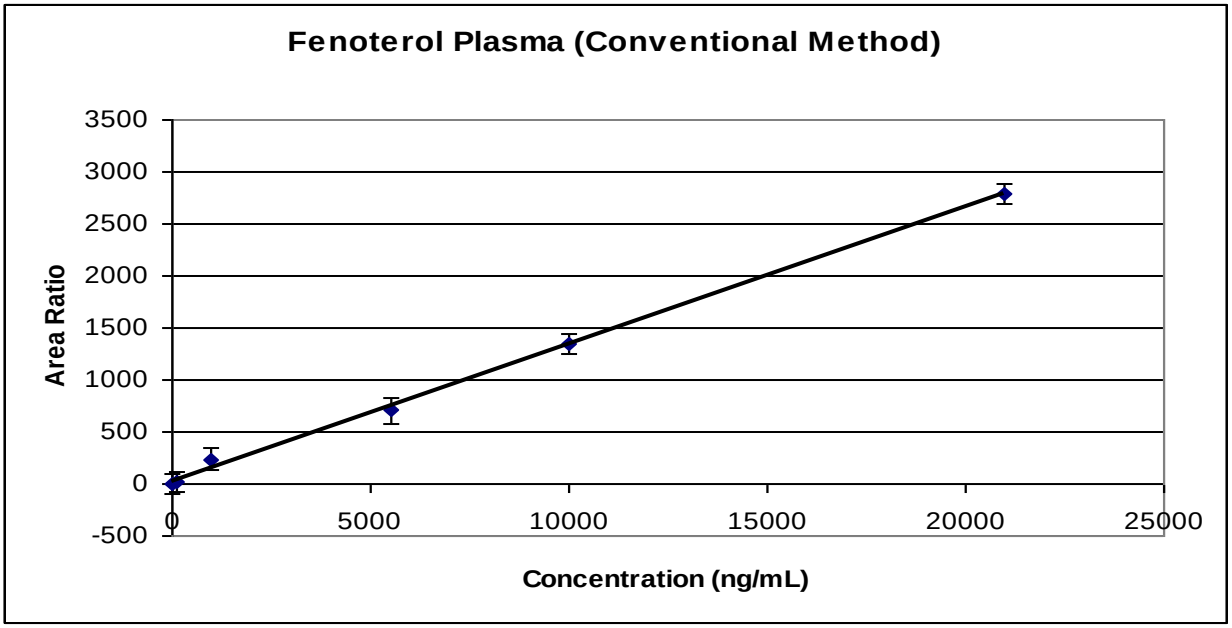

Figure 17: A plot of peak area ratio of fenoterol and the internal standard versus the concentration in the sample. The weighted equation for the line is y=0.1401x + 0.8755

	Area Ratio	C_0 (ng/mL)
Concentrations		
10 (ng/mL)	2.5E+00	1.1E+01
	2.4E+00	1.1E+01
	2.3E+00	9.8E+00
	Mean	1.1E+01
	RSD(%)	8
5000 (ng/mL)	6.9E+02	4.9E+03
	7.8E+02	5.6E+03
	7.0E+02	5.0E+03
	Mean	5.1E+03
	RSD(%)	7
20000 (ng/mL)	2.8E+03	2.0E+04
	2.8E+03	2.0E+04
	3.1E+03	2.2E+04
	Mean	2.1E+04
	RSD(%)	7

Table 17: Measured concentration using plasma protein precipitation

2.3.7 Calibration Methods Comparison

	Relative Recovery (%) (RSD, %; n=3) Phosphate Saline Solution Buffer			
Concentrations (ng/mL)	On-fibre Standardization	Dominant Pre-equilibrium Desorption	Diffusion-Based Interface Model	Equilibrium Extraction
10	94 (19)	120 (30)	91 (15)	100 (9)
5,000	104 (19)	102 (27)	100 (11)	98 (12)
20,000	95 (19)	100 (26)	105 (9)	100 (10)

Table 18: Relative recovery and standard deviation comparison in PBS buffer between 3 *in vivo* calibration methods, equilibrium extraction, and conventional method. For diffusion-based interface model, the results are based on the experimental calibration constant since it gave more accurate results.

	Relative Recovery (%) (RSD, %; n=3) Whole Blood				
Concentrations (ng/mL)	On-fibre Standardization	Dominant Pre-equilibrium Desorption	Diffusion-Based Interface Model	Equilibrium Extraction	Conventional Method: Protein Precipitation
10	94 (17)	110 (20)	93 (14)	110 (9)	110 (8)
5,000	104 (15)	106 (28)	102 (9)	98 (8)	102 (7)
20,000	100 (16)	105 (23)	100 (9)	100 (9)	105 (7)

Table 19: Relative recovery and standard deviation comparison in whole blood between three *in vivo* calibration methods, equilibrium extraction, and conventional method

Table 18 and 19 compare the three pre-equilibrium SPME calibration methods with equilibrium extraction and conventional method. Results obtained between conventional method and SPME equilibrium extraction corresponded well with each other which validated the compatibility of SPME with the conventional method. Because SPME integrates the sampling

procedure and sample preparation, it is a more time effective sampling method: after extraction, each fibre was desorbed and the desorption solvent was injected directly into the instrument for analysis. The conventional method required many sample preparation steps such as centrifugation and vortexing before the sample could be injected into the instrument. PBS buffer is comparable to protein-free plasma in terms of matrix effect due to the same ionic strength between the two matrices. Musteata et al. had proven that when a drug was spiked into PBS and protein-free plasma, the amount extracted was practically the same when using the same type of fibre and sampling conditions. In addition, it was found that the pH of the protein-free plasma remained the same when isotonic PBS was used to dilute protein-free plasma in a 10:1 ratio [26-27]. Therefore, the distribution constant of the fibre and the two matrices was the same due to similar ionic strength.

When comparing on-fibre standardization and diffusion-based interface model, the relative recoveries were very much comparable. However, standard deviation with using on-fibre standardization was slightly higher than the diffusion-based interface model. This could be due to the extra experimental steps required for loading of fibres prior to sampling for on-fibre standardization. The diffusion-based interface model clearly offered an attractive alternative in terms of fewer experimental steps and a cost-effective method since the analyte of interest can be used as a standard for calibration and fewer fibres are required. In addition, the need for additional experiments for calibration step can be eliminated altogether when the calibration constant can be calculated. The main disadvantage for the diffusion-based interface model is the need to control flow rate or the sampling speed to achieve a constant size of boundary layer. This requirement is difficult in certain applications when connection to a pump for controlling of flow rate is problematic. However, if sensitivity is not a problem, static extraction can also be performed in order to eliminate the need for a pump.

Dominant pre-equilibrium desorption is a new developed technique that has been successfully applied to fish muscle by Zhou et al. [16]. Comparing this calibration method with the other two pre-equilibrium SPME calibration methods on the same system, this method had the highest standard deviation for both whole blood and PBS buffer. Experimental errors were incurred from the fibre loading procedures due to variability of fibres even within the same batch. In addition to poorer precision, the time required was greater than the other two calibration methods. This was because extraction had to be performed first to determine n, and a separate experiment was required to determine q_0 in order to decide on the loading concentration. In addition, it was found by experiment that the sampling speed was also important for dominant pre-equilibrium desorption. In order for isotropic relationship to exist between extraction and desorption, all experimental parameters including sampling time, sample volume, temperature, and sampling speed for the two processes must be the same unless sampling was performed under static condition. This was different from on-fibre standardization since the two processes, extraction and desorption, occurred simultaneously in the same sample with the use of a single fibre.

2.4 Conclusions

In this research, three *in vivo* SPME calibration methods were compared on a flow-through system. They were evaluated in terms of precision, accuracy and experimental procedures, and were validated by equilibrium SPME extraction and a conventional method. All three calibration methods had comparable accuracy and the diffusion-based interface model had the highest precision. In addition, it did not require any other standard besides the analyte of interest and the number of fibres used for calibration was smaller in comparison with the other two calibration methods. However, a disadvantage of the diffusion-based interface model was the requirement of constant sampling rate and therefore required a pump to perform any extraction. In the case where the control of sampling rate is impossible, on-fibre standardization, which had a comparable accuracy as diffusion-based interface model, may be the next best alternative. Since desorption and extraction occurred simultaneously on one fibre, desorption was used to calibrate extraction. Dominant pre-equilibrium desorption has the same calibration principle as on-fibre standardization, however, it had the lowest precision. This could be due to the excessive number of fibre required which increased both error and cost. Since the sampling area, or the interface, was too small to fit both an extraction and a desorption fibre, two separate experiments were performed for extraction and desorption. This required more time and further reduced the precision of the results in comparison to on-fibre standardization. Therefore, based on this research, diffusion-based interface model had the most favorable results with the least experimental procedures. This clearly suggests potential use of diffusion-based interface model for future *in vivo* SPME studies.

Chapter 3: Pharmacokinetic studies of fenoterol and methoxyfenoterol in rats using semi-automated *in vivo* solid-phase microextraction sampling and diffusion-based interface calibration model

3.1 Overview

SPME can be used to sample circulating blood of animals without the need to withdraw a representative blood sample. In this study, in *vivo* SPME in combination with liquid-chromatography tandem mass spectrometry was used for pharmacokinetic studies of two drug analytes: fenoterol and methoxyfenoterol on five rats. This research illustrates, for the first time, the feasibility of a diffusion-based interface model calibration for *in vivo* SPME studies. To provide a constant sampling rate as required for the diffusion-based interface model, partial automation of SPME animal sampling was accomplished using Accusampler®, an instrument capable of automated repetitive blood sampling from rats. The use of the Accusampler® with a custom-written program allowed the automation of all *in vivo* SPME steps except the insertion and removal of the SPME probe. Each semi-automated *in vivo* SPME sampling was followed by automatic blood draw in order to enable the comparison of SPME results to traditional analysis based on blood withdrawal and plasma-protein precipitation. The results obtained show good agreement between SPME and the conventional method, indicating the utility of the proposed method. In addition, *in vivo* SPME allowed the monitoring of the metabolite methoxyfenoterol glucuronide, which could not be detected using the traditional plasma protein precipitation method. The proposed diffusion-based interface model has several advantages over other kinetic calibration models for *in vivo* sampling including: (i) It does not require any addition of standard into the sample matrix during *in vivo* studies, (ii) it is simple, rapid and eliminates the need to pre-load appropriate standard into SPME extraction phase and (iii) the calibration constant can be

calculated based on the diffusion coefficient, extraction time, fibre length and radius, and size of the boundary layer. In the current study, the experimental calibration constants of 338.9±30mm^{-3} and 298.5±25 mm^{-3} showed excellent agreement with the theoretical calibration constants of 304.2 mm^{-3} and 315.3 mm^{-3} for fenoterol and methoxyfenoterol, respectively.

3.2 Background

Asthma, a chronic inflammatory disease of the airways, is a growing public concern since it is one of the most prevalent chronic diseases with more than 100 million sufferers worldwide according to statistics from Asthma Society of Canada [1]. Consequently, much effort and resources are put into research of asthma medications each year in the hope of discovering new active compounds to target the disease.

Fenoterol, 5-[1-hydroxy-2-[[2-(4-hydroxyphenyl)-1-methylethyl]-amino]ethyl]-1,3-benzenediol, is a β_2-adrenoceptor (β_2-AR) agonist that is used for the treatment of asthma and congestive heart failure [2-3]. The prescriptive fenoterol is a racemic mixture of (R,R)-fenoterol and (S,S)-fenoterol. However, previous preclinical studies, cellular membrane affinity chromatography studies and cardiomyocyte binding and contraction assays, conducted by Wainer's group at National Institute of Health (NIH), have demonstrated that (S,S)-fenoterol is essentially inactive at the receptor while (R,R)-fenoterol is responsible for the β_2-AR agonist activity [3]. Additionally, the data from these studies have shown that (R,R)-methoxyfenoterol, a fenoterol derivative, has the same activity as (R,R)-fenoterol at the β_2-AR. Thus, it was proposed that (R,R)-methoxyfenoterol could also be used as asthma and heart failure medication. In this research, a comparative pharmacokinetic study of (R,R)-fenoterol and (R,R)-methoxyfenoterol was conducted.

In designing a preclinical study, the sensitivity of the bioanalytical assay is a primary concern. This is especially true with low bioavailability drugs such as fenoterol, with an incomplete absorption and extensive metabolism via phase II pathways [2, 4]. Therefore, the success of the study is greatly dependent on method selection. The first pharmacokinetic study of fenoterol on rats was performed by Koster et al. where fenoterol was extracted by ion-pair

extraction into ethyl acetate using BIS as a pairing agent after various routes of administration [4]. Recently, Siluk et al. performed plasma-concentration time analysis on fenoterol using solid phase extraction (SPE) [2], and Kim et al. developed an online immunoextraction method for better sensitivity and specificity extraction of fenoterol [5]. However, all these methods measured the drug concentration in plasma samples which require collection of blood samples of at least 0.150 mL [5] and further processed to plasma.

Since the invention of SPME, a novel sampling technique, *in vivo* studies such as drug dosing and pharmacokinetics have been made simpler with the integration of sampling and sample preparation [6]. SPME allows direct extraction of a drug circulating in animal blood without the need to withdraw any blood sample. This eliminates the need to separate plasma from whole blood, speeds up overall sample preparation time and minimizes inadvertent analyte losses during sample preparation because the number of sample handling steps is greatly reduced. Most importantly, SPME allows the monitoring of free or unbound concentrations of drug analyte where most conventional methods offer the total concentrations [7-8]. Measurement of free concentration is important as it is the unbound drug analyte that determines its efficacy.

The main objective of this research was to apply the diffusion-based interface calibration model for the first time to *in vivo* SPME pharmacokinetic studies in rats. However, initial pharmacokinetic studies performed on rats using *in vivo* SPME relied on a polyurethane interface connected to the carotid artery catheter and manual assisted agitation using push-pull method with a syringe [9]. This type of manual sampling would not provide sufficiently uniform agitation to enable the use of diffusion-based interface calibration, so in current research the process of *in vivo* SPME sampling was further automated using AccuSampler®, a commercial instrument automated for repetitive blood sampling, The automated system allowed for a much easier sampling procedure, minimal animal contact, and provided uniform and reproducible flow rates

within the tubing which permitted the use of diffusion-based interface calibration model for *in vivo* SPME studies for the first time. The model was successfully applied to comparative pharmacokinetic study of fenoterol and methoxyfenoterol on five rats. In addition, a conventional method based on blood withdrawal was performed to validate the SPME data obtained.

3.3 Materials and methods

3.3.1 Materials

(R,R)-Fenoterol and (R,R)-methoxyfenoterol were obtained from National Institute of Health (Baltimore, MD, US). Pseudoephedrine and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, US). The standards were diluted to different concentrations either in methanol for instrument calibration or PBS pH 7.4, for *in vitro* experiments. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, US). Fresh rat whole blood (sodium heparin as anticoagulant) and plasma were obtained from NoAb BioDiscoveries Inc. (Mississauga, ON, Canada). Rat whole blood was maintained at 4^0C for maximum 1 week and plasma was kept frozen at -20^0C until use. Deionized water was obtained from a Barnstead/Thermodyne Nano-pure ultrapure water system (Dubuque, IA, US).

3.3.2 LC-MS/MS Assay- Please see section 2.2.2

3.3.3 *In Vitro* SPME Method Development- Please see section 2.2.3

3.3.4 Animal Experiments

Ten conscious Sprague-Dawley rats (Charles River Labs, St. Constant, PQ, Canada) weighing approximately 300 g were used to conduct *in vivo* experiments: five rats for fenoterol and five for methoxyfenoterol. Animals were conscious and freely moving throughout the study.

The catheter from the animal was linked to the AccuSampler® tubing system (Dilab, Lund, Sweden). Rats were administered 5 mg/kg of either fenoterol or methoxyfenoterol. At each time point, a pre-conditioned, sterile SPME device was inserted by piercing the septum of the interface and only the coated portion of the wire housed inside the hypodermic needle was exposed to the flowing blood. The flow rate of the pump was 1.4 mL/min which was well below normal blood flow rate in the rat carotid artery for minimal disturbance. The push-pull volume was 0.35 mL. After sampling, the blood was returned to the animal and the catheter was flushed with saline to compensate for the lost blood to maintain fluid balance within the animal. For each time point, two samplings were performed: SPME sampling was performed first followed by blood withdrawal of 0.2 mL for conventional plasma analysis

3.3.5 *In Vivo* SPME Procedure

Before drug administration, a zero time probe analysis was performed followed by blood draw (0.2 mL). Drug concentration was monitored for 6 hours at time points 3, 15, 30, 60, 120, 180, 240, 300, and 360 minutes. After 4 minutes of extraction, the probe was removed from the interface and quickly rinsed with water to prevent blood clotting at the tip of the hypodermic needle. Residual water on the fibre was blotted lightly with a Kimwipe tissue. The SPME probes were stored at -20^0C until analysis was performed. A new fibre was used for each time point.

The probes collected were desorbed in 0.1 mL inserts with desorption solvent for 1 hour with vortex (2400 rpm). A 0.01 mL portion of pseudoephedrine (50 ng/mL) was added and the

resulting solution was vortexed manually. A 0.02 mL portion was injected for analysis using the chromatographic conditions as stated in section 2.2.2.

3.3.6 Conventional Plasma Sampling and Analysis

For validation purposes, 0.2 mL blood was withdrawn after SPME sampling. The time points when blood draws were performed were 7, 19, 34, 64, 124, 184, 244, 304, 364 min. The blood sample was subsequently centrifuged (6700 rpm, 5 min, 4^0C) and plasma was isolated and frozen at -20^0C in 2 mL cryovials (Wheaton Science Products, Millville, NJ, US) until analysis. For analysis, protein precipitation was first performed. 0.08 mL of plasma was mixed with 0.30 mL of HPLC grade acetonitrile in conical centrifuge vials. After vortex mixing (2400 rpm, 5 min) and centrifugation (14,000 rpm, 10 min, 4^0C), 0.01 mL of the supernatant was transferred to a 0.1 mL insert (Supelco, Bellefonte, PA, US) followed by an addition of 0.09 mL of desorption solvent for dilution and 0.01 mL of pseudoephedrine (50 ng/mL) as internal standard to control for injection volume variation. The resulting solution was vortexed manually and 0.02 mL was injected for analysis using the chromatographic conditions as stated in section 2.2.2.

3.3.7 Calibration Methods

For diffusion-based interface model calibration, the diffusion constant was determined using 100 ng/mL of 10 mL whole blood sample (n=5 determinations). The blood sample was incubated at 37^0C and sampling was performed with the AccuSampler® pump using the same sampling parameters as the *in vivo* studies on the animals. The determined diffusion-based constant was applied to calculate the circulated concentration at each time point.

3.4 Results and Discussions

3.4.1 Calibration Method - Diffusion-based Interface Model

For the diffusion-based interface model, the calibration constant was obtained by performing SPME procedure in rat whole blood spiked with known amount of fenoterol or methoxyfenoterol. To mimic the actual *in vivo* experiment, 10 mL whole blood of 100 ng/mL fenoterol or methoxyfenoterol was incubated at 37^0C and all other sampling parameters i.e. sampling rate and extraction time remained the same. Using the amount extracted and the known concentration in the sample, the calibration constant can be determined. Extractions were performed five times to obtain a better precision.

The theoretical calibration constant was determined similar to section 2.3.4.

	b (mm)	δ (K•min^2/mm^3)	Re	Sc (mm^4/min^2•K)	D_L (K•min/mm^2)	L (mm)	t (min)
Fenoterol	0.145	3.15E-4	0.540	1.05E10	1.89E-8	15	4
Methoxyfenoterol	0.145	3.14E-4	0.540	1.09E10	1.82E-8	15	4

Table 20: Parameters used to calculate calibration constant for fenoterol and methoxyfenoterol. $Sc = \nu/D_L$, where ν is the kinematic viscosity of water, and $Re = ud/\nu$, where u is the linear velocity of the sample and d is diameter of the fibre used.

The radius of the fibre was 0.145 mm. All parameters, including the kinematic viscosity of blood, sampling time, and temperatures, were the same as section 2.3.4. The molar volume to calculate the diffusion coefficient was calculated by summing the size of the atoms making up the chemical's structure [10]. For fenoterol, the molar volume is 309800 mm^3/mol and 330300 mm^3/mol for methoxyfenoterol.

	Experimental (n = 5) (mm^{-3})	Theoretical (mm^{-3})
Fenoterol	338.9 ± 30	304.2
Methoxyfenoterol	298.5 ± 25	315.3

Table 21: A comparison between experimental and theoretical calibration constant for fenoterol and methoxyfenoterol

Similar to the diffusion-based interface model calibration performed in chapter 2, the theoretical calibration constant for both fenoterol and methoxyfenoterol is either within or very close to the range of the determined experimental constant.

In terms of the calibration method, the diffusion-based interface model is convenient as it does not require any addition of standard. If literature values can be accessed easily, the calibration constant can be calculated and the calibration step can be omitted altogether. In the case where the values for the parameters can only be estimated, as in the case for fenoterol and methoxyfenoterol since these analytes are not well researched, a calibration constant obtained experimentally may be more accurate. Nevertheless, the calibration method for the diffusion-based interface model is simple and therefore offers an attractive alternative to other previously used pre-equilibrium calibration methods

3.3.2 Pharmacokinetic Profiles

The sampling procedures employed for *in vivo* SPME were much simpler compared to previous pharmacokinetic studies on rats [9]. Since the interface was connected to the Accusampler® instead of on the nape of the animal's neck, direct handling of the animal during sampling was avoided. In addition, during the sampling time of 4 minutes, the animal was allowed to move freely in the cage where as in the previous pharmacokinetic studies, the animal

was held by the analyst during the time of extraction (Figure 18). This causes extra stress on the animal during the experiment and might have possible effect on the accuracy of the results.

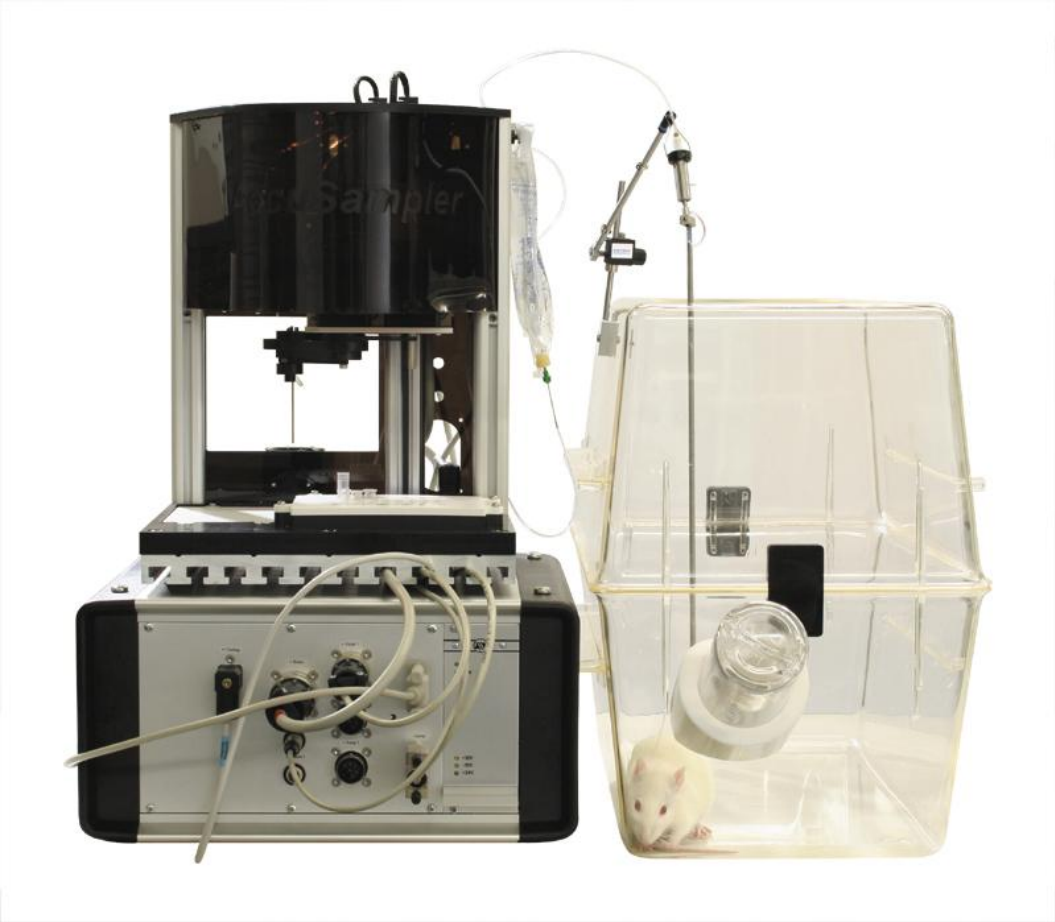

Figure 18: Connection of Accusampler ® with a rat [11]

The fenoterol (Figure 21) and methoxyfenoterol (Figure 22) concentration versus time profiles obtained by SPME sampling which measured the concentrations in whole blood were validated by conventional method, protein precipitation, which measured the analyte concentrations in plasma. The distribution of fenoterol and methoxyfenoterol are different in plasma and whole blood. Therefore, the results from plasma and whole blood cannot be compared directly for drugs whose red blood cell (RBC) to plasma partition ratio is different from 1. In current study, the RBC to plasma partition ratio was determined for fenoterol and methoxyfenoterol using SPME.

Extractions were performed in SPME and plasma sample spanning the circulated concentration for fenoterol and methoxyfenoterol. The RBC-plasma partition ratio was determined by the ratio of the slope plotted with concentration against the amount extracted (Figure 19, 20).

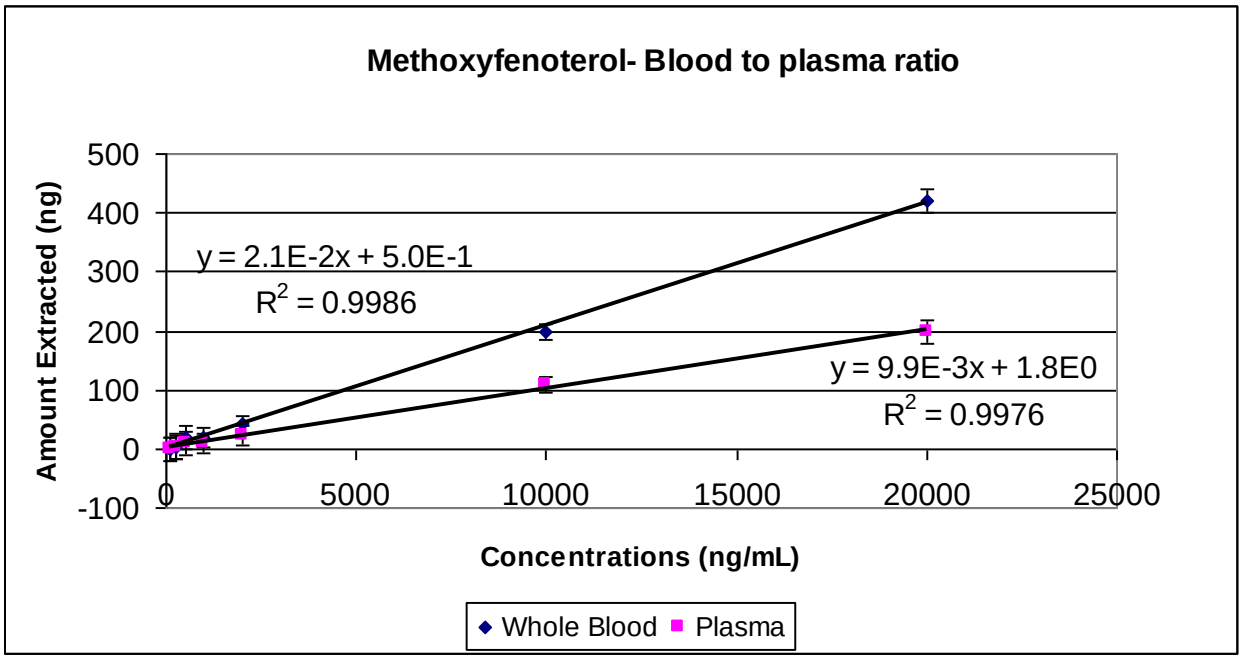

Figure 19: Determination of blood to plasma ratio of methoxyfenoterol using the slope of concentration versus amount extracted

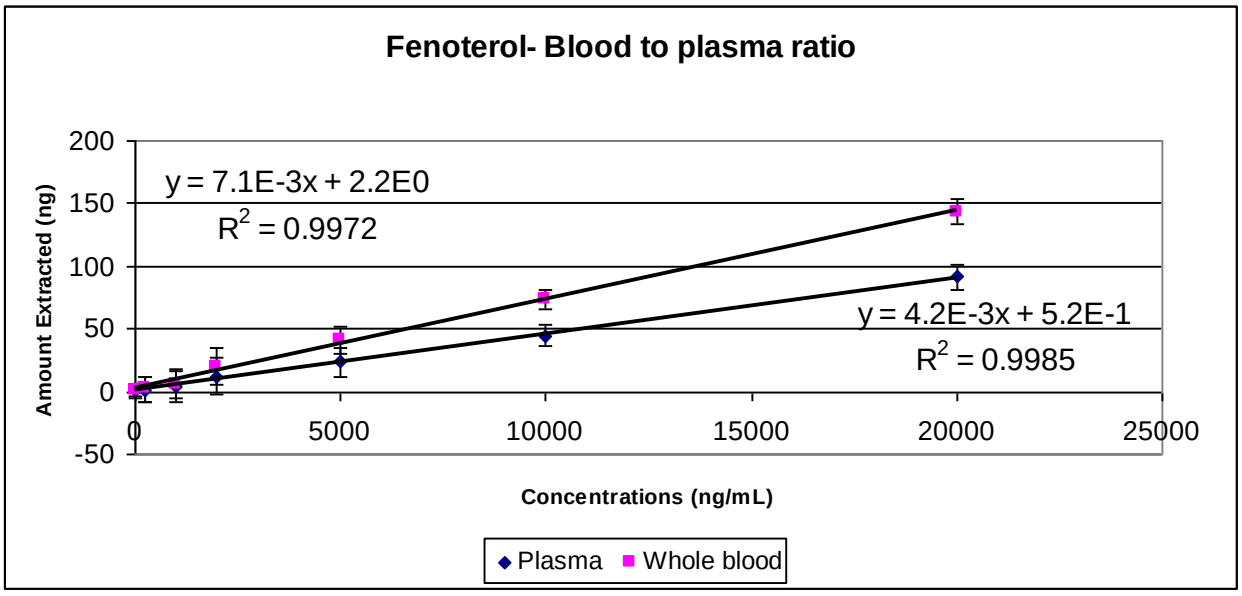

Figure 20: Determination of blood to plasma ratio of fenoterol using the slope of concentration versus amount extracted

From this experiment, the conversion factor of 1.7 and 2.1 for fenoterol and methoxyfenoterol was determined and applied to the plasma sample. Koster et al. also determined the RBC-plasma partition ratio to be 1.76±0.10 [4], which further validated the SPME method to determine the RBC-plasma partition ratio.

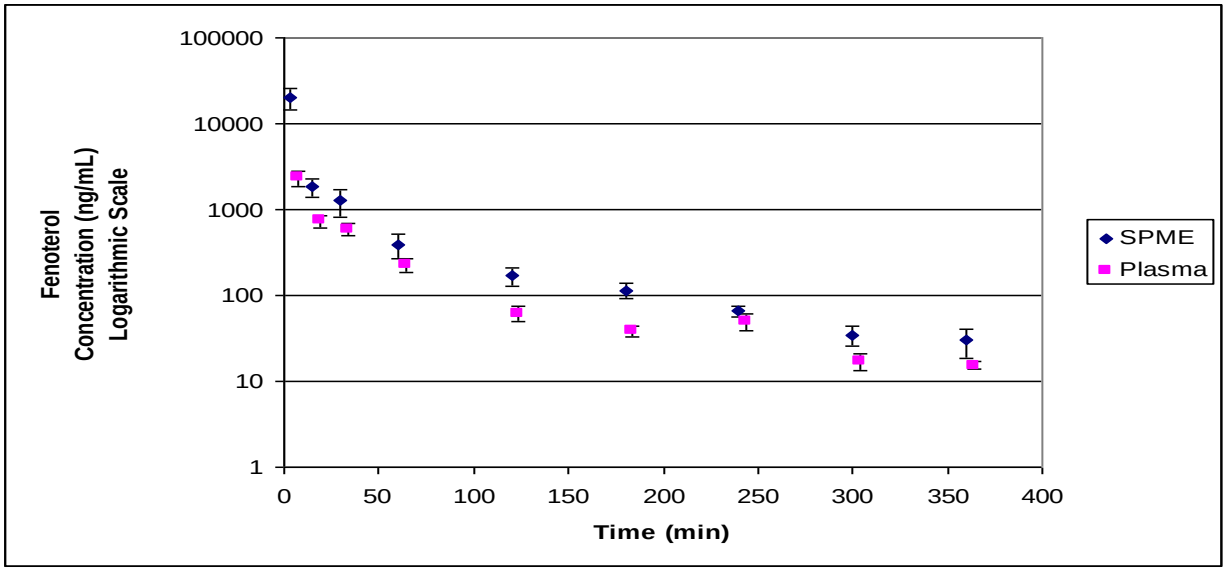

Figure 21: Mean concentration versus time profiles of fenoterol in logarithmic scale (n =5) following 5 mg/kg i.v. administration. (•) represents data points obtained by SPME and (■) represents data points obtained by conventional method. Plasma data was corrected for RBC-plasma partition ratio of 1.7 to facilitate the comparison of two methods. Diffusion-based calibration was used to calibrate results obtained by SPME.

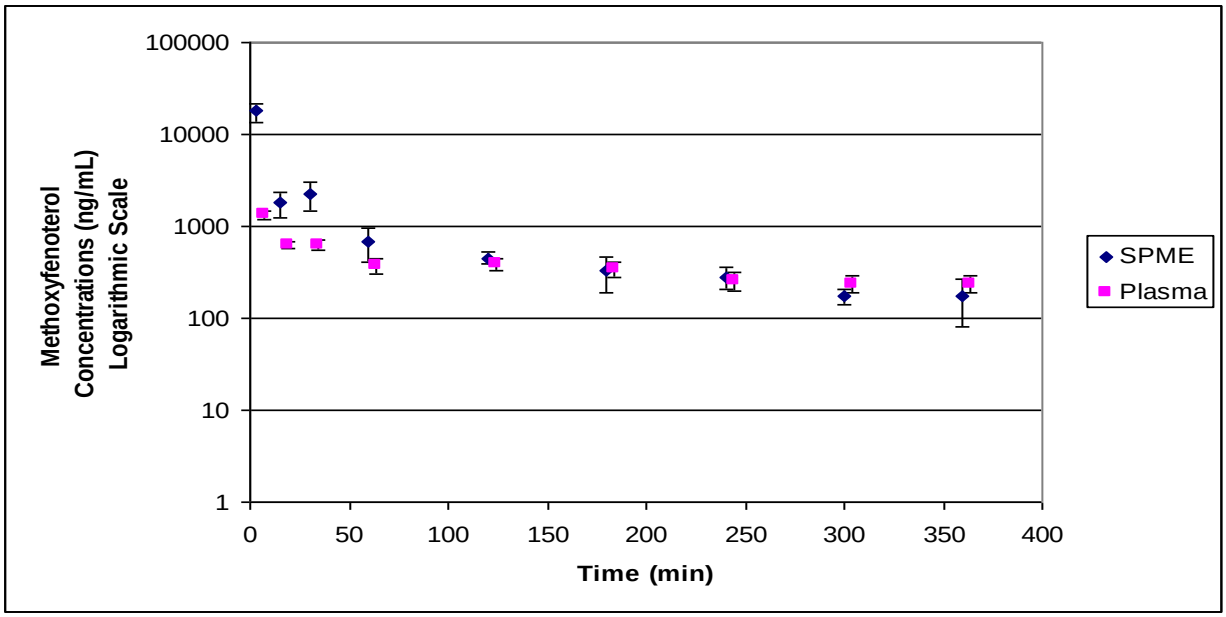

Figure 22: Mean concentration versus time profiles of methoxyfenoterol (n =5) in logarithmic scale following 5 mg/kg i.v. administration. (•) represents data points obtained by SPME and (■) represents data points obtained by conventional method. Plasma data was corrected for RBC-plasma partition ratio of 2.1 to facilitate the comparison of two methods. Diffusion-based calibration was used to calibrate results obtained by SPME.

The pharmacokinetic profiles of both analytes show a good correlation between the concentrations obtained by SPME method and conventional method except for the earlier time points where the concentrations measured by SPME were higher than concentrations measured by conventional method. This could be explained by the rapid concentration changes following drug administration, the fact that SPME sampling was performed over a 4-min period and the time difference between SPME sampling and blood withdrawals; for the conventional method, blood was withdrawn 4 minutes following SPME. As time increased, the concentration change became less dramatic, and therefore, the measured concentrations between the two sampling methods showed much better correlation at later time points. Siluk et al. also performed pharmacokinetic study on fenoterol [2]. The profiles obtained displayed similar pattern as was obtained using SPME.

In addition to measuring the concentrations of the administered analytes, the metabolites of both fenoterol and methoxyfenoterol were monitored simultaneously. Fenoterol glucuronide was not detected with SPME and conventional sampling but methoxyfenoterol glucuronide was detected only with SPME (Figure 23). This further illustrates the sensitivity of the SPME method. Due to the lack of methoxyfenoterol glucuronide standard, the exact concentrations of the detected metabolite could not be determined at this time. However, this is the first report of the use of *in vivo* SPME to sample very polar metabolites such as glucuronides thus further demonstrating the sensitivity and usefulness of SPME for the study of metabolism.

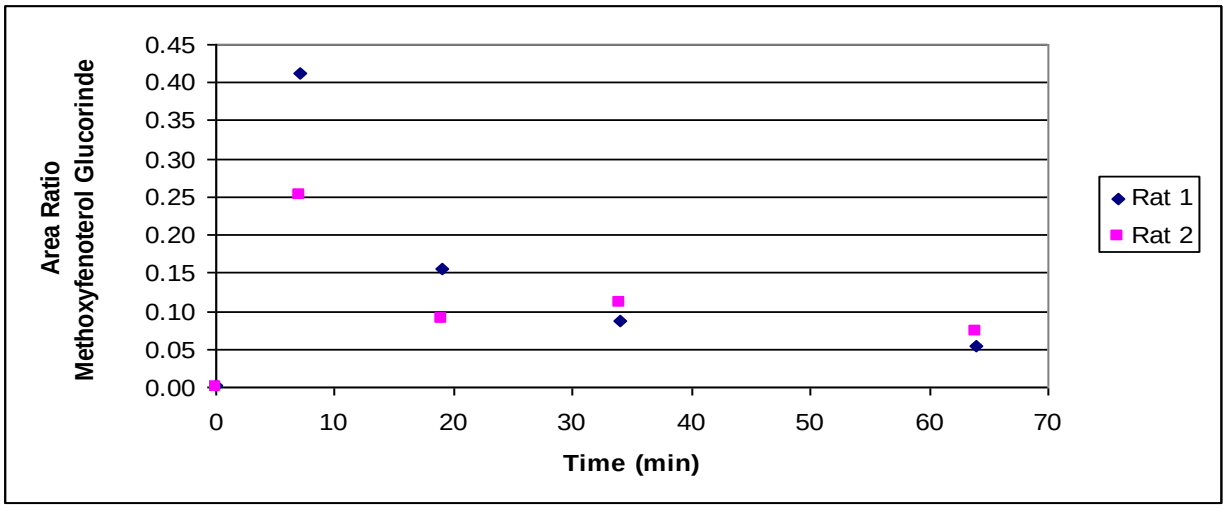

Figure 23: Detection of methoxyfenoterol glucuronide (n=2) simultaneously with methoxyfenoterol. The presence of methoxyfenoterol glucuronide was only detected at the first 4 time points i.e. 3, 15, 30, and 60 minutes for both rats.

3.4 Conclusions

Diffusion-based interface model calibration and the use of an automated SPME blood sampler, AccuSampler®, were successfully applied for the first time to *in vivo* SPME. Use of the AccuSampler® simplified the extraction procedures and minimized direct contact with the animal during the experiment. In addition, it provided a constant blood flow into the interface for extraction, a requirement for diffusion-based interface model calibration. Diffusion-based interface model calibration, unlike previously used kinetic calibration, does not require any other deuterated or radioactive compounds as standards and does not require any pre-loading steps prior to *in vivo* experiments. The calibration constant can be estimated using theoretical calculations and parameters obtained from literature or can simply be determined by extraction with a known concentration sample and using the same sampling parameters as the actual *in vivo* experiments. Therefore, this calibration approach not only extends the applicability of pre-equilibrium *in vivo* SPME to situations where no appropriate standard is available, but also greatly simplifies or even eliminates the need for experimental calibration.

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