

# Evaluation of Blood Draw Free Solid Phase Microextraction (SPME) Sampling to Determine the Pharmacokinetics of R,R-Fenoterol and R,R-Methoxyfenoterol in Conscious Rats

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## ABSTRACT

A blood draw free SPME *in vivo* sampling method was developed and compared to conventional plasma sampling for the determination of the pharmacokinetics of the  $\beta_2$ -adrenergic receptor agonist, fenoterol (Fen), and a pharmacologically active analog, methoxyfenoterol (MFeN). Five mg/kg Fen or MFeN were administered intravenously to groups of rats. For conventional sampling, plasma was isolated following collection of serial arterial blood samples. Plasma concentrations of Fen and MFeN were determined by protein precipitation and LC-MS/MS analysis. Plasma clearances ( $CL_p$ ) for Fen and MFeN were similar (2969  $\pm$  310 and 2726  $\pm$  242 mL/h/kg (n=3), respectively), whereas their steady-state volumes of distribution ( $V_{ss}$ ) differed considerably (1928  $\pm$  503 and 6374  $\pm$  720 mL/kg, respectively). The terminal half-life of Fen was slightly shorter than MFeN (2.54  $\pm$  0.21 and 3.16  $\pm$  0.54 h, respectively). The fraction of MFeN metabolized to Fen averaged 0.96  $\pm$  0.40%. For SPME sampling, prototype SPME probes (Supelco) with biocompatible extractive coating were used. A sampling interface was designed to facilitate insertion of the SPME probes for repeated sampling from arterial blood. A diffusion-based, pre-equilibrium, internal standard-free calibration method for quantitative analysis of the 2 analytes by SPME was employed. A constant sampling rate (1.4 mL/min) was accomplished by an AccuSampler<sup>®</sup>. Total blood concentrations of Fen and MFeN were determined by SPME sampling and LC-MS/MS analysis. Total blood concentrations were consistently greater than corresponding plasma concentrations determined by conventional sampling. Estimated blood clearances ( $CL_b$ ) for Fen and MFeN were lower than  $CL_p$  (1205  $\pm$  129 and 812  $\pm$  97.7 mL/h/kg (n=5), respectively), as were  $V_{ss}$  values (560  $\pm$  177 and 1592  $\pm$  491 mL/kg, respectively). This is primarily a result of red blood cell partitioning/binding since the blood to plasma concentration ratios (B/P) of Fen and MFeN were 1.71  $\pm$  0.10 and 2.03  $\pm$  0.15, respectively.  $CL_b$ 's calculated from the corresponding B/P and  $CL_p$  values were 2061  $\pm$  221 and 1649  $\pm$  198 mL/h/kg, respectively, indicating that SPME sampling holds promise as a valuable method for blood draw free sampling from rodents.

## INTRODUCTION

Solid Phase Microextraction (SPME) is a simple, fast, sensitive and convenient sample preparation technique, which minimizes solvent usage while integrating sampling and sample preparation steps [1]. In the current application, a wire with an immobilized extractive coating is introduced into a sample. The amount of an analyte extracted at equilibrium is given by:

$$n_e = \frac{C_0 K_A V_f V_s}{K_B V_f + V_s} \quad V_s \gg V_f K_B \quad n_e = C_0 K_B V_f V_s \quad (1)$$

$C_0$  = the initial sample concentration of analyte  
 $n_e$  = amount of analyte extracted at equilibrium  
 $V_s$  = sample volume  
 $V_f$  = fibre coating volume  
 $K_B$  = analyte distribution constant between fibre coating and sample matrix

When  $V_s \gg V_f K_B$ ,  $n_e$  is directly proportional to  $C_0$  and a defined sample volume is not required. This allows the direct extraction of a drug circulating in blood without the need to withdraw a blood sample. It also eliminates the need to separate plasma from whole blood, speeds up overall sample preparation time and minimizes analyte losses during sample preparation by reducing the number of sample handling steps. Most importantly, SPME allows the monitoring of both unbound and total concentrations of drug whereas most conventional methods determine only total concentrations.

Previous *in vivo* SPME studies relied on either equilibrium extraction using polypropylene, a thin extractive coating [2,3], or pre-equilibrium desorption of an isotropic standard pre-loaded on a C18 coated probe [4]. The C18 coating has a more universal extractive efficiency than polypropylene but requires a pre-equilibrium extraction method because the time for equilibrium is too long. The present study investigates the feasibility of a diffusion-based, pre-equilibrium, internal standard-free calibration method. The amount of analyte extracted by this method is controlled by analyte diffusion through a static boundary layer surrounding the submerged fibre in the sample. During pre-equilibrium extraction, diffusion of the analyte occurs from the high concentration in the bulk sample to the low concentration in the sorbent. This allows the rate of extraction to be correlated linearly to the concentration of the analyte in the sample [5]. The concentration of the bulk sample can be determined as follows:

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

where  $n$  is the amount of analyte extracted at time  $t$ ,  $b$  is the outside radius of the fibre coating,  $\delta$  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. The thickness of boundary layer can be calculated as follows:

$$\delta = 9.52(b / Re)^{0.62} Sc^{0.38} \quad (3)$$

where  $Re$  is the Reynolds number and  $Sc$  is the Schmidt number. Because the concentration depends on the thickness of boundary layer, the agitation rate during sampling must be constant in order to provide a constant boundary layer thickness throughout the experiment.

Fenoterol (Fen), which is marketed as a racemic mixture of the R,R and S,S isomers, is a selective  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) agonist in clinical use for the treatment of asthma. The R,R isomer represents the pharmacologically active component and is currently undergoing clinical trials for the treatment of congestive heart failure (CHF). An analog, R,R-methoxyfenoterol (MFeN), which has similar activity at the  $\beta_2$ -AR, is also being investigated for CHF since preliminary studies in rats indicated that MFeN may have a better oral bioavailability and longer terminal half-life than Fen [6].

The objective of the present study was to compare the pharmacokinetics (PK) of Fen and MFeN utilizing a diffusion-based, pre-equilibrium calibration method for SPME sampling which allowed the use of an extractive coating and can be used for a wide range of analytes as well as eliminated the need for an isotropic internal standard.

## METHODS

### In Vitro SPME Method Development

R,R-Fen and R,R-MFeN were provided by Dr. Irwin Wainer (NIH). Prototype SPME probes obtained from Supelco with C16-amide coating (45  $\mu$ m) were selected for *in vivo* sampling as this coating had the highest extraction efficiency (compared to C18 and cyano propyl) for Fen and MFeN [6]. To enhance extraction efficiency, probes were conditioned for at least 30 min in ACN/MeOH (80/20 v/v) prior to use. Following sampling, analytes were desorbed from the probes in 0.1 mL ACN/MeOH (80/20 v/v) for 1 h on a vortex mixer.



Prototype SPME probes from Supelco.

To determine the optimal extraction time for *in vivo* sampling, the sampling speed and extraction time was evaluated *in vitro*. Extractions were performed in whole blood (37°C) spiked with 100 ng/mL Fen or MFeN, for either 2 or 4 min at 0.6 and 1.4 mL/min.

To confirm that the amount of analyte extracted by the pre-equilibrium sampling method is much lower than that at equilibrium, extractions were performed in whole blood spiked with 100 ng/mL Fen or MFeN at 37°C for 4, 5, 10, 30, 60 and 90 min at 1.4 mL/min.

To compare plasma concentrations determined by conventional sampling to blood concentrations determined by SPME, the blood to plasma concentration ratio for Fen and MFeN were determined (10 to 20,000 ng/mL) at 37°C by SPME sampling at equilibrium.

### In Vivo Experiments

Conscious, freely moving male Sprague-Dawley rats implanted with catheters in the carotid artery and jugular vein were used. Groups of rats were administered 5 mg/kg Fen or MFeN *i.v.* For conventional sampling, blood (0.25 mL) was manually withdrawn from the arterial catheter and plasma isolated and stored frozen until analysis. For SPME sampling, a custom-made sampling interface, connected by a stainless steel connector to the arterial catheter, was used for insertion of the SPME probes into the blood [3]. For each sampling period (4 min), a pre-conditioned SPME probe was inserted into the sampling interface, the coating was exposed to the blood and the probe (0.35 mL) was successively pulled and pushed over the exposed SPME coating at a rate of 1.4 mL/min with an AccuSampler<sup>®</sup> (DILab). After sampling was completed, the SPME probe was removed from the interface and rinsed with water (30 s). The probes were stored frozen until analysis. After each sampling interval, blood remaining in the interface was returned to the animal and the interface and catheter flushed with heparin-containing saline.

The diffusion constant was determined at 37°C for 100 ng/mL of each analyte spiked in 10 mL whole blood (n=5 determinations). Sampling was performed over 4 min at 1.4 mL/min with the AccuSampler<sup>®</sup> pump. The experimentally determined diffusion constant and the amount extracted were used to calculate the concentration at each time point (Equation 2). Concentration versus time data were analyzed by noncompartmental methods using WinNonlin Pro 4.0 (Pharsight Corp., Mountainview, CA).

### Sample Analysis

Sample analysis for quantification of MFeN and Fen in whole blood and plasma was conducted using LC-MS/MS (PE Sciex API 3000 or 4000). For blood analysis, pseudophedrine was used as an internal standard and analytes were separated chromatographically on an Atlantis HILIC Silica 3  $\mu$ m column, with gradient elution. The calibration ranges were 7.5 – 20,000 ng/mL and 10 – 20,000 ng/mL, respectively, for MFeN and Fen in whole blood. Calibration standards >5000 ng/mL were diluted in desorption solvent prior to analysis. For plasma analyses, mianserin was used as the internal standard. Sample clean-up of plasma was conducted by protein precipitation with acetonitrile, and analytes were separated chromatographically on a ZORBAX XDB-C18 3.5  $\mu$ m column, with gradient elution. The calibration range was 1 – 1000 ng/mL for both analytes in plasma.

## RESULTS

### In Vitro SPME Method Development

- A sampling rate of 1.4 mL/min over 4 min provided sufficient sensitivity, based on the amount extracted, for PK studies (Fig. 1).
- The amount extracted at a sampling rate of 1.4 mL/min for 4 min was confirmed to be well below that at equilibrium (Fig. 2).
- The calibration constants for Fen and MFeN were determined experimentally (Fen = 339 $\pm$ 30 & MFeN = 299 $\pm$ 25 mm<sup>2</sup>) and are compared to those estimated mathematically by Equations 2 & 3 (Fen = 304 & MFeN = 315 mm<sup>2</sup>). The experimentally determined calibration constants were used to determine blood concentration from the amount extracted.
- The blood to plasma concentration ratios for Fen and MFeN were determined to be 1.71  $\pm$  0.10 and 2.03  $\pm$  0.15, respectively (data not shown).

## References

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Figure 1. Mean ( $\pm$  S.D., n=3) % extracted from blood as a function of sampling rate & interval.

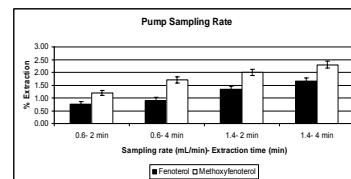
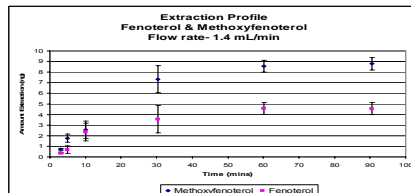


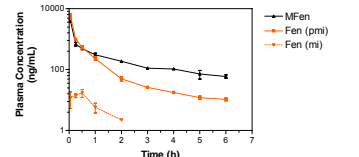
Figure 2. Mean ( $\pm$  S.D., n=3) amount extracted (ng) from blood versus time profile.



### In Vivo Experiments

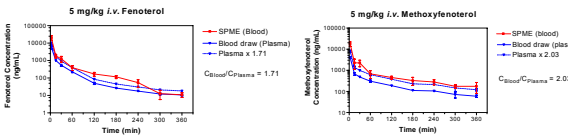
- Plasma concentration versus time profiles for Fen and MFeN suggest that the terminal half-life of MFeN is slightly longer than that of Fen (Fig. 3, Table 1), predominantly due to a significantly larger steady-state volume of distribution ( $V_{ss}$ ). No difference in the plasma clearance between Fen and MFeN was observed. The fraction of MFeN metabolized to Fen is small (0.955  $\pm$  0.395).
- The blood concentration versus time profiles measured by SPME sampling are greater than those measured in plasma (Figs. 4 and 5), consistent with blood to plasma concentration ratios (B/P) that are greater than unity for both Fen and MFeN. As a consequence, estimated systemic blood clearances for Fen and MFeN were lower than plasma clearances (Table 1), as were  $V_{ss}$  values. Systemic plasma clearances calculated by correction for the corresponding B/P values were 2061  $\pm$  221 and 1649  $\pm$  198 mL/h/kg, respectively.
- Estimated PK parameters from SPME-derived blood concentration versus time data suggest that the CL from blood is smaller, and the  $V_{ss}$  is larger for MFeN than Fen, resulting in a longer terminal  $t_{1/2}$  for MFeN than Fen in blood (Table 1).

Figure 3. Mean ( $\pm$  S.D., n=3) plasma concentrations following *i.v.* dosing of MFeN and Fen (pmi). Fen (mi) formed from MFeN is also shown. Sampling by blood draws.



Parameter	Units	Methoxyfenoterol		Fenoterol (pmi)	
		Blood draw	SPME	Blood draw	SPME
$C_0$	ng/mL	6168 $\pm$ 597	30367 $\pm$ 7062	9040 $\pm$ 2364	37764 $\pm$ 14450
Terminal $t_{1/2}$	h	3.16 $\pm$ 0.544	2.63 $\pm$ 0.764	2.54 $\pm$ 0.214	0.968 $\pm$ 0.143
AUC <sub>0-inf</sub>	h*ng/mL	1888 $\pm$ 158	6227 $\pm$ 734	1697 $\pm$ 189	4189 $\pm$ 470
CL	mL/h/kg	2660 $\pm$ 216	812 $\pm$ 97.7	2969 $\pm$ 310	1205 $\pm$ 129
MRT <sub>0-inf</sub>	h	2.64 $\pm$ 0.419	2.01 $\pm$ 0.744	0.644 $\pm$ 0.119	0.457 $\pm$ 0.105
$V_{ss}$	mL/kg	6970 $\pm$ 509	1592 $\pm$ 491	1928 $\pm$ 503	560 $\pm$ 177

Table 1. Mean ( $\pm$  S.D.) PK parameters for MFeN and Fen (pmi) in plasma (blood draw, n=3) and blood (SPME, n=5) following *i.v.* dosing of MFeN and Fen.



Figures 4 and 5. Mean ( $\pm$  S.D.) concentrations following *i.v.* dosing to groups of rats. Blood concentrations determined by SPME sampling (n=5) are compared to plasma concentrations determined by manual blood draw and isolation of plasma (n=3). Plasma concentrations corrected for the blood/plasma concentration ratios are also shown.

## CONCLUSIONS

- Differences in the PK of Fen and MFeN were more pronounced when determined by SPME than by conventional plasma sampling, largely as a result of blood to plasma partitioning/binding of Fen and MFeN. Recent data suggests that both temperature and time-dependent partitioning/binding of Fen (but not MFeN) to RBCs may complicate its PK profile.
- The data indicate that pre-equilibrium SPME sampling, utilizing the diffusion-based, internal standard-free calibration method with C16-amide type coatings, holds promise as a valuable universal tool for blood draw free sampling and the PK determination of potential drug candidates in rodents.