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Comparison and validation of calibration methods for *in vivo* SPME determinations using an artificial vein system

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ABSTRACT

The success of *in vivo* solid phase microextraction (SPME) depends significantly on the selection of calibration method. Three kinetic *in vivo* SPME calibration methods are evaluated in this paper: (1) on-fibre standardization (OFS), (2) dominant pre-equilibrium desorption (DPED), and (3) the diffusion-based interface (DBI) model. These are compared in terms of precision, accuracy, and ease of experimental use by employing a flow device simulating an animal circulatory system. In addition, the kinetic calibration methods were validated against established SPME equilibrium extraction (EE) external calibration and a conventional sample preparation method involving protein precipitation. The comparison was performed using a hydrophilic drug fenoterol as the analyte of interest. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used for the determinations. All three kinetic methods compared well with both EE extraction and the conventional method in terms of accuracy (93–119%). In terms of precision, the DBI model had the best precision in whole blood and buffered phosphate saline solution with %RSD similar to the standard techniques (9–15%). DPED had the poorest precision of %RSD (20–30%) possibly due to errors associated with uncertainty in the amount of standard loaded on-fibre and remaining on the fibre after desorption. In addition, incurred errors could result due to the greater number of fibres used in comparison to the other two calibration methods. The precision of the OFS procedure was better than for DPED primarily because the use of multiple fibres is eliminated. In terms of the ease of use for calibration, the DBI model was the simplest and most convenient as it did not require standards once it had been calibrated or the uptake constant was calculated. This research suggests the potential use of DBI model as the best kinetic calibration method for future in-vein blood SPME investigations.

1. Introduction

Solid phase microextraction (SPME) has gained popularity in recent years for *in vivo* applications due to its simple sampling technique and its advantages over conventional methods [1–7]. These include preservation of the circulating blood volume of the animal and minimal disturbance to the chemical balance of the system because, depending on SPME conditions chosen for the analysis, substance depletion can be rendered negligible as only a small amount of the free analyte is extracted. Therefore, more data points can be obtained per animal and thus inter-animal variation can be eliminated for achieving accurate results. The amount extracted with a SPME probe at equilibrium can be determined by the following equation:

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

where n_e is the amount extracted, K_{fs} is the distribution constant of the analyte between the fibre and sample matrix, V_f is the volume of the fibre, V_s is the volume of the sample and C_0 is the bulk concentration of the target analyte in the sample [8]. Eq. (1) can be simplified to Eq. (2), when the volume of the sample is large enough so that $K_{fs} \cdot V_f \ll V_s$:

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

The simplification of the equation further illustrates two advantages of equilibrium SPME for on-site or *in vivo* analysis: first, the amount of analyte extracted is directly proportional to the initial analyte concentration of the sample, and second, a defined sample volume is deemed unnecessary [8]. During extraction, the probe is exposed to the sample, and the amount of analyte extracted can be used to calculate the initial analyte concentration in the sample.

Successful use of *in vivo* SPME is dependent on the selection of calibration method. The main difficulty to calibrate *in vivo* SPME is the variability of blood flow rate in an animal which affects the amount of analyte extracted by SPME. Equilibrium extraction remains an attractive option because during *in vivo* studies in a conscious animal, the blood flow rate within the catheterized blood

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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 EE [9]. Lord et al. performed the first *in vivo* SPME pharmacokinetic studies to measure the circulating concentration of diazepam and its metabolites in beagles using an extraction time of 30 min, which was sufficient to establish equilibrium between the fibre and blood. In this work the performance of the EE was validated against standard plasma analysis procedures. However, such a long sampling time limits temporal resolution because the determined concentration would be an average of the overall sampling time period [3].

Therefore, EE is only feasible with thin coatings such as polypyrrole [1]. Unfortunately, fibres with very thin coating have not been successfully produced with very good inter-fibre reproducibility to date, which prevents their use for quantitative analysis [9]. In contrast, fibres with thicker coatings (45 μm) were recently developed by Supelco and aimed for single-use *in vivo* with good inter-fibre reproducibility [10]. For these fibres or any fibres with thick coatings [10], pre-equilibrium extraction in combination with kinetic calibration offers an alternative sampling method for *in vivo* studies [11–20]. To date several pre-equilibrium extraction calibration methods were developed and derived by a proposed theoretical model based on a diffusion-controlled mass transfer processes [11,12]. Using the proposed model, Chen et al. demonstrated the feasibility of using the desorption process to calibrate the extraction process since a symmetric relationship existed between the amount of standard desorbed from the fibre and the amount of analyte extracted from the sample matrix [13,17,18]. This kinetic calibration, called on-fibre standardization (OFS), was subsequently applied to the pharmacokinetic studies of beagles [1,3,4] and rats [5].

This calibration method, however, may not be useful when the availability of standard is limited. For those analytes with no suitable standards or when the standard is toxic towards the living system, a standard-free pre-equilibrium extraction calibration method, dominant pre-equilibrium desorption (DPED), developed by Zhou et al. can be used [21,22]. For DPED, it was found that the rate of desorption is constant when the pre-loaded amount is 4-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 [21]. Another prominent factor to consider in this calibration method is the distance between the desorption fibre and the extraction fibre. Because the pre-loaded standard is the same as the extracted analyte, these fibres must be kept apart from each other in order to prevent the analyte desorbed from the desorption fibre to enter the extraction fibre. However, the fibres cannot be too far from each other such that the matrices between desorption and extraction could be different [21].

DPED was successfully applied to pesticide extraction from Jade plants [21] and to study accumulation of pharmaceuticals in wild fish [22]. However, 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. This was the case for sampling muscle of wild fish where one fish was used for the extraction and another for desorption [22].

To address the limitations of the DPED calibration model, a diffusion-based interface (DBI) model, which was previously applied to air and water samples [14–16,23,24], was investigated for *in vivo* SPME studies. Similar to DPED, the diffusion-based interface calibration uses the analyte itself to calibrate the amount extracted. However, unlike DPED, this method requires fewer SPME fibres and the size of the sampling area is not of great concern.

In this model, the initial analyte concentration is calibrated using the molecular diffusion coefficient, the amount of analyte extracted, sampling rate, time, temperature, and fibre geometry as shown in Eq. (3) [23]. During pre-equilibrium extraction, the amount of analyte in the sorbent ($C_{sorbent}$) is practically zero. Therefore, an analyte concentration gradient exists across the boundary layer [25]. Analyte diffuses from high concentration, the bulk sample, to low concentration, the sorbent, and the rate of extraction can be correlated linearly to the concentration of the analyte in the sample. Thus, calibration can be performed according to the extraction rate [26]. The concentration of the bulk sample can be determined as follows:

$$C = \frac{n\ln(b + \delta/b)}{2\pi L t D_L} \quad (3)$$

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 [24]. The thickness of boundary layer, δ, can be calculated as follows:

$$\delta = 9.52\left(\frac{b}{\mathrm{Re}^{0.62}\mathrm{Sc}^{0.38}}\right) \quad (4)$$

where Re is the Reynolds number and Sc is the Schmidt number. An important requirement for the DBI model is the control of the convection as this has a direct effect on the boundary layer thickness and the size of the boundary layer must be constant in order for the rate of extraction to be linearly correlated to the concentration in the sample [26].

In this research, the three kinetic calibration methods were compared to SPME EE extraction and evaluated in terms of accuracy, precision and experimental procedures. No such studies comparing all of the above methods have been reported to date although Zhang et al. have previously shown that precision is different between EE and OFS [4]. Furthermore, a conventional, plasma protein precipitation method was performed to validate the results obtained by SPME. All sampling and calibration techniques were performed with a flow system simulating an animal blood circulatory system, and comparison analyses were performed for spiked samples of phosphate buffered saline and rat whole blood. The comparison was performed using fenoterol as the analyte of interest because of its relatively high polarity (log P of 1.37 [27,28]). Such hydrophilic drugs have not been commonly investigated for *in vivo* SPME applications. Analyte detection and quantification were achieved using HILIC-based liquid chromatography tandem mass spectrometry. The paper demonstrates for the first time, the advantages and disadvantages of various kinetic calibration methods and provides important insight for the selection of an appropriate calibration method for a given *in vivo* application.

2. Materials and methods

2.1. Materials

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