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Direct tissue sampling of diazepam and amitriptyline using mixed-mode SPME fibers: A feasibility study

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ABSTRACT

Here, we report on the applicability of the C18/SCX fiber (containing both C18 and strong cation exchange groups) as a direct sampling tool in tissue. An agarose gel model was used as semi-solid tissue surrogate, to mimic changes in matrix tortuosity as expected in tissue. Pork muscle was used as tissue source and was loaded with diazepam or amitriptyline using 24 h incubation in spiked phosphate-buffered saline (PBS). Diazepam is neutral and its behavior is predictable based on the octanol–water partition coefficient (K_{ow}). Amitriptyline is >99% positively charged at pH 7.4, and is likely to behave differently in both agarose gel and tissue compared to neutral compounds. The proposed SPME method yielded detectable fiber concentrations after direct sampling in agarose gel and loaded tissue, including short sampling times and different loading concentrations in tissue. Sorption affinity of both diazepam and amitriptyline is decreased when sampling from agarose gel compared to PBS, due to the presence of different binding groups in agarose. Sorption affinities from tissue are even further decreased compared to agarose gel. This indicates that tissue contains even more binding sites for these compounds compared to agarose gel. Interestingly, for both compounds, equilibration in tissue occurred faster than equilibration in agarose gel or PBS, most likely caused by direct fiber contact or through facilitated transport. Although more research is needed to obtain good quantitative results, these results show that the C18/SCX fiber is a sensitive tool to determine tissue concentrations of neutral and cationic compounds.

1. Introduction

Solid-phase microextraction (SPME) is a simple, fast passive sampling tool developed in the 1990s [1]. In recent years, *in vivo* application of SPME has gained more interest [2,3]. Most *in vivo* studies using SPME sample compounds from a liquid matrix such as blood. However, recent work has shown that SPME can also be applied in semisolid matrices such as tissue [4]. The application of SPME in tissue enables direct measurement of drug concentrations at the site of action, and allows for an empirical determination of the distribution of a compound.

The first application of SPME in tissue was in the hippocampus of mice [5]. They measured toluene after nasal inhalation by placing an SPME fiber through a cannula implanted in the brain, for 2 min. They observed a changing amount of toluene extracted after different inhalation concentrations and different time intervals. These measurements can be used to calculate pharmacokinetic parameters, such as elimination half-life. However, actual tissue concentrations were not calculated, as this requires correct calibration. Further studies on tissue concentrations focused on the sampling of neutral pharmaceuticals in fish using neutral SPME coatings, either in a laboratory setting or after exposure to wastewater effluent [6–14].

One of the main issues regarding tissue sampling using SPME is fouling and how this affects the calculation of tissue concentrations. Usually, tissue concentrations were calculated from partition coefficients generated in matrix-free solutions, such as phosphate-buffered saline (PBS) [6,7]. However, this disregards both potential fouling of the fiber and the effect of tissue density on uptake kinetics. Fouling occurs when part of the sample matrix adheres to the fiber and thereby blocks or increases the uptake of analyte [15]. The phenomenon has been extensively described for liquid samples containing protein, see e.g. [16]. Recently, a mechanistic-based mathematical model was established to estimate fouling effects in liquid matrices [17]. In semisolid matrices such as tissue,

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the influence of fouling was found to be limited for the sampling of pharmaceuticals [8] and polychlorinated biphenyls (PCBs) [18] using polydimethylsiloxane (PDMS) fibers in fish muscle. However, fouling should be evaluated for other SPME coatings as well as other tissue types.

The different density and porosity of tissue compared to aqueous phase clearly alters the sorption behavior of a compound to the SPME fiber, as diffusion is limited or hindered by the nature of the sample matrix [11,19]. This is called tortuosity, which is defined as the ratio between the length of the diffusion path and the distance between the starting and end point of the diffusing molecule. To simulate matrix tortuosity, an agarose gel model was proposed to determine rate constants of chemicals to PDMS [13], which can then be applied in unknown tissue samples to determine tissue concentrations. Using this model, different gel concentrations can be used to mimic different tissue types, for instance, 0.8% agarose gel was found to correlate best with fish muscle [13].

The aim of this paper is to evaluate the applicability of the C18/SCX coated SPME fiber in tissue sampling. The C18/SCX (mixed-mode) fiber coating consist of hydrophobic C18 chains and strong cation exchange (SCX) groups, made up of propylsulfonic acid. As many pharmaceuticals are bases, and therefore positively charged at physiological or environmentally relevant pH, the C18/SCX fiber was chosen as sampling tool. This fiber has been proven to be orders of magnitude more sensitive for organic cations than conventional SPME fiber coatings such as polyacrylate and PDMS [20]. Furthermore, a similar mixed-mode fiber has been applied in tissue before, i.e. metabolomics profiling in lung and liver tissue of pigs [21], studying the brain metabolome of rats [22] and sampling of methylprednisolone and its metabolites in liver and lung tissue of pigs [23]. However, as of yet, no work has been done on the use of the C18/SCX fiber in direct tissue sampling as a quantitative extraction tool to determine tissue concentrations.

In this paper, the C18/SCX fiber is applied as a direct sampling tool in both agarose gel and tissue models. Other research showed a decrease in diffusion rates of pharmaceuticals in agarose gel and tissue [13]. Here, we study uptake kinetics of diazepam and amitriptyline to the C18/SCX fiber from PBS, agarose gel and tissue. Experiments are performed under static conditions, so rate of diffusion will be the rate-limiting step in the sorption of compounds to the fiber. Diazepam and amitriptyline are used as model compounds. Diazepam is neutral at physiological pH and its behavior in the test systems is expected to be predictable based on hydrophobicity ($\log K_{ow}$). For amitriptyline, which is >99% cationic at pH 7.4, tissue distribution and fiber sorption are less predictable based on $\log K_{ow}$ alone. We suspect that tissue contains multiple binding sites for both chemicals, resulting in a decrease in free concentration and thus sorption to the fiber. Also, the agarose gel model might incorporate such binding groups, thereby limiting its likeness to PBS but increasing its likeness to tissue.

Ultimately, we aim to show that the C18/SCX fiber is a sensitive tool that can be applied directly in tissue, both *in vitro* and *ex vivo*. This will allow the development of an SPME method to be applied in forensic toxicology. With SPME, the collection of tissue concentrations will require less sample preparation compared to conventional methods. As SPME fibers are small and exposure of the fibers can be short, it might be an excellent tool for direct measurements on site (e.g. at a crime scene). Another benefit of SPME is that multiple consecutive measurements in the tissue or other biological matrix are possible, thereby giving insight into specific forensically important distribution phenomena, including postmortem redistribution.

2. Materials and method

2.1. Chemicals and materials

Diazepam and amitriptyline HCl (analytical grade) were obtained from Spruyt Hillen (IJsselstein, The Netherlands) and Sigma Aldrich (Zwijndrecht, The Netherlands), respectively. Stock solutions were prepared in methanol and stored at −20 °C. Ultrapure agarose was obtained from Invitrogen (Paisley, Scotland). HPLC-grade methanol and acetonitrile were from BioSolve (Valkenswaard, The Netherlands). Ammonia solution (25%) was from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) consisted of 138 mM NaCl, 8 mM of Na_2HPO_4, 1.5 mM of KH_2PO_4 and 2.7 mM KCl (all from Merck) dissolved in Milli-Q water (18.2 MΩ.cm, Millipore, Amsterdam, The Netherlands). PBS was set to pH 7.4 using 1 M HCl or 1 M NaOH. Prototype C18/SCX solid-phase microextraction (SPME) fibers were provided by Supelco, Sigma Aldrich (Bellefonte, PA, USA). This mixed-mode coating consist of silica particles coated with C18 and propylsulfonic acid. Fibers used here had a total length of 3 cm (nitinol wire) of which 1.5 cm is coated, at an average thickness of 45 μm.

2.2. Agarose gel preparation

To simulate the density of tissue, 1% agarose gel was used. To prepare the gels, 1 g of agarose is mixed with 100 mL of PBS in an Erlenmeyer flask. This flask is placed in a microwave and heated until just below boiling point. The flask is removed from the microwave and swirled gently to dissolve the agarose. Gels were cast in 5 mL vials which were spiked with stock solutions of diazepam or amitriptyline. The liquid agarose solution is added to the vial, and the vials are vortexed immediately to mix the agarose solution with the analyte. After vortexing, vials are left at room temperature for approximately 2 h to allow the gel to cool and become solid. Concentrations in agarose gel after exposure of the fiber could not be measured properly, so nominal concentrations were used for calculations and plots.

2.3. Tissue loading

Tissue was loaded with diazepam or amitriptyline using static drug exposure. Pork tissue was purchased at the local supermarket, stored at −20 °C and thawed before use. The tissue was weighed and cut into pieces ranging between 1 and 2.5 g. The tissue pieces were individually exposed for 24 h in 100 mL of PBS, spiked with the analyte of interest. For most experiments, a concentration of 10 mg/L was used. After 24 h, the pieces were removed and blotted dry before placing the SPME fibers in the tissue.

2.4. SPME procedure

SPME fibers were conditioned before use by placing them overnight in a solution of 50:50 methanol: Milli-Q water. Fibers were exposed to agarose gel or tissue by carefully inserting the fiber in the center of the gel or tissue. After a specific exposure time, usually a few hours, fibers were removed from the sample and immediately desorbed. Fibers were wiped with a paper tissue to remove any excess material attached to the fiber. Desorption fluid consisted of 90% acetonitrile and 10% Milli-Q water, with 0.1% NH_3 to reach a pH of 11. Desorption of the C18/SCX fiber reaches >96% recovery after 15 min, while a second desorption step showed that carry-over was 1–3% [20]. Prior to HPLC analysis, the desorption fluid is acidified using 0.1 M HCl (ratio 2:1 desorption fluid: HCl). A triplicate was used for each data point, i.e. three individually loaded tissue pieces with one SPME fiber exposed to each piece. All experiments incorporated blanks (e.g. fibers exposed to unspiked agarose gel or to tissue pieces placed in blank PBS for 24 h) to account for the extraction of potential endogenous compounds and for potential carry-over.

2.5. Drug concentration in tissue

Drug concentrations in tissue after exposure of the fiber were determined to obtain distribution coefficients between tissue and the SPME fiber. Tissue samples were extracted using a liquid–liquid extraction, using acetonitrile at subzero temperatures as described by Yoshida and Akane [24]. For this method, a single smaller sample (approximately 0.5 g) of each piece of loaded tissue was taken and placed in 2 mL of Milli-Q water. The tissue is then homogenized using an Ultra Turrax (IKA, Staufen, Germany). Of this homogenate, 125 μL is transferred to an Eppendorf tube, mixed with 375 μL acetonitrile and vortexed. To precipitate all solids from the sample, it is centrifuged at 18,000 rcf for 10 min. The sample is then placed at −20 °C for at least 30 min. The aqueous phase and the acetonitrile will separate and the analytes of interest will be found in the upper organic layer [24]. Phase separation cannot be visually observed, so care should be taken to only obtain the upper organic layer. For HPLC analysis, 125 μL of the top acetonitrile layer is transferred to an HPLC vial. Recovery (and standard deviation) of diazepam and amitriptyline was 75 ± 6% and 89 ± 5%, respectively, based on a $n = 10$ recovery test.