Introduction

- Determination of free circulating drug is important in establishing the pharmacokinetic activity. In most cases, drug-protein complexes are formed thus affecting the active level of circulating drugs.
- Techniques used for determining drug protein binding levels consist of ultrafiltration, ultracentrifugation and microdialysis. Automation can be used in the case of microdialysis, but processing may be greater than 6 hours for equilibrium to be reached.

In this study, a novel BioSPME microextraction device is evaluated as a rapid means of determining drug protein binding affinities from plasma. Here, the SPME LC-Tips with C18 fiber chemistry were compared to Thermofisher Scientific Rapid Equilibrium Dialysis (RED) device, for speed and simplicity in measuring binding affinity in rat plasma samples.

Experimental

Single Use SPME LC-Tips with C18 Chemistry

The approach of the BioSPME technique is based upon the adsorption mechanism of Fick’s Law, where differential migration exists between free analytes in solution and analytes that partition into the fiber coating. The rate of this differential migration is dependent upon the affinity of the analyte for the phase coating compared to the affinity for the matrix.

Distribution Constant $K_{fs}$

$V_f : \text{volume of fibre coating} $

$K_{fs} = \frac{C_{\infty f}V_f}{C_{\infty s}Vs}$

Adsorption Mechanism on BioSPME Extraction

- The rate of this differential migration is dependent upon the affinity of the analyte for the phase coating compared to the affinity for the matrix. BioSPME is not an exhaustive technique and extraction is governed by distribution constants dependent on an analytes affinity for the coating as compared to the sample matrix. After a given amount of time, an equilibrium is achieved between the concentration of analytes in the matrix and the fiber coating.
- In the case of the BioSPME fibers, the polymeric binder used to adhere the C18 functionalized particles onto the fiber acts as a shield that prevents large molecular weight (i.e. proteins) from adsorbing onto the fiber, thus allowing for only the free fraction/unbound analyte to be extracted by the fiber coating.
- In this study a model set of protein binding drugs were selected to compare with BioSPME approach with the equilibrium dialysis technique. Drugs with reference binding affinities ranging from 20%-99% were selected for comparison of the sampling devices.

LC-MS/MS Conditions

- Mobile phase: (A) 5 mM ammonium formate, (B) 5 mM ammonium formate in 90:10 acetonitrile/water
- Flow rate: 500 µL/min
- Temp: 40°C
- Dil: MS/MS, ESI (+), MRM transitions
- Injection: 2 µL
- Gradient: 0% B to 70% B in 3 minutes, then to 95% B in 0.1 minute, hold 95% B for 0.9 minutes
- Instrument: Agilent 1290 Infinity II with Agilent 6460 QQQ

MRM Transitions

- Compound Name | Sigma Part Number | Precursor Ion | Product Ion | Fragmentor | Collision Energy
- Codeine | C-005 | 202.2 | 152.1 | 105 | 5
- Codeine-d5 | C-005 | 303 | 152.1 | 105 | 76
- Dextrometorphan | D-067 | 296.1 | 191.1 | 145 | 32
- Dextrometorphan-d8 | D-067 | 296.1 | 191.1 | 145 | 32
- Diclofenac | D5850 | 296 | 214 | 90 | 32
- Diclofenac-d15 | D5850 | 343.1 | 285.1 | 50 | 5
- Fentanyl | 00502 | 296.1 | 191.1 | 145 | 32
- Fentanyl-d15 | 00502 | 343.1 | 285.1 | 50 | 5
- Quinidine | C0628 | 265.2 | 81.2 | 150 | 26
- Quinidine-d8 | C0628 | 265.2 | 81.2 | 150 | 26
- Warfarin | W-003 | 301.1 | 163 | 100 | 8
- Warfarin-d15 | D-7050* | 314.1 | 163 | 100 | 8

Chromatogram of Binding Analytes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>r²</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>0.9887</td>
<td>1.60</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.9859</td>
<td>748.4</td>
</tr>
<tr>
<td>Propranolol</td>
<td>0.9948</td>
<td>6.14</td>
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<tr>
<td>Warfarin</td>
<td>0.9846</td>
<td>5.14</td>
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<tr>
<td>Diclofenac</td>
<td>0.9861</td>
<td>5.12</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.9885</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Sample Preparation

- Stock solution of binding analytes was prepared at 10 ng/mL in methanol.
- Rat plasma stabilized with K3EDTA (BioReclamation, IVT, Hicksville, NY USA) was spiked at 200 ng/mL of binding analytes and allowed to equilibrate for 3 hours at 37°C prior to extraction studies.
- Phosphate buffered saline (PBS, pH = 7.4) was prepared at the following concentrations (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4).
- Spiked PBS was prepared at 200 ng/mL of binding analytes, this was used for the SPME extraction studies.
- Blank PBS was used for the RED device binding studies.

SPME C18 Extraction Protocol

1. Fibers were conditioned by soaking in methanol for 10 minutes
2. Fibers were then equilibrated by soaking in water for 10 minutes
3. The samples were prepared by placing 800 µL of plasma and buffer samples into a 2.0 mL Nunc® 96-well plate (Sigma-Aldrich Z717266)
4. The extraction procedure was conducted by placing the SPME C18 fibers directly into 800 µL of plasma and buffer samples and agitated for 30 minutes at 500 rpm using Corning® LSE™ Digital Microplate Shaker. Sample replicates of N=5
5. Fibers were then transferred from the samples and placed directly into a 600 µL conical 96-well plate (Corning® Scientific 391-01-201) that had been prefilled with 300 µL of internal standard desorption solvent (50 ng/mL in ACN). The well plate was agitated for 10 minutes at 500 rpm using Corning LSE Digital Microplate Shaker.
6. The SPME fibers were removed and the well plate was capped, vortexed and analyzed directly.

Calibration Curve

Summary

- In the case of the BioSPME analysis, the free fraction of the analyte is measured in both the reference (PBS) and the plasma sample. This technique simplifies the calculation of determining protein binding.
- Protein binding affinities for both the BioSPME and the equilibrium dialysis devices closely matched the referenced range for all analytes. In the case of quinidine, a lower binding affinity was observed for both techniques as compared to the reference data. This may be specific to the plasma sample use in the study.
- Drug binding levels were determined using the BioSPME approach in less than 60 minutes, thus a 4X reduction in analysis time over the equilibrium dialysis device. This was a significant time savings as compared to membrane techniques.
- The BioSPME technique allows for direct sampling of the plasma sample, eliminating the need for protein precipitation as in the equilibrium dialysis device. This also minimizes concern associated with matrix interference.
- The BioSPME technique for directly determining free fraction of drug within plasma proved to be simpler and faster technique over traditional dialysis membrane techniques.
- Additional studies are planned to further reduce the extraction time for the BioSPME.

Reference


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