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Rapid Determination of Drug Protein Binding Affinity using Solid Phase Microextraction

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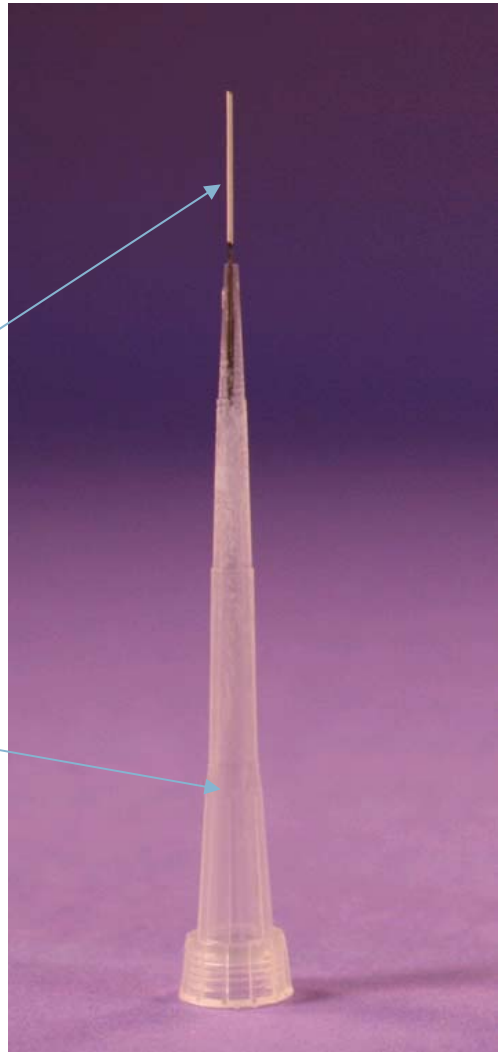
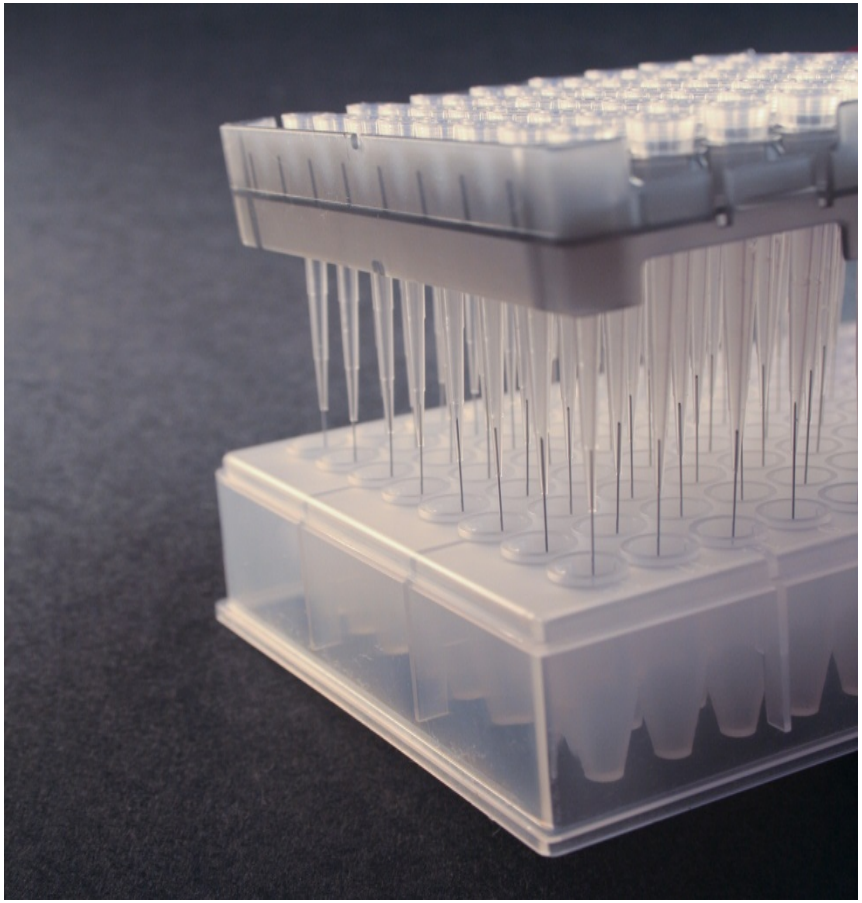
Introduction

- 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 Equilbrium Dialysis (RED) device, for speed and simplicity in measuring binding affinity in rat plasma samples.

Experimental

Single Use SPME LC-Tips with C18 Chemistry





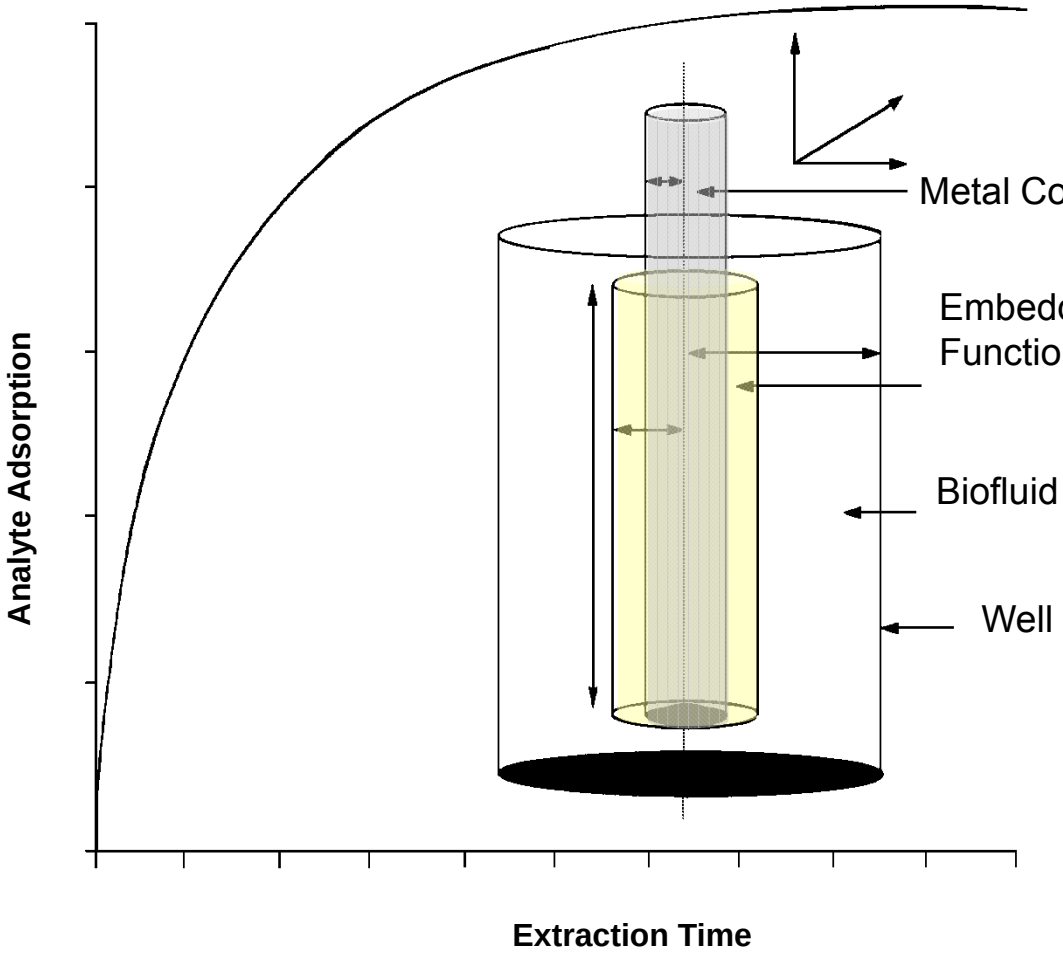
The approach of the BioSPME technique is based upon the adsorption mechanism of Fick's Law, where differential migration exists between free analytes in solution and analytes that partition into the fiber coating. The rate of this differential migration is dependent upon the affinity of the analyte for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

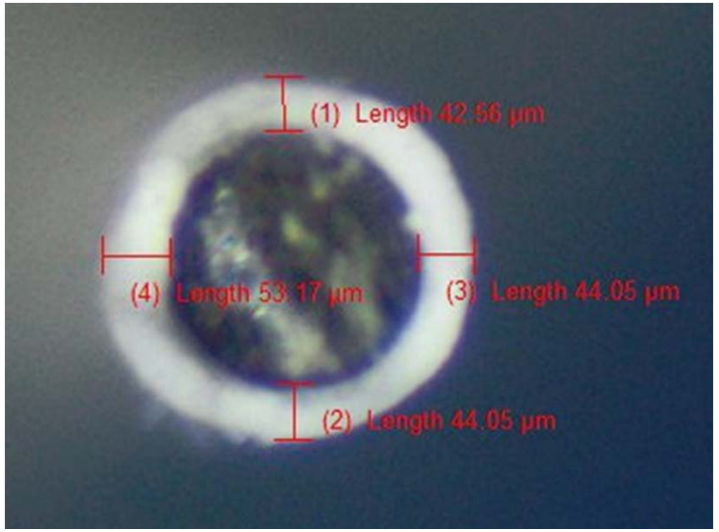
Distribution Constant K_{fs}

$$K_{fs} = C^{\infty}_{f} V_f / C^{\infty}_{s} V_s$$

V_s : volume of sample
V_f : volume of fibre coating
C^{∞}_{f} : equilibrium concentration on the fiber
C^{∞}_{s} : equilibrium concentration in the sample

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 particle onto the fiber core acts as a shield that prevents large molecular weight (i.e. proteins) from absorbing onto the fiber, thus allowing for the 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.

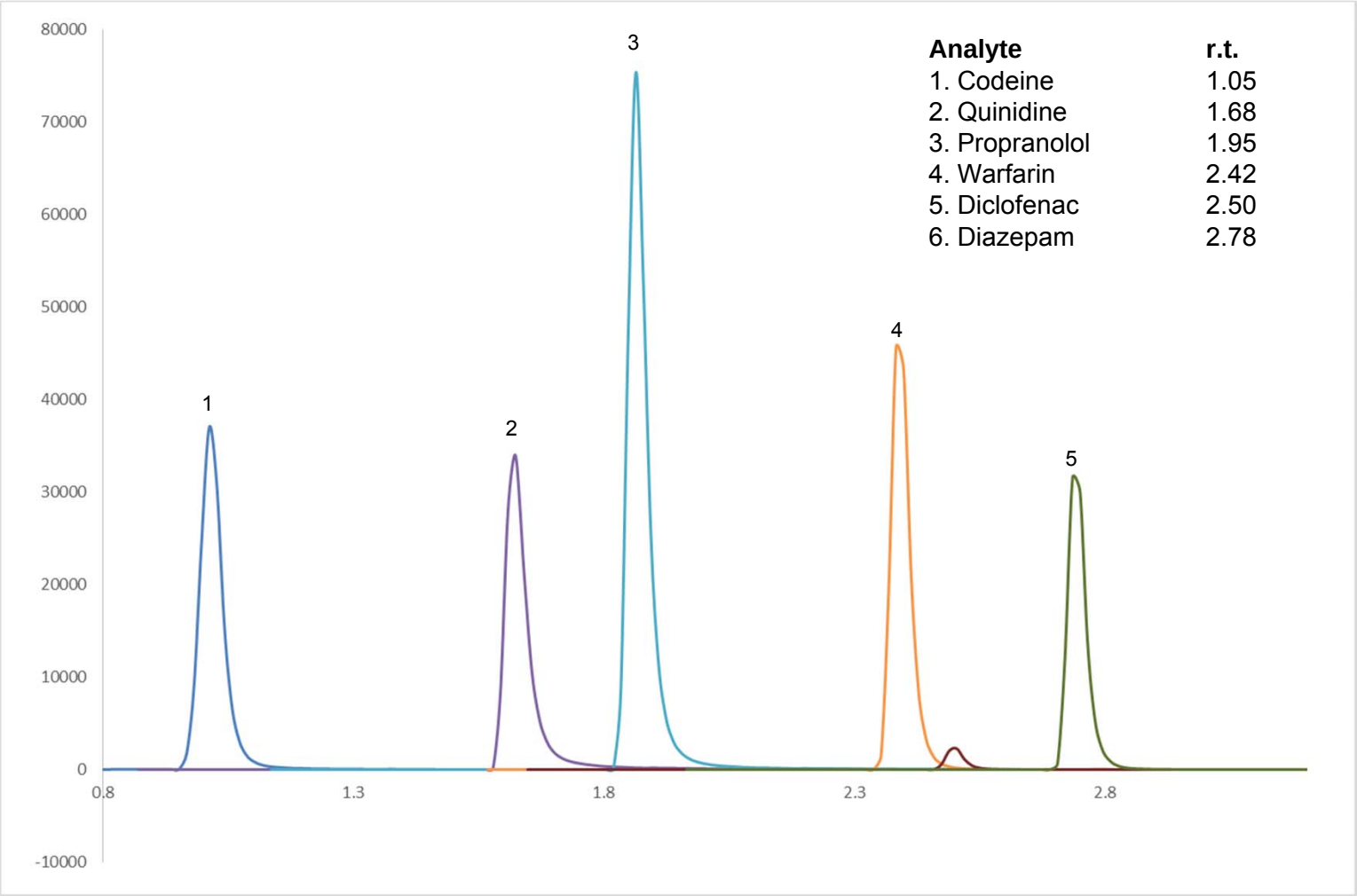
LC-MS/MS Conditions

column:	Ascentis® Express C18, 5 cm x 2.1 mm I.D., 2.7 µm particles
mobile phase:	(A) 5 mM ammonium formate
	(B) 5 mM ammonium formate in 90:10 acetonitrile:water
flow rate:	500 µL/min
temp.:	40 ºC
det.:	MS/MS, ESI (+), MRM transitions
injection:	2 µL
gradient:	5%B to 70%B in 3 minutes, then to 90%B in 0.1 minute, hold 90%B for 0.9 minutes
instrument:	Agilent® 1290 Infinity II with Agilent 6460 QQQ

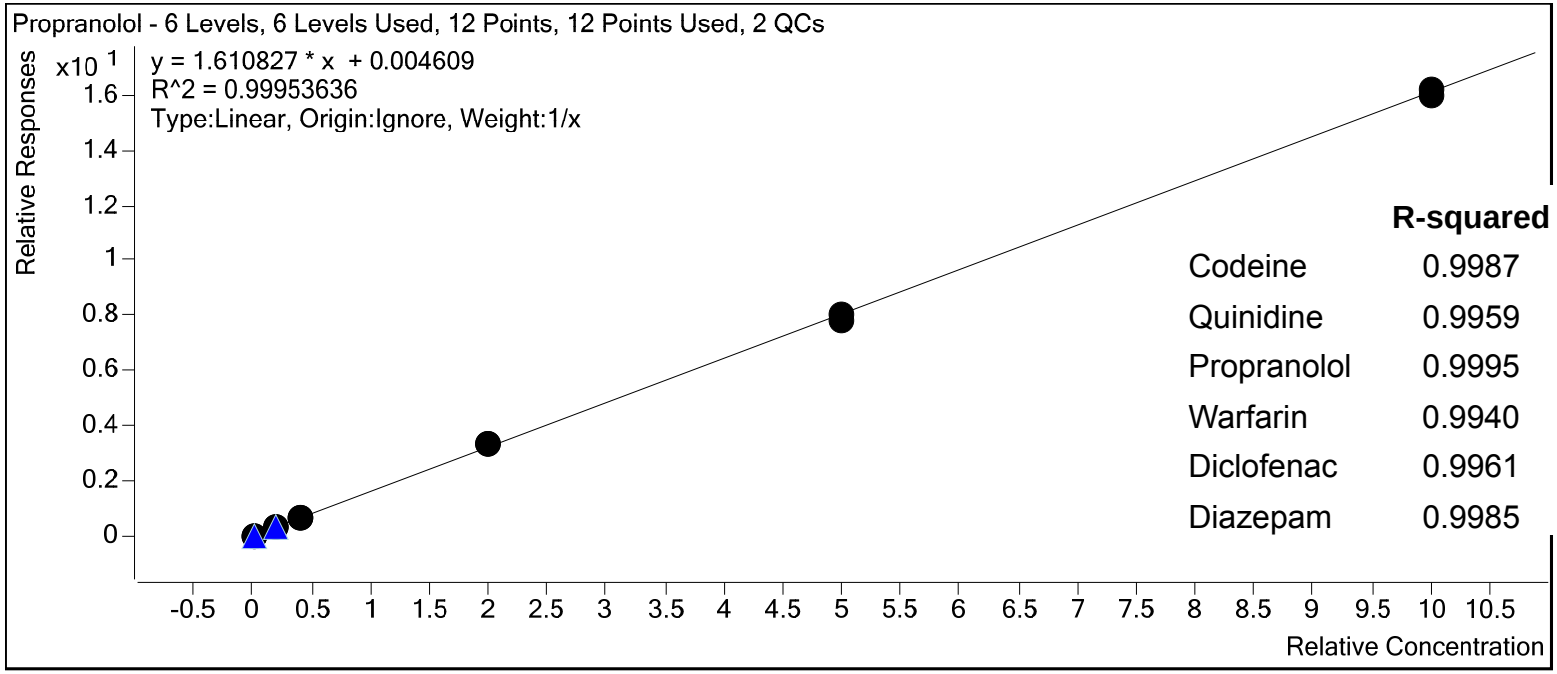
MRM Transitions

Compound Name	Sigma Part Number	Precursor Ion	Product Ion	Fragmentor	Collision Energy
Codeine	C-006	300.2	152.1	125	76
Codeine-d_3	C-005	303	152.1	125	76
Diazepam	D-907	285.1	193.1	166	32
Diazepam-d_5	D-902	290.11	198	145	32
Diclofenac	D6899	296	214	90	32
Firocoxib-(5,5-dimethyl-d_6)	35362	343.15	289.1	80	8
Propranolol	P-055	260.2	56.2	120	28
Propranolol-d_7	P-085	267.2	56.2	120	28
Quinidine	Q3625	325.2	81.2	150	36
Warfarin	W-003	309.1	163	100	8
Warfarin-d_5	D-7080*	314.15	163	105	8

Chromatogram of Binding Analytes



Calibration Curve



	R-squared	Slope
Codeine	0.9987	1.60
Quinidine	0.9959	748.8
Propranolol	0.9995	1.61
Warfarin	0.9940	57.9
Diclofenac	0.9961	5.12
Diazepam	0.9985	1.09

Sample Preparation

- Stock solution of binding analytes was prepared at 10 µg/mL in methanol.
- Rat plasma stabilized with K_2EDTA (BioReclamation, IVT, Hicksville, NY USA) was spiked at 200 ng/mL of binding analytes and allowed to equilibrate for 3 hours at 37 ºC 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 Na_2HPO_4, 1.8 mM KH_2PO_4).
- 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

Supelco *SPME LC Tips Part No. 57234-U*

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 SPME extraction 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 (Axygen® 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.

Analyte Binding Calculation for BioSPME Technique

$$\% \text{ protein bound} = \frac{(\text{concentration PBS} - \text{concentration plasma}) \times 100}{\text{concentration PBS}}$$

RED Device Protocol

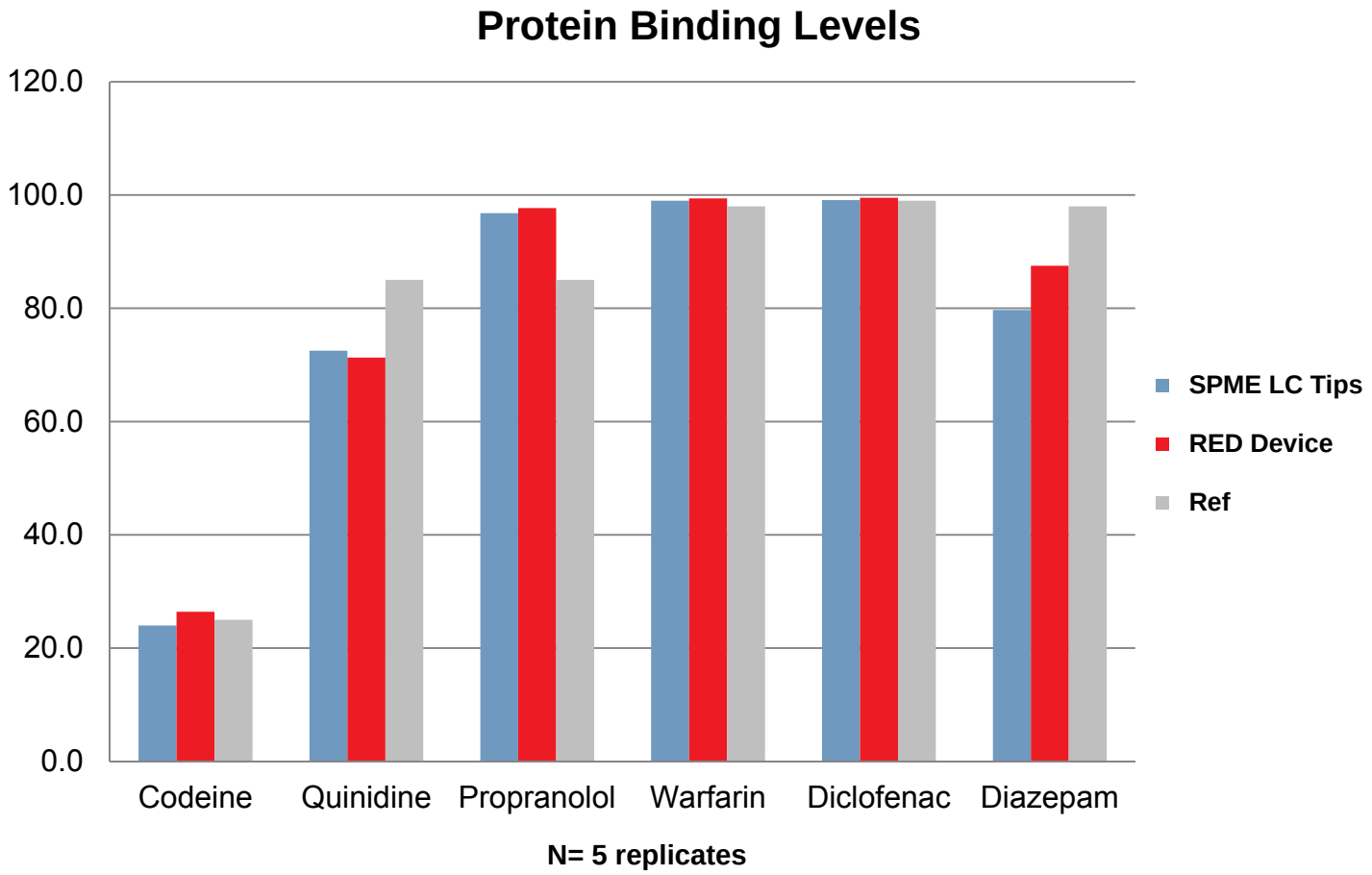
Single-Use RED plate with inserts, Thermo PI90006

1. The samples were prepared by placing 200 µL of plasma into the plasma compartment of the RED device and 350 µL of buffer into the buffer compartment.
2. The RED device was capped with a sealing mat and placed on Eppendorf® ThermoMixer C at 37 ºC at 250 rpm for 4 hours.
3. After equilibration step, 50 µL aliquots were taken from each compartment. 50 µL of plasma was added to the buffer compartment aliquot and 50 µL of buffer was added to the plasma compartment aliquot.
4. After mixing, 300 µL of ice cold acetonitrile with 50 ng/mL internal standard was added.
5. Samples were vortexed at 1200 rpm for ~ 5 minutes and then centrifuged at 15,000 rpm for 10 minutes.
6. The supernatant was decanted into a glass HPLC vial for analysis.

Analyte Binding Calculation for RED Equilibrium Dialysis Technique

$$\%\ \text{protein bound} = 100\% - \left(\left(\frac{\text{concentration PBS chamber}}{\text{concentration plasma chamber}} \right) X100\% \right)$$

Binding Affinity Comparison



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 level.
- 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 approach 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.

Summary (contd.)

- The BioSPME approach 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

1. Journal of Laboratory Automation February 2011 16: 56-67.