Supelco®
Analytical Products

Application Note

Comparison of Supel™ BioSPME 96-Pin Device to Rapid Equilibrium Dialysis Technique for Determination of Plasma Protein Binding

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Introduction:

An important aspect of drug discovery is understanding the interaction of the drug candidate with plasma proteins and lipids. The portion of drug bound to proteins and lipids is referred to as the plasma protein binding (PPB or F_b). Molecular attributes of the drug can provide insight on the number of interactions with protein. In general, organic acids have a single binding site with albumin, whereas, organic bases will have multiple bindings sites associated with glycoproteins.[1] In addition to albumin, other proteins commonly associated in drug binding are alpha-1-acid glycoprotein (AAG) and lipoproteins such as very high-density lipoprotein (VHDL) and low-density lipoprotein (LDL).[2] When it comes to the pharmacologic effectiveness of a drug, it is the free fraction or unbound fraction (F_u) of a drug that is generally responsible for drug activity as described by the free drug hypothesis.[3,4,5]

Determining protein binding properties of a drug is important to understand the amount of free drug available in blood. Equilibrium membrane dialysis has been the traditional technique to measure drug protein binding. This technique involves equilibration of the drug rich plasma sample with the drug-free buffer across a membrane, allowing for free drug to migrate across the membrane and preventing the transfer of the protein bound drug into the buffer. This equilibrium is achieved in excess of 24 hours. Other techniques such as rapid equilibrium dialysis further reduce the workflow time down from >24 hours to 6 hours using specifically designed devices.

In this study, the Supel™ BioSPME 96-Pin device is utilized to measure drug protein binding. Supel™ BioSPME 96-Pin devices have been developed using Solid Phase Microextraction technology for extraction of free unbound analytes from biological fluids. These devices consist of a 96-pin plate, where the tips of the pins are coated with a thin layer of adsorbent particles. The patented binder within the coating allows for small analytes of interest to bind, while larger macromolecules are excluded. This allows for a robust and selective non-exhaustive extraction of free analyte that can be employed in both qualitative and quantitative applications. The 96-pin configuration allows for direct sampling from 96 well plates, and is compatible with robotic liquid handling systems providing a fully automated high-throughput methodology.

In this study, the Supel™ BioSPME 96-Pin device workflow is compared with the rapid equilibrium dialysis technique for measuring drug protein binding. A series of compounds with molecular weights in the range of 230-750 Da and a hydrophobicity (logP) range of 1.5-5 were utilized for comparison of protein binding values from each technique. In addition to measured values and accuracy, the study compares overall sample cleanliness and workflow time from each technique.

Experimental:

Protein Binding Determination using Supel™ BioSPME 96-Pin Device:

Human plasma and buffer were spiked at a therapeutically relevant concentration and incubated for one hour at 37 ºC while shaking at 300 rpm. After the incubation, 200 µL of plasma and buffer were loaded into separate columns of an extraction well plate (n = 8). The protein binding determination workflow using the Supel™ BioSPME 96-Pin device was conducted with an automated robotic liquid handling system. Briefly described in **Figure 1**, the pin device is conditioned for twenty minutes static (without shaking) in isopropanol, then is transferred into a new well plate containing water for 10 seconds (wash step). This is followed by the extraction step, where the pin device is transferred into the preloaded extraction plate and analyte extraction takes place while shaking at 1200-1250 rpm at 37 ºC for 15 minutes. The pin device is returned to the water solution for a 60 second wash, and is then transferred into a desorption plate for the final step. The desorption solution is a mixture of 80:20 methanol:water, and the pin device undergoes desorption for 20 minutes under static conditions. **Figure 2** shows the Supel™ BioSPME 96-Pin device being maneuvered by the automated liquid handing system gripper. **Figure 3** represents the extraction of free unbound analyte onto the Supel™ BioSPME 96-Pin device.

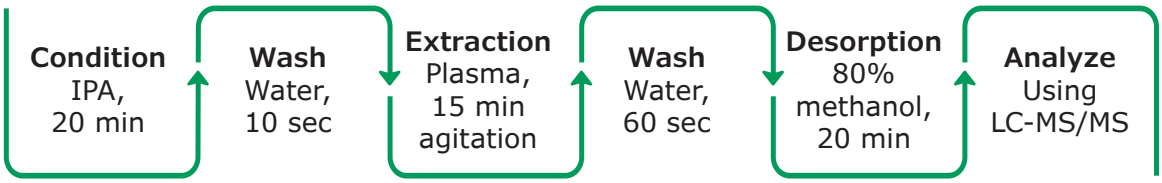

Figure 1. Overview of the steps for the Supel™ BioSPME 96-Pin device determination of free fraction of drug in human plasma.

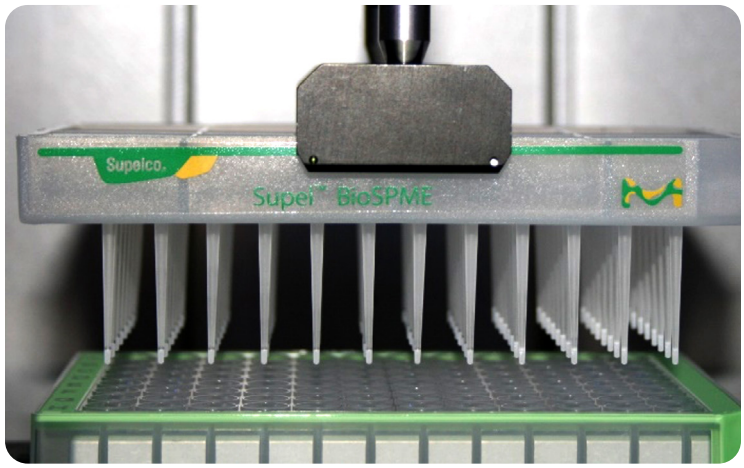

Figure 2. Supel™ BioSPME 96-Pin device maneuvered by automated liquid handing system gripper.

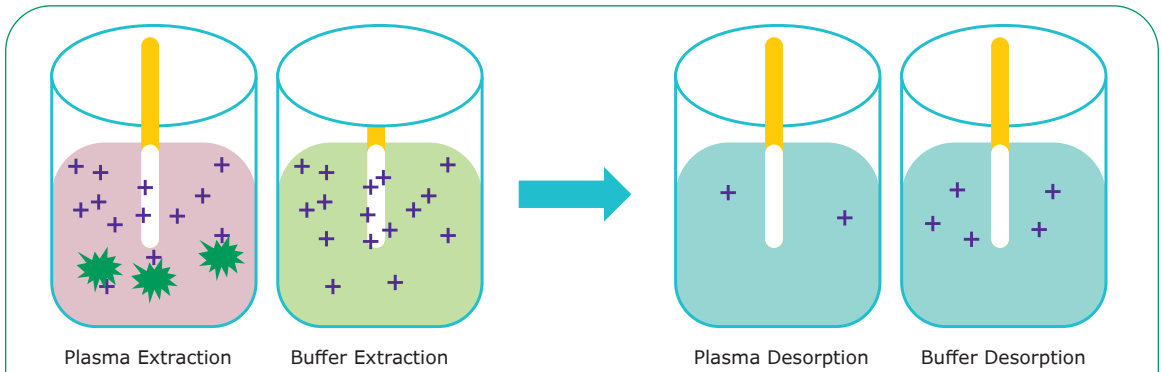

Figure 3. Representation of the extraction step (left) removing free analytes from plasma (pink) and buffer (green) and the analytes releasing into the desorption solution (blue). The amount extracted does not greatly impact the concentration of free analyte which is termed non-depletive. As the buffer solution is considered 100% free, Supel™ BioSPME 96-Pin device will extract more from buffer than from the plasma.

The extraction plates used in this study included both plastic and glass-coated plates. The choice of the plate depended on the compound properties and how well the compound behaved in buffer solution. More hydrophobic compounds, such as ketoconazole and imipramine were found to exhibit non-specific binding to plastic and had better extraction efficiency from glass-coated 96-well plates. Extraction for erythromycin and propranolol were performed from glass-coated plates as well, as higher extraction efficiency values were obtained from glass in comparison to extraction from plastic plates.

Protein Binding Determination by Rapid Equilibrium Dialysis

Rapid equilibrium dialysis was performed as directed by the instruction sheet. Briefly, 200 µL of human plasma "spiked" at a therapeutically relevant concentration and 400 µL of phosphate buffered saline (PBS) were loaded into corresponding chambers in at least triplicate sets. Dialysis proceeded for at least 4 hours while covered and shaking at 300 rpm and 37 ºC on an Eppendorf shaker. At the end of dialysis, 50 µL of the spiked plasma was mixed with 50 µL of clean (unspiked) PBS, and 50 µL of the dialysate (buffer compartment) was mixed with 50 µL of clean plasma. This was done to ensure matrix consistency. Next, 300 µL of ice-cold acetonitrile was added to each sample before centrifugation at 5,000 rpm for 10 minutes at 4 ºC. Finally, the supernatant was transferred into glass vials for analysis by LC-MS/MS.

Samples prepared using both the Supel™ BioSPME 96-Pin device and the rapid equilibrium dialysis device were analyzed by LC-MS/MS. The chromatographic and mass spectrometric analysis was performed on an Agilent 1290 / AB Sciex 6500 LC-MS/MS system following the conditons described in **Tables 1** and **2**. Quantitation was performed using a matrix-matched external calibration in the desorption solution.

Table 1. LC-MS/MS Conditions for monitoring analytes for protein binding determination

Column:	Ascentis® Express Biphenyl column (10 cm x 2.1 mm, 2.7 mm)
Mobile Phase:	[A] 5 mM ammonium acetate, 0.1% acetic acid in 95% water and 5 % acetonitrile [B] 5 mM ammonium acetate, 0.1% acetic acid in 95% acetonitrile and 5% water
Gradient:	Initially start at 10% B and hold for 0.5 min, increase to 90% B over 2.5 min, hold at 90% B for 2 min, decrease to 10% B in 0.1 min and hold for 2 min at 10% B
Flow Rate:	0.4 mL/min
Column Temp:	40 ºC
Detector:	MS, ESI(+) Scheduled MRM (See **Tables 1** and **2**)
Injection:	Dependent upon analyte; 5 – 20 µL

Table 2. Analyte description and LC-MS/MS parameters

	MW	Log P	pKa	Physiological Charge	Analyte Calibration Curve (ng/mL)	q1	q3	dwell (ms)	DP	EP	CE	CXP
carbamazepine	236	2.45	13.9	0	0.5 – 100	237.1	194.2	40	21	10	29	26
diazepam	284.7	2.82	2.92	0	1 – 100	285.0	193.2	40	91	10	43	28
imipramine	280.4	4.8	9.4	1	2 - 100	281.1	58.2	40	41	10	61	10
prednisolone	360.4	1.6	12.59	0	5 - 250	361.1	147.1	50	36	10	33	10
propranolol	259	3.48	9.42	1	2.5 - 100	260.2	183.1	40	66	10	25	34
warfarin	308.3	5	6.33	-1	0.05 - 10	309.1	163.0	40	256	10	21	12
zolpidem	307.4	3.15	5.65	0	0.5 – 100	308.2	235.2	40	36	10	49	16
nalidixic acid	232.2	1.59	5.95 4.68	-1	1 - 100	233.1	187.0	25	32	7.5	15.5	32
erythromycin	734	2.6	8.88	1	0.5 – 100	734	576.3	40	121	10	27	10
Ketoconazole	530	4.35	6.75	0	1 – 250	531.2	82.1	50	51	10	59	10
Buspirone	385	1.78	4.12	1	1 – 100	386.2	122.1	40	51	7.5	45	10

Results & Discussion

Calculation of %Free Fraction (F_U) by Supel™ BioSPME 96-Pin Device

The Supel™ BioSPME method determines the free concentration of analyte in plasma by comparing it with the extraction of the analyte from buffer samples where 100% of the analyte is considered to be free of protein binding.

The percent free or percent unbound is determined in **Eq. 1**:

Eq. 1 $$\text{Free Fraction } (F_U) = \frac{\text{concentration free}}{\text{concentration total}} \times 100\%$$

where concentration free represents the unbound concentration of the analyte in the matrix (in this case plasma) and concentration total represents the total concentration of analyte. The amount extracted is independent of units and can be calculated using preferred quantities (e.g. nanograms or moles) M_{free}, and extraction volume of plasma, V_{plasma}. The concentration of analyte in the desorption solution is quantified by an external calibration curve, and if the desorption volume is equal to the plasma and buffer extraction volumes, the concentrations from desorption will be equal to the extracted concentrations as shown in **Eq. 2** & **Eq. 3**.

Eq. 2 $$\text{concentration extracted from plasma, } P = \frac{M_{E,Plasma}}{V_{plasma}}$$

Eq. 3 $$\text{concentration extracted from buffer, } B = \frac{M_{E,Buffer}}{V_{Buffer}}$$

M_E represents the mass extracted

The bound and free fractions, FB and Fu, can be determined from the extracted concentrations as shown in **Eq. 6** and **7**.

As the buffer solution is considered 100% free, Supel™ BioSPME 96-Pin device will extract more from buffer than from the plasma.

Eq. 4 $$\text{Bound Fraction } (F_B) = 100\% - \text{Free Fraction } (F_U)$$

Eq. 5 $$\text{Bound Fraction } (F_B) = \frac{\text{concentration total} - \text{concentration free}}{\text{concentration total}} \times 100\%$$

Eq. 6 $$\text{Bound Fraction } (F_B) = \frac{B-P}{B} \times 100\%$$

Eq. 7 $$\text{Free fraction } (F_U) = \frac{P}{B} \times 100\%$$

In cases where depletion of compounds from plasma was pronounced upon Supel™ BioSPME 96-Pin device extraction (extraction exceeded 5% of total spiked analyte), a correction to the calculated Bound Fraction was required as described below:

Eq. 8 $$\text{Bound Fraction } (F_B) = \frac{\left[P^0 - \frac{[(B^0 - B) \times P]}{B} - P\right]}{P^0 - P}$$

where B and P, represent the respective amounts extracted from buffer, B, and plasma, P. B^0 represents the concentration the samples were spiked originally. **Eq. 8** accounts for the concentration in solution after extraction on the fiber; the depletion of the analyte from sample.[6] **Eq. 6** and **Eq. 7**, do not take this consideration into account. However, they provide accurate values when the extracted amount is less than 5%.

Using the equations, **Eq. 5** and **Eq. 6**, the values in **Table 3** for analyte-protein bindings were determined from Supel™ BioSPME 96-Pin device extractions.

Table 3. Protein Binding Values for the nine compounds from plasma using Supel™ BioSPME 96-Pin device and 200 µL sample volumes (n=8).

Analyte	Concentration Spiked (ng/mL)	Ave. F_B(%)	RSD F_B (%)	Ave. F_U (%)	RSD F_U (%)	Ave. Buffer Extracted (ng/mL)	Buffer RSD (%)	Ave. Plasma Extracted (ng/mL)	Plasma RSD (%)
Carbamazepine	100	76.4	2.6	23.6	8.3	34.7	7.4	8.2	9.4
Diazepam	100	97.3	0.4	2.7	15.3	71.4	1.3	1.9	14.8
Imipramine	100	92.6	0.6	7.4	0.5	65.4	5.5	12.2	5.5
prednisolone	100	78.2	2.6	21.8	9.2	62.1	5.1	13.5	8.2
Propranolol	100	90.5	1.3	9.5	12.6	18.3	12.5	1.7	19.2
Warfarin	2500	99.8	<0.1	0.2	8.8	10.0	6.0	<0.1	22.4
Zolpidem	100	96.9	0.4	3.1	13.7	68.4	12.0	2.1	8.5
Nalidixic Acid	2000	97.0	0.5	3.0	15.9	16.35	14.1	0.5	14.9
Erythromycin	100	81.8	4.2	18.2	2.8	35.0	22.4	6.5	7.8
Ketoconazole	500	96.8	0.7	3.2	0.5	282.4	5.6	20.0	7.9
Buspirone	100	81.6	1.7	18.4	7.3	47.4	6.3	14.2	6.8

Comparison of rapid equilibrium dialysis versus Supel™ BioSPME 96-Pin device

In **Table 4**, the measured values for analyte protein binding that were determined using the Supel™ BioSPME 96-Pin device and the rapid equilibrium dialysis device were compared with published literature values. The values from the BioSPME method are in good agreement with values determined using rapid equilibrium dialysis devices and the reported literature values. These values are compared graphically in **Figure 4**.

Table 4. Protein Binding Values (F_B) for the nine compounds from plasma using Supel™ BioSPME and 200 µL sample volumes (n=8).

Analyte	Concentration Spiked (ng/mL)	Supel™ BioSPME F_B(%)	Rapid equilibrium dialysis F_B (%)	Literature Values F_B (%)
Carbamazepine	100	76.4	75.0	70-80%
Diazepam	100	97.3	98.2	98-99%
Imipramine	100	92.6	94.4	63-95%
Prednisolone	100	78.2	86.1	65-91%
Propranolol	100	90.5	89.7	67-94%
Warfarin	2500	99.8	99.7	98.1-99.6%
Zolpidem	100	96.9	99.5	92%
Nalidixic Acid	2000	97.0	91.4	90-95%
Erythromycin	500	81.8	81.7	90%
Ketoconazole	500	96.8	99.0	84-99%
Buspirone	100	81.6	82.3	86-95%

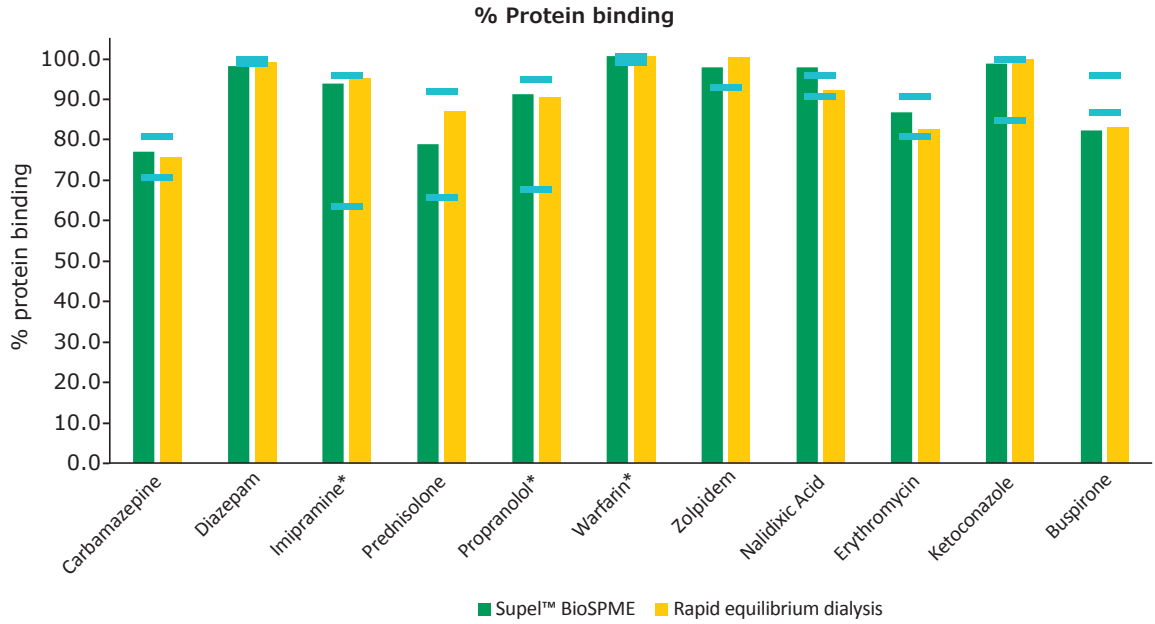

Figure 4. Comparison of protein binding values between Supel™ BioSPME 96-Pin device and rapid equilibrium dialysis methods. The blue lines indicate the protein binding literature values interval. Compounds with stars are charged at physiological pH.

Removal of Phospholipids (Matrix Effects)

Phospholipids are a problematic contaminant found in most biological samples, and it is oftentimes crucial to eliminate their presence in the sample to improve data quality. The remaining levels of phospholipids present after processing by either Supel™ BioSPME or rapid equilibrium dialysis were compared, with results shown in **Table 7** and **Figure 5**.

Samples from both devices were analyzed on an AB Sciex-3200 QTRAP® mass spectrometry with an Agilent 1290 LC using the method described in **Table 5**. The phospholipids that were monitored are listed in **Table 6**.

Table 5. LC-MS/MS conditions for monitoring phospholipids

Column:	Ascentis® Express C8 column (10 cm x 2.1 mm, 2.7 mm)
Mobile Phase:	[A] 5 mM ammonium acetate, 0.1% acetic acid in water [B] 5 mM ammonium acetate, 0.1% acetic acid in 95% acetonitrile and 5% water
Gradient:	80% A, 20% B held for 1.5 min; to 100% B in 1.5 min; the flow is increased to 0.6 mL/min in 0.1 min and held at 100% B for 12 min; in 0.1 min the flow is decreased back to 0.4 ml/min and 20% B, and held for 3 min.
Flow Rate:	0.4 to 0.6 mL/min
Column Temp:	40 ºC
Detector:	MS, ESI(+) Scheduled MRM (See **Table 1** and **2**)
Injection:	2 µL

Table 6. Phospholipid MRM Transitions Monitored, LPC – lysophosphatidylcholine, PC – phosphatidylcholine

Analyte	Precursor	Product	Dwell Time (msec)	DP	CE
Choline	184.1	104.1	40	120	80
LPC 16:0	496.4	184.1	40	120	80
LPC 18:0	524.4	184.1	40	120	80
PC 30:1	704.4	184.1	40	120	80
PC 34:2	758.4	184.1	40	120	80
PC 36:2	786.4	184.1	40	120	80
PC 38:6	806.4	184.1	40	120	80

Analyte	Precursor	Product	Dwell Time (msec)	DP	CE
LPC 18:2	520.4	184.1	40	120	80
LPC 18:1	522.4	184.1	40	120	80
PC 36:1	788.4	184.1	40	120	80
PC 38:5	804.4	184.1	40	120	80
PC 34:1	760.4	184.1	40	120	80
PC 36:3	784.4	184.1	40	120	80

Table 7. Phospholipid Remaining in analyte by method

Method	# of samples	Average % Phospholipid remaining	RSD
Supel™ BioSPME	5	<0.1	<0.01
Rapid equilibrium dialysis	5	56	7.8

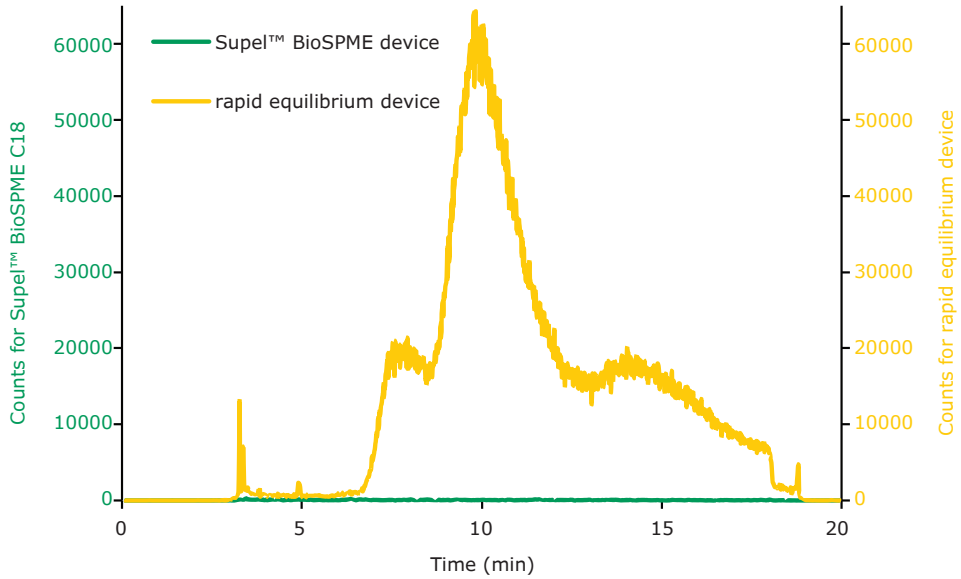

Figure 5. Phospholipid removal: Supel™ BioSPME device vs rapid equilibrium device

Figure 5 illustrates a comparison of chromatograms of the Supel™ BioSPME 96-Pin device prepared sample versus an acetonitrile protein precipitated sample prepared using rapid equilibrium dialysis. As shown in **Table 7**, the Supel™ BioSPME 96-Pin device removes over 99% of phospholipids in the samples processed. Since the rapid equilibrium dialysis process includes only an acetonitrile protein precipitation, phospholipids are fully present in the final injected sample.

Comparison of Workflow Time: Supel™ BioSPME Device Vs Rapid Equilibrium Dialysis

As high throughput laboratories are always interested in optimizing efficiencies as much as possible, the time to perform each one of the workflows was evaluated for comparison purposes. The Supel™ BioSPME 96-Pin device workflow (<2 hours) takes one third of the amount of time as the rapid equilibrium dialysis workflow (6 hours). This results in the ability to increase throughput by 3 times to free up automation instrumentation and scientist time for other assays.

Table 8. Comparison of time requirement by method

Supel™ BioSPME Method	Step Time (min)	Rapid Equilibrium Dialysis Method	Step Time (Min)
Prepare and Incubate Samples	60	Prepare Samples	60
Condition	20	Dialysis	240
Wash	0.2	Post sample preparation	40
Extraction	15	Centrifugation	10
Wash	1	Transfer for into vials for analysis	10
Desorption	15		
Total	**<2 Hours**	**Total**	**6 Hours**

Conclusion

The Supel™ BioSPME 96-Pin device technique has been demonstrated to provide significant timesaving for protein binding determination when compared with the rapid equilibrium dialysis method. The workflow for the Supel™ BioSPME 96-Pin device is less than two hours and provides triple the throughput as compared to the rapid equilibrium dialysis method. In addition, the format of the Supel™ BioSPME 96-Pin device allows for a fully automated robotic method, without the need for additional hardware for centrifugation as needed for the rapid equilibrium dialysis method. This translates to increased laboratory productivity and reduced manual steps. The accuracy of the protein binding values obtained using the Supel™ BioSPME 96-Pin device compare well to those from the rapid equilibrium dialysis method; as demonstrated with 10 compounds with varying Log P values. In addition, the Supel™ BioSPME 96-Pin device also offers cleaner samples in comparison to those from rapid equilibrium dialysis devices. The patented binder and coating of the Supel™ BioSPME 96-Pin device allows for selective extraction of target analytes, while excluding larger macromolecules to provide a fast and accurate drug protein binding measurement.

References

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Materials

Cat No.	Description
59680-U	Supel™ BioSPME 96-Pin device
59686-U	Holder for Supel™ BioSPME 96-Pin device
CLS3357	Corning Costar 350 µL V-bottom 96-well plate
P8241	Nunc 1 mL 96-well plate
Z369659-100EA	SealPlate film
0030131517-80EA	Eppendorf Plate Lid
P5368	Phosphate buffered saline
1.2781	2-Propanol, LC-MS grade
1.15333	Water, LC/MS grade
1.06035	Methanol, LC/MS grade
64065-U	Ascentis® Express Biphenyl column (10 cm x 2.1 mm, 2.7 mm)
C053	Carbamazepine solution, 1 mL, 1 mg/mL in methanol, CRM
C094	Carbamazepine-d10, 1 mL, 100 µg/mL in methanol, CRM
D-907	Diazepam solution, 1 mL, 1 mg/mL in methanol, CRM

Cat No.	Description
D-910	Diazepam-d5, 1 mL, 100 µg/mL in methanol, CRM
I-902	Imipramine hydrochloride solution, 1 mL, 1 mg/mL (as free base) in methanol, CRM
I-903	Imipramine-d3 maleate, 1 mL, 100 µg/mL (as free base) in methanol, CRM
P-121	Prednizolone solution, 1 mL, 1 mg/mL in acetonitrile, CRM
P-055	Propranolol solution, 1 mL, 1 mg/mL in methanol (as free base), CRM
W-003	Warfarin solution, 1 mL, 1 mg/mL in acetonitrile, CRM
Z-017	Zolpidem solution, 1 mL, 1 mg/mL in methanol, CRM
97023	Nalidixic acid analytical standard, 100 mg
PHR1039	Erythromycin, Pharmaceutical Secondary Standard, CRM, 1 g
B-054	Buspirone hydrochloride solution, 1 mL, 1 mg/mL in methanol (as free base), CRM
K-004	Ketoconazole solution, 1 mL, 2 mg/mL in methanol, CRM

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