In association with:

Understanding drug–protein binding and ADME studies for DMPK

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Technology Digest: understanding drug–protein binding and ADME studies for DMPK

by Vivian Xie
Digital Editor, Bioanalysis Zone

DMPK: what is it?

In drug development, DMPK studies are performed throughout the development process in order to determine the pharmacological characteristics of a drug candidate, with particular focus on a potential drug's absorption, distribution, metabolism, excretion (ADME) and pharmacokinetic properties. Drug metabolism refers to the process by which a molecule is converted into other related compounds throughout the body [1]. Pharmacokinetics is the quantitative study of a potential drug's ADME – the measurement of how much of a drug candidate is available throughout the human body over time [2].

One of the fundamental parameters utilized to develop pharmacokinetic/pharmacodynamic (PK/PD) relationships, predict drug–drug interactions and evaluate drug candidate toxicity is understanding a drug candidates protein binding. A drug's distribution and protein binding capability changes over its lifetime within the body [3]. As such, proteins exist beyond plasma composition in the bloodstream and bind with drugs in the skin, tissue and organs, understanding how these binding interactions influence the bioavailability and distribution of a drug's active compounds aid in determining its therapeutic effect [4].

The importance of preclinical DMPK/ADME studies

Preclinical DMPK studies can differ slightly from clinical DMPK studies, most notably in the types of studies conducted and the methods of analysis utilized [1].

Throughout the preclinical study phase, drug discovery and development often involves the utilization of in vitro and in vivo experimental models, ranging from test tube experiments, cell cultures, animals or human subjects that are healthy or otherwise do not exhibit the disease/condition targeted by the drug candidate [5]. ADME parameters obtained during the preclinical stage provide insight into important chemical characteristics [1]. In vitro studies elucidate a compound's apparent permeability, metabolic stability and protein binding. In vivo studies in animal and healthy human subjects can aid in determining oral availability, exposures, distribution and toxicity [5].

A drug candidate only progresses to the clinical stage of drug development once all preclinical safety studies have been cleared [1]. While there are currently no specific regulatory requirements regarding when ADME studies should be conducted during clinical development, the 2012 European Medicines Agency's guidelines offers the following guidance: "the results of the mass-balance study should generally be available before starting Phase III" [6]. Such studies can include evaluations of drug safety, dosage, side effects, efficacy and drug–drug interactions [7].

> *QPS provides a 1-week turnaround timeline program (10–20 compounds per week) to meet all the sponsor's drug screening project needs - commented Lata Venkatarangan, Director and Head of DMPK, QPS, LLC (DE, USA).*

Understanding the role of protein binding in drug development

A drug compound's protein binding affinity strongly influences its PK/PD behavior [8]. Distribution and protein binding of a compound changes throughout its lifetime within the human body. Drug–protein binding, specifically plasma protein binding, occurs following the absorption of the compound into the bloodstream. Consideration must also be given to the drug–protein binding that occurs outside of the bloodstream, wherein the compound interacts with molecules in skin, bodily fluids, tissues and organs [3]. While bound drug–protein compounds form a reservoir of the drug, only the unbound drug compound is free to exert the desired therapeutic effect on the targeted area [4].

For these reasons, ADME studies that consider drug–protein binding must be an integral part of the drug development process in the preclinical and clinical stages, with consideration given to ADME programs that provide the proper bioanalytical capabilities to support regulatory submissions. Services provided by industry specialists, such as QPS, LLC (DE, USA), offer programs such as radiolabeled ADME studies to effectively execute preclinical and clinical ADME programs during the drug development process [9]. Other quantitative and qualitative methods include quantitative whole-body autoradiography (QWBA) in support of a wide range of studies for drug development including ADME [10].

> *QPS can provide the excellent service for compounds with challenging time-to-equilibrium issues, they are experienced with the protein binding determining from several different CROS, which helps the sponsor move the projects long smoothy - explained Jovita Saquing, Senior Research Scientist, QPS, LLC (DE, USA).*

Preclinical radiolabeled and QWBA ADME studies: the turning point

Radiolabeled ADME studies are typically conducted through the administration of a single dose of the drug compound containing a radioactive nuclide. Plasma and excretion samples are then collected and analyzed for total radioactivity and the profile of drug-related material in urine, feces and plasma, allowing for the quantitation of all drug-related material in biological samples. Radiolabeled ADME studies, in contrast to traditional analytical methods such as MS, is dependent only on the specific activity of the analytes, which is constant between the initial drug compound and the subsequent metabolites containing the radioactive nuclide. Radiometric HPLC does not, in fact, even require the structural identity of the metabolite to characterize them [7].

QWBA can similarly be utilized in the drug development process to determine the distribution and concentration of radiolabeled test compounds, providing information on PK/PD, accumulation and retention [11]. As a technique that can be performed separately or combined with radiolabeled mass balance and plasma PK studies, it provides high-resolution data in the ADME properties of a drug candidate [10].

Service providers will ideally support a range of ADME programs that include QWBA and radiolabeled ADME studies, including in vitro and ex vivo protein binding determining from drug discovery to GLP utilizing non-radiolabeled and radiolabeled compounds, such as small molecules, peptides and oligonucleotides [9]. Depending on the compound behavior, protein binding can thus be determined in a variety of biological matrices, including but not limited to plasma, serum and CSF, or tissues, such as brain, liver, lung or pancreas [10]. These services form an integral component of the ADME package required for a new drug application submission and the subsequent progression of a drug candidate throughout the development pipeline.

> “*They [QPS] deliver high-quality data for the most challenging ex vivo protein binding determination methods (i.e. ultracentrifugation) even for the most difficult compounds, which may not be possible with other techniques - concluded Timothy Snow, PhD, Principal Research Consultant, Snow Scientific Consulting (FL, USA).*”

Summary

Protein binding ADME studies form a key role in determining the PK/PD of a potential new drug and are important considerations not only for the health and safety of all patients but also for weighing time and cost balances throughout a drug development program [3]. Implementing a well-rounded ADME program for the investigation of drug–protein binding can ensure a detailed assessment of the total fate of a drug candidate and support regulatory and new drug application submissions [10].

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QPS White Paper

Plasma Protein Binding

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Introduction

Drugs bind to plasma proteins including albumin, α1-acid glycoprotein, and lipoproteins with different affinities and selectivities depending on the physical/chemical properties of the drug. It is generally accepted that the bound and unbound or free drug in plasma is in rapid equilibrium with each other, but it is the free drug that is biologically active, i.e., determines the biological activity of the drug. Therefore, it is important to determine the free fraction of drugs at different stages of drug development including discovery, preclinical, and clinical.

At the discovery stage, plasma protein binding studies can be used to differentiate structural leads with different extent of binding. At the preclinical stage, the free concentration in different species can be used to estimate exposure multiples using data generated in preclinical efficacy models, GLP-compliant toxicology studies, and anticipated clinical dose range.

Factors that can alter the amount of free drug in plasma may include the concentration of the drug, concentration of the metabolite(s), the quantity and quality of the plasma proteins (e.g., malnutrition, infection, liver disease, renal disease, malignancy), and co-administration of two or more drugs. If a compound is highly bound to plasma proteins, it is important to determine which plasma protein the compound is bound to, e.g., albumin, α1-acid glycoprotein, lipoproteins, or other carrier proteins found in plasma. If the compound is highly bound to lipoproteins, it may be necessary to determine, using density gradient ultracentrifugation, which lipoprotein fraction, e.g., high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), etc. is involved.

Plasma protein binding drug-drug interactions (DDIs) can change the safety and efficacy profiles of a drug. This is especially clinically relevant for drugs with a narrow therapeutic index that are highly bound to plasma proteins, in which small changes in the free fraction can result in unexpected and often undesirable side effects. For example, if a drug has a therapeutic index of 5 and a free fraction of 1% (i.e., 99% bound), increasing the free fraction to 2% (i.e. 98% bound) could lower the therapeutic index to 2.5 due to a doubling of the free fraction.

Experimental Conditions

Protein Binding Methods

Plasma protein binding determinations can be performed *in vitro*, *ex vivo*, or *in vivo* with radioactive or non-radioactive compounds using various methods including ultrafiltration, equilibrium dialysis, and microdialysis.

The choice of ultrafiltration, equilibrium dialysis or microdialysis depends on the type of data to be generated, *in vitro*, *ex vivo* or *in vivo*, and the physical/chemical properties of the compound being evaluated. A compound that binds non-specifically to equipment would dictate that equilibrium dialysis is used. Conversely, if the compound is unstable in plasma, ultrafiltration is the method of choice. However, if *in vivo* free concentration is the desired outcome, then microdialysis is the only option.

Methods of Quantification

A robust, sensitive, and specific bioanalytical method, usually LC-MS/MS, or scintillation counting using a radioactive ligand, is required to quantify low, free concentration of the study drug.

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Other Factors to Consider

When scintillation counting is chosen as the method of quantification, a radiolabeled test article can be spiked into the plasma samples collected. The amount of radiolabeled material added should be trace amount that does not change the binding characteristics. The purity of the radiolabeled material should be sufficiently high as not to affect the testing results.

Generating reliable plasma protein binding data requires: assessment of the compound's stability during the duration of the protein binding study, amount of time required to reach protein binding equilibrium, and the extent of non-specific binding of the compound to the equipments.

Using equilibrium dialysis, the extent of plasma protein binding of the test article is usually determined at 37°C for 4 – 6 hours in an apparatus that rotates to maximum interactions between the test article and the plasma protein binding sites.

Plasma samples can be obtained from preclinical studies, or from phase I, phase II, or phase III clinical studies conducted in healthy subjects or patients. The plasma may contain drug alone, drug with metabolites or drug and metabolites with co-administered drug(s) and its metabolite(s). The number of samples per subject or patient will depend on the study protocol's specifics on number of subjects per dose, number of samples collected per subject and volume of blood collected at each collection time point.

Although the study design for a protein binding study is relatively straightforward for most compounds, the exact experimental conditions can be a challenge when there are specific questions being asked.

The protein binding studies described in this White Paper show how *ex vivo* binding studies can answer some important clinical questions.

Example 1

Question: *What is the free fraction of Compound A in plasma of healthy subjects after a single dose?*

Method: Plasma samples were obtained from a single dose escalation study in healthy subjects. Plasma samples were collected at 3, 4, and 6 hours post dosing from subjects who received the same dose. Pilot studies indicated that Compound A was stable in plasma at 37°C and was not bound to the ultrafiltration device. The *ex vivo* protein binding study was conducted at 37°C using ultrafiltration and the test article was quantified using LC-MS/MS.

Results:

Time (hours)	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Mean ± SD
3	5.01	3.57	4.14	4.51	2.93	3.92	4.03 ± 0.81
4	4.93	4.00	4.16	4.77	3.76	3.90	4.32 ± 0.50
6	4.73	4.22	6.75	3.78	5.07	4.55	4.91 ± 1.14
Mean ± SD	4.89 ± 0.14	3.93 ± 0.33	5.02 ± 1.50	4.35 ± 0.51	3.92 ± 1.08	4.89 ± 0.14	4.42 ± 0.52

Table 1. The free fraction of Compound A in plasma of healthy subjects.

Conclusion: Compound A was highly bound to plasma proteins of healthy subjects with a free action averaging 4.42%. The percent free was independent of the time when the plasma sample was collected (p=0.345), suggesting it is independent of the plasma concentration of the test article and the concentration of the metabolite(s), if present.

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Example 2

Question: *Is the free fraction of Compound B in plasma of healthy subjects after a single dose different from the free fraction after multiple doses?*

Method: Plasma samples were obtained from a multiple dose escalation study in healthy subjects. Blood samples were collected at 1 hour post dosing from the same subjects on Study Day 1 and Study Day 9. Pilot studies indicated that Compound B was stable in plasma at 37°C and equilibrium was established after 3 hours of incubation. The *ex vivo* protein binding study was conducted at 37°C for 3 hours using equilibrium dialysis and the test article was quantified using LC-MS/MS.

Results:

	Subject								
Study Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Mean ± SD**
1	8.53	7.32	7.84	6.36	9.24	5.92	9.16	6.95	4.03 ± 0.81
9	6.31	10.60	7.67	7.10	5.24	8.29	15.30	15.02	9.44 ± 3.86
Mean	7.42	8.96	7.76	6.73	7.24	7.11	12.23	10.99	

Table 2. The free fraction of Compound B in plasma of healthy subjects after single and multiple doses.

Conclusion: Compound B was moderately bound to plasma proteins of healthy subjects with a free fraction averaging 7.67% ± 1.25 on Study Day 1 and 9.44% ± 3.86 on Study Day 9. There was no significant difference in free fraction between Study Day 1 and Study Day 9 ($p=0.256$).

Example 3

Question: *Does renal impairment affect the free fraction of Compound C?*

Method: Plasma samples were obtained at 3 hours post dosing from a single dose study comparing the pharmacokinetics of Compound C in healthy subjects, and renal impaired patients. The *ex vivo* protein binding study was conducted at 37°C using ultrafiltration, and the test article was quantified using LC-MS/MS.

Results:

Group	Renal Function (Based on Measured Creatinine Clearance)	N	% Bound (Mean ± SD)
1	Normal	6	59.85 ± 4.50
2	Mild Renal Impairment	6	65.63 ± 4.06
3	Moderate Renal Impairment	6	62.84 ± 6.34
4	Severe Renal Impairment	6	61.23 ± 6.27
5	End Stage Renal Failure	6	55.69 ± 3.92

Table 3. Protein binding of Compound C in plasma of healthy subjects and renal impaired patients.

Conclusion: Compound C was moderately bound to plasma proteins of healthy subjects with a free fraction averaging 40.15%. The free fraction of Compound C in patients with different levels of renal impairment was similar to the free fraction determined in healthy subjects.

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Example 4

Question: *What is the free fraction of Compound D and its major metabolite X?*

Method: Plasma samples were obtained at 2 and 4 hours post dosing from a singe dose, escalation study. The *ex vivo* protein binding study was conducted at 37°C using equilibrium dialysis, and the test article was quantified using LC-MS/MS.

Results:

	N	Compound D	Major Metabolite X
Concentration Range (ng/mL)	80	64.9 - 2380	19.4 - 951
Free Fraction (%)	80	0.213 ± 0.053	0.243 ± 0.059

Table 4. The free fraction of Compound D and its major metabolite X in plasma of healthy subjects.

Conclusion: Compound D and its metabolite X were tightly bound to plasma proteins of healthy subjects with a free fraction averaging 0.213% ± 0.053 and 0.243% ± 0.059, respectively. The free fraction of Compound D and metabolite X appeared to be independent of their plasma concentrations over a 33-fold and 49-fold range, respectively.

Example 5

Question: *Does Compound E affect the free fraction of Compound F?*

Method: Compound F was administered to healthy subjects once daily for 19 days, and Compound E was co-administered with Compound F three times daily on Study Days 15 to 19. Plasma samples were obtained at various time points on Study Day 14 (steady state plasma concentrations of Compound F) and Study Day 19 (steady state plasma concentrations of Compound E and Compound F). The *ex vivo* protein binding study was conducted at 37°C using equilibrium dialysis, and the Compound F was quantified using LC-MS/MS.

Results:

	Compound F	Compound F & Compound E
Free Fraction (%)	0.864 ± 0.146	0.930 ± 0.147
Range of Free Fraction (%)	0.584 - 1.25	0.657 - 1.46
Free Concentration (ng/mL)	3.052 - 11.332	2.358 - 13.139

Table 5. The free fraction of Compound F in the absence and presence of Compound E in plasma of healthy subjects.

Conclusion: Compound F was highly bound to plasma proteins of healthy subjects with a free fraction averaging less than 1%. The free fraction of Compound F, as well as the free concentrations of Compound F, were not changed in the presence of Compound E. These results indicate the lack of protein binding interactions after co-administration of Compound E and Compound F.

Summary

The *ex vivo* protein binding studies presented in this White Paper show the type of data that can be generated, and the value of knowing the extent of plasma protein binding in guiding the design and execution of the clinical development plan for a drug.

Research Article

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Improving the accuracy of unbound fraction measurement of drug–protein binding by preconditioning the RED membrane inserts

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Aim: The objective of this study was to evaluate the rapid equilibrium dialysis (RED) device in protein binding assays in diluted protein matrices and to improve the accuracy of the unbound fraction (f_u) measurement. **Methodology:** Protein binding assays of drug compounds in bovine serum albumin solutions and human plasma with different folds of dilution were performed using the RED device with and without preconditioning of the dialysis membrane inserts, and the results were compared with those using other approaches in this study. **Results & conclusion:** Preconditioning of the RED membrane inserts improved the f_u data accuracy of drug–protein binding assay in matrices with relatively low protein contents and such impact could be compound dependent.

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Keywords: bovine serum albumin • dialysis membrane • drug–protein binding • equilibrium dialysis • membrane preconditioning

Drug–protein binding assays are often conducted at different stages of drug discovery and development. In the discovery phase, plasma protein binding (PPB) assay is performed along with preclinical pharmacokinetic studies to evaluate the unbound drug exposure and clearance, in the selection of drug candidates for further lead optimization. The unbound fraction (f_u) is an important parameter for establishing pharmacokinetic/pharmacodynamic models, projecting clinical efficacious doses, predicting drug–drug interaction potentials, etc [1,2]. Protein binding assay is also conducted in various other matrices, for example incubation media for *in vitro* drug metabolism and pharmacological assays to better understand the target potency of drug candidates and their mechanism of actions, since free drug instead of total drug concentration is often needed for dose-response correlation and data interpretation [3].

Equilibrium dialysis is one of the most commonly used methods for measuring drug–protein binding [3–15]. The free drug is separated from the protein-bound drug by passing through a semi-permeable membrane. Over the last few decades, the development of dialysis devices in 96-well formats has greatly improved the assay throughput. Rapid equilibrium dialysis (RED) device is a commercially available apparatus that has been widely used in protein binding assays [8–13,15]. The RED device uses a base plate containing 48 compartments and disposable dialysis membrane inserts. Such design simplifies the assembly/preparation of the device and eliminates the potential for crosstalk or leakage. The standard 96-well plate footprint of the base plate is suitable for automation using robotic liquid handlers. The RED device has been validated for PPB assay and produced results consistent with literature values [8,9]. However, there is a lack of reference information on its performance in protein binding assays in other matrices such as incubation media with low concentrations of proteins (e.g., serum albumin). Due to the significant impact of protein binding on a drug's pharmacological activity, target potency, clinical dose prediction, etc., it is important to ensure accurate f_u data are generated.

In this work, protein binding assays of drug compounds in bovine serum albumin (BSA) solutions and human plasma with and without dilution were performed using the RED device and the data were compared with those obtained using other methods. We observed that preconditioning of the RED membrane inserts, a step not instructed by the vendor, significantly improved the f_u data accuracy of drug–protein binding measurement in matrices with relatively low protein contents. Experiments were also performed for identification of the potential

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interferences from the dialysis membrane which could have possibly contributed to the inaccurate determination of f_u.

Experimental

Chemicals & materials

Analytical standards of the test compounds, BSA, alpha 1-acid glycoprotein (AAG) from human plasma, glycerol and polyethylene glycol (PEG) standards were purchased from Sigma (MO, USA). The internal standard was a proprietary compound synthesized in-house. Dimethyl sulfoxide (DMSO), HPLC-grade methanol and acetonitrile were obtained from Sigma. Deionized water was produced using a Milli-Q Water Purification System (Millipore SAS, France). Human plasma (K_2EDTA) was from BioIVT Inc. (NY, USA). The RED base plates and membrane inserts (MWCO: 8 kDa) were from Thermo Fisher Scientific (MA, USA). The high-throughput dialysis (HTD) device and membranes (MWCO: 6 to 8 kDa) were from HTDialysis (CT, USA). The ultrafiltration devices (Microcon Ultracel YM-10 centrifugal filter units, MWCO: 10 kDa) were from Millipore (MA, USA). The dialysis phosphate buffer (100 mM sodium phosphate and 150 mM sodium chloride, pH 7.4) was prepared in-house. BSA solutions and diluted human plasma were prepared by adding an appropriate amount of the BSA or human plasma into the phosphate buffer. The working solutions of the test compounds were prepared in DMSO. Standards of glycerol and PEG were prepared in deionized water. The internal standard solution was prepared at 50 ng/mL in acetonitrile.

For all the assays using equilibrium dialysis and ultrafiltration as described below, the test compounds were individually spiked into a specific matrix (protein solution, plasma, or diluted plasma) at 1 μM by adding appropriate amount of the 200 μM DMSO solution of each compound into the matrix and mixed well (final DMSO content 0.5%). The experiments were performed in triplicate.

Protein binding assay using RED device

A 200 μl aliquot of the spiked sample was added to the donor chamber and 350 μl of the phosphate buffer was added to the receiver side of the RED plate with membrane inserts. The loaded device was sealed with an adhesive membrane and incubated with shaking for 18 h at 37°C in a CO_2 incubator with 85% humidity. Time zero samples were prepared by adding an aliquot of the spiked sample prior to dialysis to an equal volume of buffer and extracted with the internal standard solution in acetonitrile. After incubation, an aliquot of the sample from the donor or receiver chamber of the RED device was added to an equal volume of the opposite blank matrix and extracted with the internal standard solution.

Preconditioning of the RED membrane inserts

A 550 or 800 μl aliquot of deionized water was added to the donor or receiver side of the membrane inserts. The inserts were soaked for 30 min before removal of the water. The procedure was repeated once. The preconditioned membrane inserts were placed into a RED base plate for the dialysis assay. For identification of interferences, the rinsing water was collected and pooled each time after soaking of the membrane inserts. A 15 mL aliquot of the pooled water sample was evaporated to dryness, reconstituted with 300 μl of deionized water and analyzed by LC–MS.

Protein binding assay using HTD device

The dialysis membranes were soaked in deionized water for 1 h and in 20% ethanol in water for 20 min. The membranes were then rinsed thoroughly with deionized water and soaked in the phosphate buffer prior to use. The device was assembled according to the manufacturer's instruction [7]. A 150 μl aliquot of the spiked sample was added to the donor chamber and 150 μl of the phosphate buffer was added to the receiver chamber of the HTD device. The loaded device was sealed with an adhesive membrane and incubated with shaking for 18 h at 37°C in a CO_2 incubator with 85% humidity. Time zero plasma samples were prepared by adding an aliquot of the spiked sample to an equal volume of buffer and extracted with the internal standard solution in acetonitrile. After incubation, an aliquot of the sample from the donor or receiver chamber was added to an equal volume of the opposite blank matrix and extracted with the internal standard solution.

Table 1. Properties and multiple reaction monitoring transitions of the test compounds.

Test compound	Class	cLogD (pH 7.4)	MRM transition
Warfarin	Acidic	1.7	309 → 163
Oxaprozin	Acidic	1.1	294 → 103
Verapamil	Basic	2.8	455 → 165
Bosentan	Acidic	4.0	552 → 202
Glibenclamide	Acidic	2.9	494 → 369
Zafirlukast	Acidic	5.5	576 → 319
Atorvastatin	Acidic	2.4	559 → 440
Efavirenz	Neutral	4.5	314 → 244

MRM: Multiple reaction monitoring.

Protein binding assay using ultrafiltration

After incubation of the spiked plasma at 37°C for 1 h, a 450 µl aliquot of the sample was loaded to the top reservoir of the Microcon ultrafiltration device and centrifuged at 1000 × g in an Eppendorf 5424R centrifuge (NY, USA) at 37°C for 15 min. To determine the volume of filtrate, the collection tube of each ultrafiltration unit was weighed on a Sartorius balance (Alert Scientific, CT, USA) before and after centrifugation. An aliquot of the initial spiked sample was added to an equal volume of the phosphate buffer and extracted with the internal standard solution. After centrifugation, an aliquot of the retentate or ultrafiltrate sample was added to an equal volume of the opposite blank matrix and extracted with the internal standard solution.

Nonspecific binding measurement for ultrafiltration

To assess the nonspecific binding (NSB) loss during ultrafiltration, compounds were spiked into the phosphate buffer at 1 µM and a 450 µl aliquot of the spiked buffer was centrifuged through the ultrafiltration device at 1000 × *g* for 15 min. Aliquots of the initial spiked buffer sample before centrifugation and the ultrafiltrate samples were extracted with the internal standard solution.

Sample extraction & LC–MS/MS analysis

The sample extraction mixtures were vortexed thoroughly and centrifuged at 3600 r.p.m. for 20 min using a Beckman Allegra centrifuge (Beckman Coulter, CA, USA). The supernatants were transferred to 96-well plates for LC–MS/MS analysis.

The LC–MS/MS analysis was performed on an API 5500 QTrap or 6500+ triple quadrupole mass spectrometer (AB Sciex, CA, USA) coupled with Agilent 1100 or 1290 Series binary pumps (Agilent Technologies, CA, USA) and a CTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The drug compounds were eluted on a Unisol C18 column (2.1 × 30 mm, 5 µm; Agela Technologies, DE, USA) using a previously established gradient method [16]. Mobile phase A was 10 mM ammonium acetate in water (pH 4). Mobile phase B was acetonitrile/methanol (1:1, *v/v*). Glycerol was eluted on an Atlantis HILIC Silica column (2.1 × 50 mm, 5 µm; Waters Corporation, MA, USA), with mobile phase A of 10 mM ammonium acetate in water (pH 4) and mobile phase B of acetonitrile.

The compounds were ionized in positive or negative electrospray ionization (ESI) mode and detected by multiple reaction monitoring transitions (Table 1). Representative LC–MS/MS chromatograms are shown in Supplementary Figures 1 & 2. Glycerol was monitored using the transition of m/z 93 → 93. The ESI source temperature was 600°C. The nebulizer and heater gas were set at 45 and 60 psi, respectively. The ion spray voltage was 5000 and -4500 V for positive and negative ionization, respectively. The data were processed using Analyst 1.6.2 or 1.7.1 software.

Data analysis

Quantitation was based on the peak area ratio of the compound versus the internal standard. For equilibrium dialysis assay, the f_u and recovery were calculated using Eq. 1 and Eq. 2. For PPB assay in diluted plasma, Eq. 3 was used to calculate f_u (extrapolated) in the undiluted plasma based on the measured f_u in diluted plasma and the dilution factor.

$$f_u = A_{r,t}/A_{d,t} \qquad \text{(Eq. 1)}$$

Table 2. Unbound fraction (% mean ± SD) of warfarin and oxaprozin in bovine serum albumin solutions.

% BSA	Warfarin			Oxaprozin		
	RED	HTD	f_u ratio (RED/HTD)	RED	HTD	f_u ratio (RED/HTD)
0.125	63.8 ± 3.2[†]	34.0 ± 0.3	1.9	44.7 ± 3.2[†]	13.0 ± 1.3	3.4
1.0	6.68 ± 0.39	6.22 ± 0.79	1.1	2.30 ± 0.11[†]	1.48 ± 0.10	1.6
2.0	2.94 ± 0.05	2.75 ± 0.26	1.1	0.85 ± 0.06	0.88[‡]	1.0
4.0	1.64 ± 0.14	1.83 ± 0.20	0.9	0.49 ± 0.10	0.46 ± 0.06	1.1

[†]$p < 0.01$ (comparing f_u data measured using the RED and HTD devices)
[‡]n = 1.
BSA: Bovine serum albumin; RED: Rapid equilibrium dialysis.

$$\text{Recovery (\%)} = \left(V_r A_{r,\,t} + V_d A_{d,\,t}\right) \times 100 / \left(V_d A_{0h}\right) \tag{Eq. 2}$$

$$f_u\,(extrapolated) = \frac{1/D}{[(1/f_u) - 1] + 1/D} \tag{Eq. 3}$$

$A_{r,\,t}$ and $A_{d,\,t}$: peak area ratio of the compound in the receiver and donor samples after incubation, respectively

A_{0h}: peak area ratio of the compound in the time zero sample

V_d: donor volume (μl); V_r: receiver volume (μl)

D: plasma dilution factor

For ultrafiltration assay, the NSB, f_u and recovery were calculated using Eq. 4, Eq. 5, and Eq. 6, respectively. For assay in diluted plasma, f_u (extrapolated) in the undiluted plasma was calculated using Eq. 3.

$$NSB = \left(A_{b,\,total} - A_{b,\,f}\right)/A_{b,\,total} \tag{Eq. 4}$$

$$f_u = A_f / \left\{(1 - NSB) \times A_{p,\,total}\right\} \tag{Eq. 5}$$

$$\text{Recovery (\%)} = (V_f A_f + V_p A_p) \times 100 / (V_{p,\,total} A_{p,\,total}) \tag{Eq. 6}$$

$A_{b,\,total}$: peak area ratio of the compound in the spiked buffer before ultrafiltration

$A_{b,\,f}$: peak area ratio of the compound in the ultrafiltrate

A_f, A_p, and $A_{p,\,total}$: peak area ratio of the compound in the ultrafiltrate, plasma retentate and the spiked plasma before ultrafiltration, respectively.

V_f, V_p, and $V_{p,\,total}$: volume of the ultrafiltrate, plasma retentate and the initial spiked plasma, respectively

All the f_u data are expressed in percentage (Tables 2–8). Differences between mean f_u were determined by the Student's t-test using Microsoft Excel (Microsoft Corporation, WA, USA). Data were considered significantly different when $p < 0.01$.

Results & discussion

Protein binding of warfarin & oxaprozin in BSA solutions

In the *in vitro* assays, BSA is commonly added to cell incubation media to support cell growth, increase compound solubility, or reduce NSB of the compounds to assay apparatus, etc. BSA could be a major component in the media that contributes to the compound protein binding. Warfarin and oxaprozin are two acidic drugs that are highly bound to serum albumin. The unbound fractions of the two compounds in BSA solutions (0.125 to 4%) were measured using the RED and HTD devices. HTD is also a commonly used equilibrium dialysis device for protein binding assays [5–7,12,14], and it has been used for comparative analysis for validation of the RED device in PPB assay [8].

As shown in Table 2, f_u (%) values of warfarin were similar using the two devices in solutions containing 1 to 4% BSA. However, at 0.125% BSA, f_u of warfarin obtained from RED was about two-times of that from HTD. For oxaprozin, the f_u (%) values were similar at 2 or 4% BSA using the two devices, while f_u from RED was higher than that from HTD at 0.125% and 1% BSA. The recovery values of the compounds in both RED and HTD assays were mostly within 100 ± 20%. Overall, difference in f_u data was observed for the two compounds in assay with low BSA concentrations, with the data from RED significantly higher than those from HTD ($p < 0.01$).

Table 3. Unbound fraction (% mean ± SD) of oxaprozin in bovine serum albumin solutions using the rapid equilibrium dialysis device (with and without membrane preconditioning).

% BSA	RED	RED (preconditioned)	f_u ratio
0.125	29.4 ± 2.1†	11.8 ± 0.3	2.5
0.25	17.7 ± 1.7†	6.19 ± 0.18	2.9
0.50	6.01 ± 0.20†	3.04 ± 0.04	2.0
1.0	2.19 ± 0.06†	1.57 ± 0.04	1.4

†$p < 0.01$ (comparing f_u data measured using the RED device with and without membrane preconditioning).
BSA: Bovine serum albumin; RED: Rapid equilibrium dialysis.

Table 4. Unbound fraction (% mean ± SD) of verapamil in human AAG solutions.

AAG (mg/ml)	RED	RED (preconditioned)	HTD
1.0	86.3 ± 5.0†	19.6 ± 1.8	19.3 ± 1.1
3.0	33.3 ± 9.0	6.07 ± 0.30	6.49 ± 0.46

†$p < 0.01$ (comparing f_u data measured using the RED device with and without membrane preconditioning).
HTD: High-throughput dialysis; RED: Rapid equilibrium dialysis.

We speculated that there could be some interferences from the RED device that could impact the compound protein binding, for example by competing for the protein-binding sites with the compounds and thus increasing their unbound fractions, especially in matrices with lower protein contents (e.g., 0.125% BSA solution) where the binding sites could be more easily saturated. One possible source of interferences would be from the dialysis membrane. For both RED and HTD devices, the dialysis membranes were made of regenerated cellulose [6,9]. Based on the user instructions, the membranes for the HTD device need to be soaked in water, aqueous ethanol and then rinsed with water or dialysis buffer prior to use [5–7], while preconditioning of the membrane inserts is not required for the RED device [9]. However, the instruction for the RED device is based on PPB assay in undiluted plasma [8,9]. Whether it could be applied to assays in matrices with lower protein contents would need to be evaluated.

We tested preconditioning of the RED membrane inserts by soaking the inserts in deionized water twice prior to the assay (see Experimental for the details). As shown in Table 3, f_u values of oxaprozin measured using the preconditioned RED device (i.e., RED base plate loaded with the preconditioned membrane inserts) were generally lower than those without preconditioning, and the data were comparable with those using the HTD device at the same BSA concentrations (Table 2). Such impact of membrane preconditioning on f_u measurement was likely due to the removal of interferences from the dialysis membrane by soaking in deionized water.

Protein binding of verapamil, a basic drug known to bind to AAG, was measured in solutions containing 1.0 and 3.0 mg/ml of human AAG. The AAG concentration in human plasma typically ranges from 0.55 to 1.4 mg/ml, and it can be elevated in certain disease states to concentrations as high as 3 mg/ml [1]. Table 4 shows that the f_u data obtained using the preconditioned RED device were similar to those using the HTD device, and were much lower than those without preconditioning. This shows that RED membrane preconditioning could also impact the f_u measurement of drug binding to AAG.

PPB of oxaprozin

For highly bound compounds, plasma dilution method can be used to accelerate the reaching of assay equilibrium and facilitate the detection of the compounds in the dialysates [12,15]. The f_u in undiluted plasma can be obtained by extrapolation of the measured f_u in diluted plasma using Eq. 3. Binding of oxaprozin was measured in human plasma with up to 100-fold of plasma dilution using the RED device. For PPB of highly bound compounds, there could be large difference in compound concentrations between the receiver (dialysate) and donor samples, especially for assay in undiluted plasma where the free concentration could be very low. To assess the linearity of response in the concentration range of interest, oxaprozin was spiked in plasma in the range of 0.2–2000 nM, added with equal volume of buffer and extracted with the internal standard solution. The correlation coefficient (r) of the calibration curve was >0.995 with a weighting factor of $1/x^2$ for linear regression.

As shown in Table 5, in the undiluted plasma assay, the f_u values of oxaprozin were similar using RED device with and without membrane preconditioning and the data were consistent with the literature f_u value of 0.10% in human plasma [17]. However, in diluted plasma (≥10-fold dilution) the f_u (%) values without preconditioning

Table 5. Unbound fraction (% mean ± SD) of oxaprozin in human plasma.

Plasma dilution factor	RED	RED (preconditioned)	HTD	Ultrafiltration
1	0.12 ± 0.01	0.14 ± 0.03	0.26 ± 0.06	0.17 ± 0.03
10	1.58 ± 0.04[†]	0.94 ± 0.02	0.92 ± 0.02	0.85 ± 0.05
20	4.89 ± 0.13[†]	2.00 ± 0.06	2.26 ± 0.11	1.82 ± 0.06
50	26.5 ± 0.7[†]	5.40 ± 0.26	6.63 ± 0.23	5.03 ± 0.28
100	65.5 ± 4.8[†]	12.5 ± 0.6	13.6 ± 0.8	10.6 ± 0.4
f_u (extrapolated)				
10	0.16 ± 0.00[†]	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.01
20	0.26 ± 0.01[†]	0.10 ± 0.00	0.12 ± 0.01	0.09 ± 0.00
50	0.72 ± 0.06[†]	0.11 ± 0.01	0.14 ± 0.01	0.11 ± 0.01
100	1.90 ± 0.42[†]	0.14 ± 0.01	0.16 ± 0.01	0.12 ± 0.00

[†]$p < 0.01$ (comparing f_u data measured using the RED device with and without membrane preconditioning).
HTD: High-throughput dialysis; RED: Rapid equilibrium dialysis.

of the membrane inserts were significantly higher than those using the preconditioned RED device ($p < 0.01$), suggesting the impact of membrane preconditioning on the f_u measurement of oxaprozin in diluted human plasma.

Binding of oxaprozin in human plasma was also measured using the HTD device and ultrafiltration. Oxapozin did not show NSB loss to the filter membrane during ultrafiltration of spiked buffer. The recovery values of oxaprozin in the RED, HTD and ultrafiltration assays were mostly within 100 ± 20%. The f_u data obtained with HTD and ultrafiltration were comparable with those using the preconditioned RED device (Table 5), which further demonstrated the improvement of f_u data accuracy by preconditioning the RED membrane inserts.

The extrapolated f_u values of oxaprozin were comparable across the plasma dilution conditions tested, based on the f_u data measured using the preconditioned RED device, HTD device and ultrafiltration (Table 5). In the RED assay without membrane preconditioning, the mean extrapolated f_u (%) from the 100-fold diluted plasma (1.90%) was more than ten times higher than that in the undiluted plasma (0.12 %). Without comparing the data to those using the preconditioned RED device or other methods, such difference in f_u could be interpreted as saturation of the binding sites for oxaprozin in the diluted plasma and thus could cause misunderstanding of the protein binding behavior of this compound. Therefore, it would be essential to precondition the RED membrane inserts for compounds like oxaprozin for generating accurate f_u data in matrices with diluted protein contents.

Binding of drugs in diluted protein matrices

To further assess the impact of RED membrane preconditioning on the f_u measurement, a group of drug compounds with a wide range of lipophilicity (see cLogD values in Table 1) were assessed for their binding in the 0.125% BSA solution and 100-fold diluted human plasma. For warfarin, bosentan, glibenclamide and zafirlukast, the f_u values in the 0.125% BSA assay using the preconditioned RED device were significantly lower than those without preconditioning ($p < 0.01$), and the data were comparable with those using the HTD device. For efavirenz, the f_u values from RED without membrane preconditioning were slightly higher than those measured using the preconditioned RED device and were not significantly different from those using the HTD device. For atorvastatin, the f_u data obtained from RED with and without membrane preconditioning were similar and were comparable with those from HTD (Table 6). Similar trends were observed for these compounds in the 100-fold diluted human plasma assay (Table 7).

The results show that the impact of RED membrane preconditioning on the f_u measurement could depend on the specific compound. We speculated that there could be some difference in the protein binding sites/mechanisms or binding affinity for these compounds, and so in some cases the interference (if indeed present on the membrane) would have limited or no impact. Further investigation is needed to better understand such differences.

Exploratory investigation on potential interferences from the RED membrane inserts

Regenerated cellulose dialysis membranes can be made using cellulose acetate, organic acids, modification agents (e.g., polypropylene glycol), glycerol, etc. Our hypothesis was that some residual components on the dialysis

Table 6. Unbound fraction (% mean ± SD) of compounds in the 0.125% bovine serum albumin solution.

Compound	RED	RED (preconditioned)	HTD
Warfarin	64.0 ± 4.3†	34.5 ± 1.6	33.5 ± 3.2
Bosentan	52.7 ± 3.9†	17.6 ± 0.4	21.6 ± 0.4
Glibenclamide	19.0 ± 1.2†	5.29 ± 0.43	4.50 ± 0.45
Zafirlukast	2.45 ± 0.32†	0.86 ± 0.13	0.95 ± 0.18
Atorvastatin	69.2 ± 6.1	65.7 ± 9.2	65.7 ± 9.5
Efavirenz	27.0 ± 1.1	23.7 ± 0.4	28.8 ± 0.6

†$p < 0.01$ (comparing f_u data measured using the RED device with and without membrane preconditioning).
HTD: High-throughput dialysis; RED: Rapid equilibrium dialysis.

Table 7. Unbound fraction (% mean ± SD) of compounds in the 100-fold diluted human plasma.

Compound	RED	RED (preconditioned)	HTD
Warfarin	85.3 ± 1.5†	45.9 ± 1.7	42.4 ± 3.1
Bosentan	88.3 ± 2.6†	54.2 ± 3.2	45.4 ± 1.8
Glibenclamide	47.0 ± 4.2†	9.54 ± 0.13	8.45 ± 0.20
Zafirlukast	6.38 ± 0.49†	1.01 ± 0.19	1.08 ± 0.19
Atorvastatin	83.2 ± 7.1	70.3 ± 8.1	77.5 ± 4.2
Efavirenz	47.7 ± 2.2	36.9 ± 3.0	43.6 ± 1.2

†$p < 0.01$ (comparing f_u data measured using the RED device with and without membrane preconditioning).
HTD: High-throughput dialysis; RED: Rapid equilibrium dialysis.

Table 8. Unbound fraction (% mean ± SD) of oxaprozin in bovine serum albumin solutions with different levels of glycerol.

Glycerol (mM)	0.25% BSA	2% BSA
0†	21.8 ± 1.6	0.92 ± 0.08
0	7.51 ± 0.27	0.81 ± 0.06
100	7.09 ± 0.36	0.79 ± 0.03
200	6.74 ± 0.18	0.77 ± 0.04
500	7.44 ± 0.16	0.80 ± 0.03
1000	7.71 ± 0.21	0.94 ± 0.05

†RED assay without membrane preconditioning.
BSA: Bovine serum albumin; RED: Rapid equilibrium dialysis.

membrane from the manufacturing process could possibly interfere with drug–protein binding by displacement of drugs from the protein binding sites or other interactions and thus increase their unbound fractions.

Glycerol

Glycerol is added to cellulose membranes during manufacturing as a humectant to preserve membrane integrity during storage [6,9]. To estimate the glycerol level from membrane, a 200 μl aliquot of 2% BSA solution was added to the donor chamber of the membrane inserts (with or without preconditioning) to be dialyzed against 350 μl buffer in the RED device. The measured glycerol levels in the 18-h donor and receiver samples from the RED device without membrane preconditioning were very similar (~200 mM), which also suggests that glycerol does not bind to BSA. Glycerol levels in the samples from the preconditioned RED device were much lower (mostly below 20 mM), indicating substantial removal of glycerol by presoaking the membrane inserts in deionized water.

To assess its impact on protein binding of oxaprozin, glycerol was added to 0.25% or 2% BSA solutions (prespiked with oxaprozin at 1 μM) at concentrations up to 1000 mM. The spiked samples were dialyzed against buffer in the preconditioned RED device. Table 8 shows that at 0.25% BSA the f_u values of oxaprozin were consistent across the glycerol levels tested and were much lower than those obtained without preconditioning of the membrane inserts. At 2% BSA, the f_u values of oxaprozin were consistent throughout the assay conditions. Therefore, glycerol did not appear to affect the binding of oxaprozin to BSA under the conditions tested.

Polyethylene glycol

Other residual components on the dialysis membrane could possibly be the modification agents (e.g., PEG) used in the membrane preparation process. Some PEG compounds can interact with proteins via their hydrophobic moiety [18]. For PEG identification, the rinsing water from membrane inserts was evaporated to dryness and reconstituted with deionized water (see Experimental section for details). The reconstituted samples were analyzed by LC–MS with precursor ion scan of m/z 89 and m/z 177, as well as full scan in the Q1 mass range of m/z 50–1250. The chromatograms of the samples were compared with those of the reconstituted deionized water (blank control) and PEG standards with average molecular weights ranging from 200 to 1500. A group of parent ions were detected in the positive ion mode with a mass interval of 44 Da and those were also observed for some PEG standards (e.g., PEG-400, PEG-600). A late eluting peak with high intensity was observed on the chromatograms of the samples and it was not detected in any of the PEG standards tested. The peak also consisted of a group of ions with a mass interval of 44 Da and it could possibly be from PEG derivatives with high hydrophobicity and strong retention on the C18 column. The intensity of these monitored ions decreased significantly in the reconstituted water samples from the twice-soaked membrane inserts as compared to those being soaked only once.

To assess the impact of PEG on protein binding of oxaprozin, PEG-400 or PEG-1500 was added to 0.125% BSA solution (pre-spiked with oxaprozin at 1 μM) at concentrations up to 100 μM and incubated in the preconditioned RED device. The f_u values of oxaprozin were consistent throughout the conditions tested. The mean f_u (%) of oxaprozin was 9.4% and 9.2% in the presence of 100 μM of PEG-400 and PEG-1500, respectively, and the data were very similar to that in the BSA solution without PEG (mean f_u of 9.5%). Therefore, these two PEGs did not appear to affect oxaprozin-BSA binding under the conditions tested. However, it is unknown whether any PEG derivatives (if present on the membrane) could possibly interfere with drug–protein binding. Future investigation using high resolution mass spectrometry could be helpful for identification of interferences from the dialysis membrane. If there could be other contaminants (e.g., heavy metals, sulfur compounds) possibly present on the membrane, their potential impact on drug–protein binding would need to be investigated.

Implications for protein binding assay using RED device

Based on the current RED device user instructions, preconditioning of the dialysis membrane inserts is not required [9]. In two previous publications on PPB assay using the RED device [8,11], the membrane inserts were soaked in water for ≥10 min twice prior to use. However, the impact of membrane preconditioning on f_u data accuracy was not reported. It appears to be generally appropriate to perform the assay in undiluted plasma or matrices with sufficiently high protein contents using the current instruction, as also shown in this work. However, our findings indicate that the use of membrane inserts without preconditioning for assays in matrices with relatively low protein contents could lead to biased f_u data and such impact could be compound dependent. Therefore, the RED assay protocol should be assessed and modified as needed to incorporate the procedure of dialysis membrane preconditioning for protein binding assay of compounds of interest in specific matrix to ensure accurate and consistent f_u data are generated.

After the preconditioning step, there could be residual excess water on the membrane inserts if it is not sufficiently removed prior to the assay. For assay with low sample volume (e.g., 50 μl) in the donor chamber, the dilution of the sample by the residual water would not be negligible and could impact the data accuracy. The water could be removed by centrifugation of the inverted RED plates loaded with the preconditioned membrane inserts at a low-to-moderate speed (e.g., 800 r.p.m.) for a short period of time (e.g., 3 min). Given the impact of adding the laborious preconditioning procedure on the assay throughput, it would be important to identify the true cause for the biased f_u data from the RED assay without preconditioning of the membrane inserts. If indeed the bias in the f_u measurement is due to interferences from the dialysis membranes, the manufacturing process is suggested to be improved to produce interference-free membrane inserts for generating accurate protein binding data while maintaining the assay throughput.

Conclusion

In this work, we demonstrated the improvement of f_u data accuracy of drug–protein binding assay in diluted protein matrices by preconditioning the RED membrane inserts with deionized water. Such improvement was likely due to the removal of interferences from the dialysis membranes. The impact of membrane preconditioning on the f_u measurement appeared to be compound dependent. Based on our findings, we recommend that the RED device protocol be evaluated and modified as needed to incorporate the membrane preconditioning procedure for accurate

f_u measurement of drug–protein binding especially in matrices with relatively low protein contents (incubation media with albumin, diluted plasma, etc.).

Future perspective

The approach in this study can be applied to assessing the impact of dialysis membrane preconditioning on other binding assays including hepatocyte, liver microsomal and tissue homogenate binding using the RED device. Further investigation on the identification of potential interferences on the f_u measurement would help optimize the manufacturing process to produce interference-free membrane inserts for generating accurate f_u data for high-throughput protein binding assays.

Summary points

Background
- The rapid equilibrium dialysis (RED) device has been validated for plasma protein binding assay in undiluted plasma. However, there is a lack of reference information on its performance in protein binding assays in diluted protein matrices.

Experimental
- Protein binding assays of drug compounds in bovine serum albumin (BSA) solutions and human plasma with different folds of dilution were performed using the RED device with and without preconditioning of the dialysis membrane inserts, and the data were compared with those measured using other methods in this study.

Results & conclusion
- The f_u data of the compounds were similar using the RED and high-throughput dialysis (HTD) devices in matrices with higher protein contents (e.g., 4% BSA solution). However, in assay with lower protein contents, the f_u data obtained from RED were significantly higher than those from HTD. Using the preconditioned RED device, the f_u data were comparable to those from HTD and ultrafiltration.
- Preconditioning of the RED membrane inserts with deionized water improved the accuracy of f_u data in drug–protein binding assay in diluted protein matrices and such impact could be compound dependent.
- The RED device protocol is suggested to be evaluated and modified as needed to include the membrane preconditioning procedure for measuring drug–protein binding in specific matrices.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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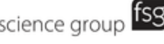

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Bioanalysis

Solid-phase microextraction for assessment of plasma protein binding, a complement to rapid equilibrium dialysis

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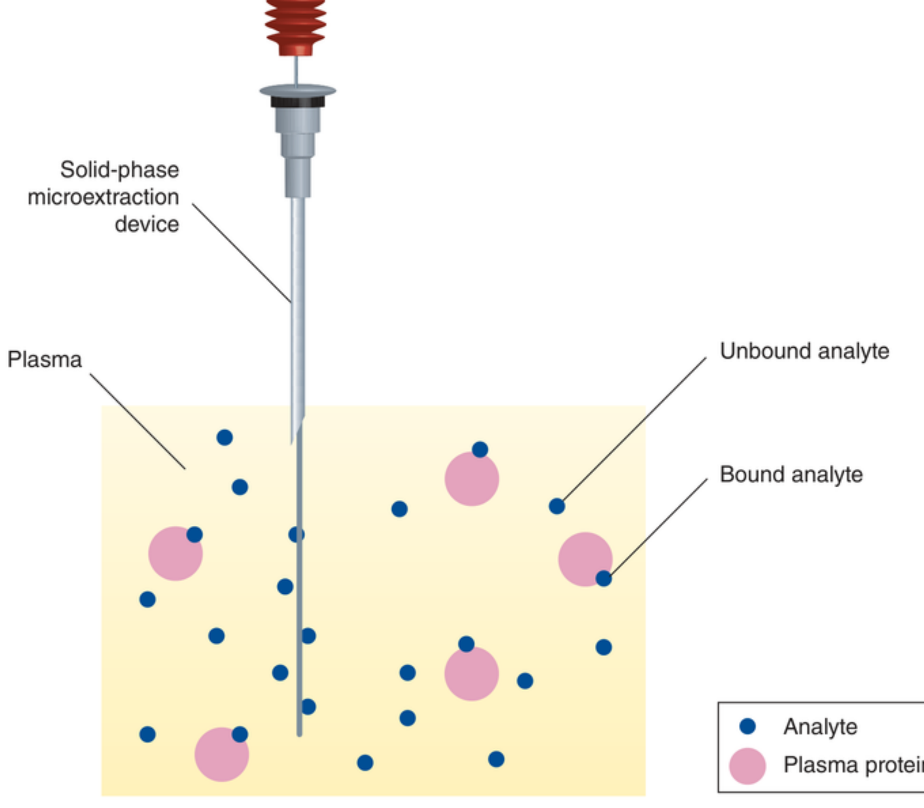
Aim: Determination of plasma protein binding (*PPB*) is considered vital for better understanding of pharmacokinetic and pharmacodynamic activities of drugs due to the role of free concentration in pharmacological response. **Methodology & results:** Solid-phase microextraction (SPME) was investigated for measurement of *PPB* from biological matrices and compared with a gold standard approach (rapid equilibrium dialysis [RED]). **Discussion & conclusion:** SPME-derived values of *PPB* correlated well with literature values, and those determined by RED. Respectively, average protein binding across three concentrations by RED and SPME was 33.1 and 31.7% for metoprolol, 89.0 and 86.6% for propranolol and 99.2 and 99.0% for diclofenac. This study generates some evidence for SPME as an alternative platform for the determination of PPB.

Graphical abstract:



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Keywords: free concentration • microsampling • plasma protein binding • rapid equilibrium dialysis • solid-phase microextraction

Administered drugs can partition between the red blood cell and plasma components of circulating blood, yet blood plasma is preferred over blood for drug concentration assays [1]. According to the well-established free drug hypothesis, only the free drug concentration at the site of action (i.e., receptor or drug target) can affect biological activity and cause efficacy and toxicity [2]. Hence, accurate determination of free drug concentration (i.e., unbound to plasma proteins) is essential for therapeutic drug monitoring, specifically for drugs with a narrow therapeutic window [3]. Despite the importance of free drug concentration, due to reasons of convenience and precedence, the majority of bioanalytical assay techniques in current use measure the total (free and bound) drug concentration, rather than the potentially more relevant concentration of free drug [4]. The sole use of total drug levels might be misleading and may not reflect the true significance of the relationship between clinical pharmacokinetics (PK) and pharmacodynamics (PD) of a drug [2,5].

Though methods of indirect assessment of protein binding exist, such as computational approaches [6,7], in drug discovery, *in vitro* experiments are commonly used to directly determine drug plasma protein binding (*PPB*) [8]. This can be expressed as fraction unbound or free fraction of drug (i.e., drug which is free concentration in comparison to total concentration) [9]. of the value for free fraction can then be used to extrapolate free concentration of drug from the total concentration, which is typically reported in bioanalytical assays. Several regulatory authorities recommend the determination of *PPB* prior to clinical trials to support the assessment of drug–drug interactions [9].

The most widely used *in vitro* methodologies for direct determination of *PPB* of drugs are equilibrium dialysis (including rapid equilibrium dialysis [RED]), ultrafiltration and ultracentrifugation. Each technique displays a variety of advantages and disadvantages in terms of speed, data quality and complexity. Comparative evaluations of each method have been reported in the literature [2]. Several analytical challenges are known to be associated with some of these techniques. For example, ultrafiltration is a rapid and simple method, where a size exclusion filter is utilized to filter the analyte from a matrix. However, the analyte may bind to the filter and cause disturbance to the equilibrium which in turn will impact the quality of the data [10]. Ultracentrifugation, on the other hand, requires the use of a powerful centrifuge (up to 250,000 *g*) along with lengthy centrifugation periods (∼16 h) to separate the binding matrix, which lowers the throughput of the method [11].

The most frequently used method in the pharmaceutical industry is equilibrium dialysis, the 'gold standard' means of protein-binding assessments [9,10,12]. A survey published by the European Bioanalysis Forum in 2014 showed that 82% of responders were using equilibrium dialysis in early-phase drug discovery, with the technique remaining the most commonly used during *in vitro* drug development and *ex vivo PPB* studies. This technique involves the use of two compartments, one with the matrix sample and one with a suitable buffer such as phosphate-buffered saline (PBS), separated by a membrane. The free drug concentration is determined when equilibrium is reached between the two compartments [13]. RED has been developed as high-throughput determination approach that can decrease the time required to reach protein-binding equilibrium, although assay times of 6 h are still required [8]. Furthermore, performance of sample clean-up is often necessary in order to prepare the samples generated by RED into a format that is suitable for LC–MS analysis. One such technique that can potentially overcome these limitations, in addition to providing a faster assay time, is solid-phase microextraction (SPME).

SPME, first established in the early 1990s, is a sampling method that combines sampling, sample preparation and extraction in one step [14]. The amount of analyte extracted by SPME is directly proportional to the concentration of unbound analyte present in the sample matrix [15]. Typically, SPME extracts in a nonexhaustive extraction that leaves the bulk drug concentration of the sample relatively unchanged. Thus, SPME may offer benefits by not disturbing the drug protein-binding equilibrium during drug extraction [15]. Analyte extraction from the matrix is independent of sample volume when the fiber is exposed to a sample volume larger than the coating capacity. The determination of *PPB* by SPME is based on establishing the free concentration of drug in plasma in the presence of proteins, compared with total drug concentration measured by SPME in the absence of proteins [16]. The percentage of drug binding to plasma proteins is calculated from the total and free concentrations of the drug

as shown below:

$$PPB = \frac{C_{total} - C_{free\ plasma}}{C_{total}}$$

Practically, it is not necessary to calculate a concentration of analyte extracted by SPME. Instead, the peak area counts of the respective analyte peaks can be used to assess *PPB*, provided the instrumental method used is suitable. *PPB* can then be calculated as follows:

$$PPB_{(\%)} = \frac{Peak\,Area_{total} - Peak\,Area_{free\ plasma}}{Peak\,Area_{total}} \times 100\%$$

PPB can also be expressed as fraction unbound (f_u), reflecting the drug concentration that is unbound rather than the degree of *PPB* present. This can be calculated as shown below:

$$f_u = 1 - PPB$$

The SPME approach has been used to determine *PPB* values *in vitro* [16–18] and could be used to characterize the distribution of small molecules in the plasma compartment during drug development, while also overcoming the issues of volume change and membrane sorption associated with RED. This manuscript builds upon this body of evidence by investigating the utility of SPME as a rapid and accurate tool for the *in vitro* determination of *PPB* by comparing it to the RED method for three selected drugs. The compounds cover a range of binding values (30–99%) in rat plasma. Three concentrations were assessed for each drug across a physiologically relevant range using qualified bioanalytical methods.

A number of SPME fiber phases have been applied to determination of drug binding to macromolecule, including mixed mode [18], polyacrylate [17], polydimethylsiloxane [19] and polypyrrole [16]. Often, these fibers are produced in-house and are customizable to the analysis being performed. Several studies have performed comparisons to an existing technique or published data when determining small molecule and macromolecular binding [16–20]. Although the latter approaches have established SPME as a tenable route to study drug *PPB*, the use of a generic fiber phase such as C18 potentially simplifies the SPME workflow for adoption within the pharmaceutical industry. Additionally, regulatory concerns may be more easily addressed when a generic approach is used. Several challenges exist for adoption of a generic SPME fiber phase. Certain analytes may possess low affinity for the SPME fiber phase, giving poorer analytical sensitivity as a result. Charged and/or polar molecules are of concern, as they possess a lower affinity for the fiber phase in comparison to uncharged and less polar molecules. The novelty, and aim of the current work, was to develop and benchmark against the well-validated, industry standard RED methodology, a rapid, generic SPME workflow for *PPB* determination using commercially available C18 fibers.

Experimental

Chemicals & materials

Metoprolol tartrate, propranolol hydrochloride, diclofenac sodium salt and diclofenac $^{13}C_6$ sodium salt 4.5-hydrate were purchased from Sigma-Aldrich (Dorset, UK); metoprolol-d_7 and propranolol-d_7 were acquired from Toronto Research Chemicals (ON, Canada). BioSPME silica probes consisting of a titanium wire coated with a biocompatible C18 extraction phase, housed inside hypodermic needle (medical grade, stainless steel, 22-gauge outer tubes) were supplied by Supelco (PA, USA); each fiber has a thickness of 45 μm and 15 mm length of coating. Control rat plasma containing K2-EDTA to prevent coagulation was obtained from B&K Universal (Grimston, UK). All animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. PBS tablets, dimethylformamide and formic acid (reagent grade ≥95%) were purchased from Sigma-Aldrich. Methanol, acetonitrile, propranolol and water were of HPLC gradient grade and obtained from Fischer Scientific Ltd. (Loughborough, UK).

Preparation of standard stocks, working solutions & test samples

Primary stock solutions for each test compound (metoprolol, propranolol and diclofenac) and their stable label isotopes utilized as internal standards (IS) were prepared in dimethylformamide (1 mg/ml). Serial dilutions of each analyte's stock solution were performed in acetonitrile/water (1:1, v/v) to give working standard concentrations of

1, 10 and 100 μg/ml. IS working solutions for each analyte were prepared from the primary stock solution to give a final concentration of 100 ng/ml in acetonitrile.

RED & SPME procedure for analysis of PPB & subsequent data transformation

PPB of the test compounds (metoprolol, propranolol and diclofenac) was examined *in vitro* using SPME and was compared with data obtained using a single-use RED device loaded with 8 kDa MWCO inserts (ThermoFisher Scientific, Hemel Hempstead, UK).

PBS solution was prepared by dissolving one PBS tablet into 200 ml of deionized water (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). SPME fibers were preconditioned with methanol for 15 min to activate the C18 sorbent, followed by water for 15 min. Appropriate volumes of analyte working solutions were spiked into fresh rat plasma and into PBS at target concentrations of 10, 100 and 500 ng/ml. Spiked plasma samples were left for 1 h to equilibrate. Nonmatrix volumes used to spike the samples were less than 5% of the total sample volume. Spiked rat plasma was gently mixed on a roller mixer (Progen Scientific, UK) for 15 min at 37°C.

One set of SPME fibers (n = 6) was immersed into 200 μl aliquots of spiked plasma and a second set was placed into 200 μl aliquots of spiked PBS for each target concentration. SPME extraction was conducted following 30 min incubation at 37°C by removing the fibers from the samples, rinsing them with water for 30 s and desorbing them in 200 μl of 100% acetonitrile containing 100 ng/ml of the appropriate IS for 15 min. All extracts were subsequently analyzed by LC–MS/MS. The entire SPME extraction procedure was performed with constant agitation at 500 rpm. The percentage of binding to plasma proteins was calculated from the total and free analyte response as follows:

$$\%PPB^{SPME} = \left(\frac{Analyte : IS\ Peak\ Area\ Ratio_{PBS} - Analyte : IS\ Peak\ Area\ Ratio_{Plasma}}{Analyte : IS\ Peak\ Area\ Ratio_{PBS}}\right) \times 100$$

A single-use RED plate preloaded with 48 equilibrium dialysis membrane inserts was utilized and 300 μl aliquots (n = 6) of spiked rat plasma in addition to 300 μl aliquots (n = 6) of control blank plasma were placed into sample chambers of the RED device. This was dialyzed against 500 μl aliquot (n = 6) of PBS added into the buffer chambers. The RED unit was covered with self-adhesive plate seal and incubated at 37°C on a flat-bed orbital shaker (MS 3 Digital, IKA) set at 300 rpm for approximately 6 h as per manufacturer's instructions for reaching equilibrium. After 6 h, dialysis was stopped and 25 μl aliquots were taken from each compartment, placed into 1.4 ml matrix tubes (Micronics, Platinastraat, The Netherlands), and an equal volume of dialyzed blank plasma was added to the PBS aliquot and 25 μl of dialyzed PBS was added to the spiked plasma compartment aliquot to ensure matrix matching of samples prior to extraction and analysis.

RED samples were extracted by protein precipitation through addition of 200 μl of 100% acetonitrile containing 100 ng/ml of IS. All tubes were vortex mixed for 5 min and centrifuged (5810R, Eppendorf, Germany) at 3000 *g* for 10 min. The supernatant was transferred into clean tubes and injected onto the LC–MS/MS.

Analyte-binding calculation for the RED approach was performed as shown below:

$$\%PPB^{RED} = \left(\frac{Analyte : IS\ Peak\ Area\ Ratio_{Plasma} - Analyte : IS\ Peak\ Area\ Ratio_{PBS}}{Analyte : IS\ Peak\ Area\ Ratio_{Plasma}}\right) \times 100$$

Following determination of *PPB*, the data were transformed into values of unbound fraction (f_u). This was performed by using the following equation:

$$f_u = 1 - \frac{PPB}{100}$$

Data are expressed within this text as both % *PPB* and fraction unbound. Further data transformation took place in order to calculate the apparent affinity constant (logK) as per methodology previously published for RED assessments [8]. These values are presented within the Supplementary Material (Supplementary Table 4), and were

Table 1. Summary of MS/MS parameters for the analysis of test compounds.

Analyte	Q1 mass (amu)	Q3 mass (amu)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Cell exit potential (V)
Metoprolol	268.3	116.2	78	10	26.4	13
Metoprolol-d_7	275.3	191.0	78	10	26.4	13
Propranolol	260.0	183.0	125	12	28	20
Propranolol-d_7	267.0	183.0	125	12	28	20
Diclofenac	296.0	214.0	93	12	49	30
Diclofenac-$^{13}C_7$	302.0	220.0	93	12	49	30

calculated using the following equation:

$$logK = \log\left(\frac{1 - f_{\mu}}{f_{\mu}}\right)$$

LC–MS/MS analysis

Chromatographic separation was achieved using an Acquity UPLC system (Waters, MA, USA) equipped with a sample manager, sample organizer, a binary solvent manager and column oven. Analytes were separated using an Acquity C18 BEH column 50 × 2.1 mm i.d., 1.7 µm particle-size (Waters) kept at 50°C and a gradient elution applied employing the mobile phases; deionized water containing 0.1% formic acid (mobile phase A) and 100% acetonitrile (mobile phase B). Following sample injection (4 µl), the mobile phase was held at 95% A for 0.5 min followed by rapid gradient to 10% A at 1.10 min. The composition was kept at an isocratic period to 1.30 min and was ramped to 95% A at 1.50 min and finally held at the same composition to 2.00 min, re-equilibrating the column prior to the next cycle. The flow rate was 0.8 ml/min and HPLC effluent was diverted to waste for the first 0.5 min of chromatographic run time using a divert switching valve (Rheodyne MX Series II™). Details of method calibration ranges for all analytes are given in the Supplementary Material (Supplementary Tables 1–3).

MS detection was achieved using an API-5000 tandem quadrupole mass spectrometer (AB Sciex, USA) equipped with TurboIonSpray™ interface. The instrument was operated in positive ion mode with the source temperature set at 500°C and an ion spray voltage of 5.5 kV. The analysis was performed using multiple reaction monitoring mode using instrument settings as described in Table 1. All gases used were nitrogen, dwell time of 100 ms was employed for ion monitoring and unit resolution was applied to both Q1 and Q3.

HPLC–MS/MS data were acquired and processed (integrated) using Analyst software (v1.6.1 Applied Biosystems/MDS Sciex, Canada).

Results & discussion

The suitability of the LC–MS method to quantify the three analytes was assessed, with a calibration line and accompanying set of six quality control (QC) samples analyzed by extracting the compounds of interest from rat whole blood. The accuracy and precision of each group of QC samples are shown with the accompanying determined concentrations of the samples within this group. Accuracy and precision are observed to be under 15% at each of the concentrations assessed when considering the data generated when extracting metoprolol. These data are included in the supplementary data sheet.

The utility of SPME fibers for measuring *PPB* was demonstrated by *in vitro* extraction of drug from both a protein-free matrix (PBS) and rat plasma. The amount of drug extracted from each matrix was compared in order to calculate *PPB*. A comparison to *PPB* values determined when using the RED device, a well-established technique for *PPB* determination, was then made. The results in Tables 2 & 3 display the calculated *PPB* and f_{μ} values for the three drugs metoprolol, propranolol and diclofenac across a range of concentrations (10, 100 and 500 ng/ml) using SPME and RED [21,22].

The calculated bound percentage (*PPB*) (and unbound fractions, f_u by SPME) correlated well with bound values determined by the RED device, indicating that SPME can generate similar values for drug *PPB* within a complex biological matrix such as plasma. It was found that consistent results were obtained by SPME for each analyte across all three concentrations with ≤15% difference in determined % *PPB* between concentrations.

The percentage difference in determined % *PPB* between the two techniques, SPME and RED was within 15% across all analytes and concentrations. In the case of diclofenac, the magnitude of the difference between RED and

Table 2. Comparison of protein-binding values for metoprolol, propranolol and diclofenac across a concentration range of 10–500 ng/ml obtained using RED and SPME. Data represent mean ± SD, n = 6 determinations.

Analyte concentration (ng/ml)	RED (f_u)	SPME (f_u)	RED (% *PPB*[†])	SPME (% *PPB*[†])	% Difference % *PPB*[‡]	Ref.
Metoprolol/literature values for % *PPB* = ~30%						[21]
10	0.657 ± 0.006	0.682 ± 0.017	34.3 ± 0.336	31.8 ± 0.784	7.3 ± 0.027	
100	0.664 ± 0.008	0.684 ± 0.012	33.6 ± 0.415	31.6 ± 0.562	6.0 ± 0.022	
500	0.685 ± 0.004	0.682 ± 0.023	31.5 ± 0.180	31.8 ± 1.09	-1.0 ± 0.035	
Propranolol/literature values for % *PPB* = ~90%						[22]
10	0.107 ± 0.00009	0.086 ± 0.0001	89.3 ± 0.0742	91.4 ± 1.01	-2.4 ± 0.011	
100	0.1 ± 0.00009	0.09 ± 0.0007	90.0 ± 0.0816	91.0 ± 0.735	-1.1 ± 0.008	
500	0.124 ± 0.00007	0.226 ± 0.003	87.6 ± 0.0504	77.4 ± 0.961	11.6 ± 0.012	
Diclofenac/literature values for % *PPB* = ~99%						[22]
10	0.013 ± 0.000008	0.015 ± 0.0001	98.7 ± 0.0589	98.5 ± 0.941	0.203 ± 0.010	
100	0.006 ± 0.000002	0.005 ± 0.00004	99.4 ± 0.0367	99.5 ± 0.857	-0.100 ± 0.009	
500	0.006 ± 0.000002	0.009 ± 0.00007	99.4 ± 0.0363	99.1 ± 0.721	0.302 ± 0.007	

[†] Errors were based on standard deviation and calculated using error propagation methodologies.
[‡] $\%Difference = \frac{\%PPB_{RED} - \%PPB_{SPME}}{\%PPB_{RED}} \times 100$ (variance not reported as <0.1% in all cases).
f_u: Fraction unbound; PPB: Plasma protein binding; RED: Rapid equilibrium dialysis; SPME: Solid-phase microextraction.

Table 3. Literature values for molecular weight, logP, pKa and physiological charge of the three molecules of interest within this study.

	Molecular weight	LogP	pKa	Physiological charge
Metoprolol	267.4	1.88	9.44	1
Propranolol	259.4	3.03	9.6	1
Diclofenac	296.2	4.98	3.8	-1

SPME was less than 1%. All results also correlated well with average protein-binding values quoted in the literature for each compound [21,22]. The small differences between the values obtained in this study and protein-binding values previously published in the literature can be explained by interanimal variations in plasma protein content or due to typical analytical experimental errors.

A paired *t*-test was conducted to compare the *PPB* values obtained using RED for all three analytes with *PPB* values measured using SPME. There was no significant difference in the values for RED (mean = 73.8, variance = 948.7) and SPME (mean = 72.5, variance = 977.7) conditions; t (crit) = 2.11, p = 0.05. This suggests that data obtained using SPME are equivalent to the data obtained using the RED device and therefore a suitable alternative method allowing more rapid analytical throughput.

A two-way analysis of variance was also performed to understand the influence of two independent variables, namely the concentration of analyte and the effect of the analytical technique on the *PPB* values. The analyte concentration included three levels (10, 100 and 500 ng/ml) and analytical techniques consisted of the RED and SPME. Neither effect was statistically significant at the 0.05 significance level. The effect of analyte concentration yielded F = 1.02, $p > 0.05$, indicating that the effect of concentration was not significant. The impact of the analytical technique yielded F = 2.89, $p > 0.05$, indicating that there is no significant difference between the analytical techniques.

The variability of the SPME assay is higher than when the RED device is used. However, this variability is still acceptable within the scope of bioanalytical methods (<15%). This variability could be, in part, due to the quality of the fibers used and the interfiber variability associated with it [23]. Interestingly, the variability of both assays was higher for metoprolol, a drug which has lower *PPB* in comparison to diclofenac and propranolol. A similar phenomenon was observed when applying mixed mode SPME fibers to the study of tramadol-binding affinity to bovine serum albumin, a drug which is 15–20% plasma protein bound, whereby variability in the assessment of tramadol binding was higher than the variability associated with the other compounds assessed within the study [24].

It was noted that the difference between the % *PPB* values for the SPME and RED techniques was greater, when the more highly protein-bound drugs propranolol and diclofenac were assessed. This was magnitude of the

unbound drug fraction (f_u) being a much smaller numerical value than the % *PPB* (i.e., the f_b). For example, a difference in f_μ was observed for propranolol at 500 ng/ml when using the RED and SPME approaches (0.006 vs 0.009), resulting in a difference of 50% between the two techniques. Practically, however, this is a small difference in the absolute magnitude of the f_u. Indeed, the difference between f_u for RED and SPME was lower in magnitude than the difference between f_u measured at the highest and lowest concentration levels for the RED technique alone.

The LC–MS method used in this study was not fully validated; however, example chromatograms are presented that demonstrate that the signal to noise ratio of chromatograms at the LLQ was greater than 5:1 for all three compounds extracted from plasma using SPME as shown in Figure 1. Additional data showing qualification of the analytical method are provided in the Supplementary Information.

Detection of analyte was achieved at drug concentrations as low as 10 ng/ml in plasma. Significant protein binding in the case of propranolol and diclofenac, which reduces the amount of analyte available for extraction by SPME, was also unproblematic with respect to quantification of free, unbound drug.

The use of a C18 fiber, an uncharged extraction phase, is of interest for the three drugs assessed within this study, as the three compounds are charged at physiological pH. This reduces the interactions that occur between analyte and extraction phase, compared the case if the analytes were uncharged, and reducing the amount of analyte extracted onto the fiber and entering the LC–MS instrumentation as a result. Previous studies have made use of a number of SPME fiber phases for *PPB* assessment [18], however, this currently necessitates the use of prototype fibers, or the use of in-house-derived fibers. Both options may not be suitable for wider application within the pharmaceutical industry. The performance of this study with a commercially available C18 fiber to extract charged, and polar, molecules, therefore, is of interest.

The data obtained in this study suggest that SPME can be employed to assess unbound drug fractions, which is in agreement with several previous reports [15,25]. The current work has generated some evidence for SPME's suitability for a rapid-throughput, standardized drug development analytical technique. The technique uses an extraction phase that adsorbs analyte and reduces adhesion of large molecules, resulting in a form of sample preparation being performed as the drug is extracted from the sample [18]. This provides a simpler approach for the measurement of drug *PPB* and f_u, which is a key parameter for the interpretation of compound bioavailability and its pharmacodynamic action.

In the data generated within this study, the depletion of the free concentration of drug from the matrix was negligible with SPME, such that the equilibrium between the bound and unbound concentration of the analyte within the matrix is potentially unaffected [15]. This may not always be the case, particularly for compounds which have high affinity for the SPME fiber phase. In these instances, non-negligible extraction of analyte occurs, resulting in depletion of free concentration, such that additional drug becomes unbound from the protein within the sample [18]. One approach to overcome this is to use a lower amount of SPME extraction-phase material, either by reducing the length or thickness of the coating. This can provide faster sampling of analyte, and reduced time to reach sampling equilibrium, however, a lower of amount of analyte is then extracted. Similarly, the use of pre-equilibrium SPME extraction, whereby the SPME extraction does not reach a drug partitioning equilibrium between fiber and sample, could be applied to overcome this issue [26]. In both of these instances, sensitive analytical instruments are required.

The disadvantages of this approach include greater analytical variability and a lower amount of analyte extracted by the SPME fiber, and subsequently entering the analytical instrument. This is a disadvantage in comparison to the use of the RED device, which involves a greater amount of analyte going onto the LC–MS system due to the nature of the sample preparation (i.e., a greater amount of analyte on-column). However, this disadvantage can be overcome by using sensitive LC–MS instrumentation that allows for successful detection of low analyte concentrations. Outside of bioanalysis performed within the pharmaceutical industry, which makes wide use of LC–MS instrumentation, access to these instruments may be problematic. However, for the application suggested within this manuscript this is unlikely to be an issue.

Notably, the data presented here were determined without requiring concentrations of analyte to be determined in this study. Instead, relative response ratios were compared between samples that contained drug incubated with plasma versus PBS (protein free vs protein containing). LC–MS as a technique can suffer from matrix effects, a phenomenon whereby nonanalyte components of the sample can suppress or enhance the analyte signal at a given concentration. Therefore, comparisons between samples must be performed in matrices that are as similar as possible. In the case of the RED device, samples matrices are matched by performance of blank extractions in

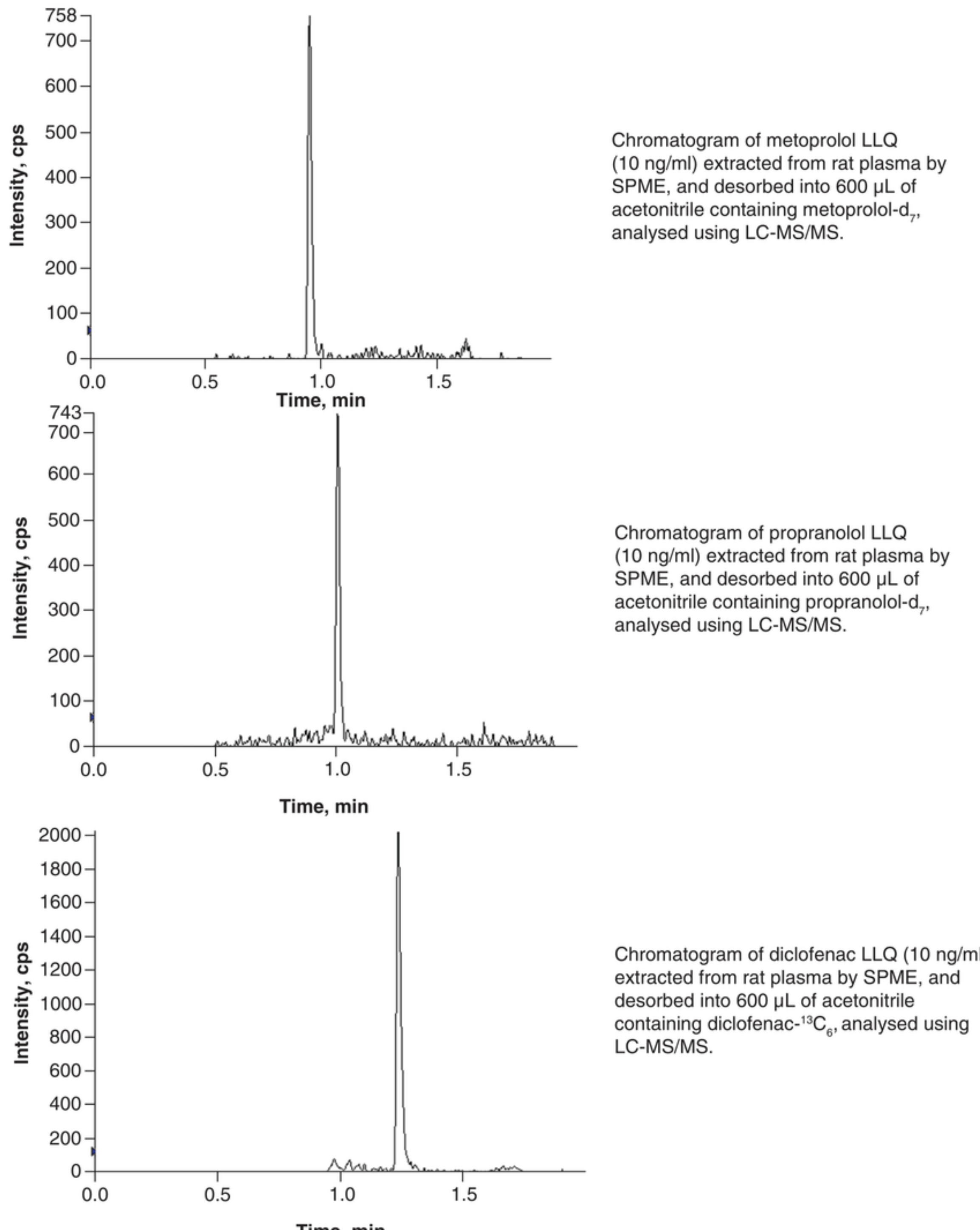

Figure 1. Example chromatograms of the LLQ (10 ng/ml) extracted from plasma using solid-phase microextraction and fiber desorbed into acetonitrile containing the internal standard.

buffer and plasma, and aliquots of these blank samples then added to the drug-containing samples. This is not the case with the SPME protocol used here. It may be of benefit if SPME extractions from samples containing no analyte are cross-mixed with samples containing analyte, in order to provide a more closely matched matrix sample, as per a similar step within the RED device protocol. This may not be a significant issue as SPME extracts a small amount of sample, providing a cleaner extract as a result (i.e., with fewer matrix components). However, for wider

adoption of the technique to *PPB* studies with drugs of varying physiochemical properties, this may be an area that warrants further investigation.

Overall, the experimental findings of the current study provide some evidence that SPME is an approach that could be utilized for *in vitro* determination of the binding affinity or partition coefficient of a compound in a biological matrix. The use of SPME facilitated determination of *PPB* values for a small number of analytes with a range of binding affinities which can be classified as low, medium and highly bound compounds (30–99% bound). Compared with the RED device, SPME offers several advantages for use in *PPB* measurements including short analysis times of less than 1 h for SPME compared with greater than 6 h for RED, and the ability to study complex matrices such as plasma directly without the need for additional sample preparation in the form of dilutions or subsequent extractions (i.e., no solvent extractions, solid-phase extraction, liquid extraction, centrifugation required). These advantages could be further exploited by development of automated SPME handlers as found in the literature, increasing throughput and assay speed further [27]. Evaluation of a wider range of drug physiochemical and protein-binding properties would generate further evidence of the applicability of SPME for drug *PPB* determination. Additionally, evaluation of matrix effects associated with the SPME assay could be of benefit when a wider range of drug molecules are assessed.

Conclusion

The impact of measuring the degree of protein binding is high when trying to understand the relationship between the PK and PD of drugs. Although RED is predominantly used for this application, SPME offers advantages in the form of increased assay speed and reduced potential of RED membrane binding. A direct comparison between SPME and RED is presented within this study. This investigation demonstrated the use of SPME for the measurement of *PPB in vitro* and highlighted its potential to replace existing techniques. The data obtained using SPME show that this approach provides accurate estimates of *PPB* values across a range of bound drug levels (30–99%) at a several physiologically relevant concentrations. The use of a commercially available C18 phase to extract multiple charged analytes generates evidence, which supports wider adoption of C18 SPME for determination of drug *PPB*. Compared with RED, SPME offered many benefits including simplicity as well as short equilibration and analysis time, where the overall procedure for SPME was completed within 1 h compared with 6–8 h using RED. SPME also offers the future possibility of automation that will enhance throughput and increase the speed of sample processing.

Future perspective

The determination of *PPB* is likely to remain an important feature of the drug development pathway. The use of SPME over the widely used RED device provides an alternative workflow with benefits of speed and simplicity. There is a growing body of evidence that supports the application of SPME to *PPB* determination, however, further validation of the technique is required before widespread adoption can take place. Though this current study demonstrates the advantages of speed and simplicity of SPME for *PPB* determination for several small-molecule drug compounds, wider validation of the approach would be of benefit. This would need to include a greater range and higher number of compounds that encompass varying charge states, protein-binding values, logP and pKa.

Summary points

- Plasma protein binding *(PPB)* is an important characteristic of a drug molecule, which is important to assess during drug development.
- The most widely used approach to *PPB* determination makes use of rapid equilibrium dialysis (RED), however, this can require long analyte equilibration times (>6 h), which can limit the throughput of the method, and may require sample preparation on assay samples generated. Alternative workflows may be of benefit.
- Solid-phase microextraction (SPME) is a nonexhaustive extraction technique that extracts analyte via the unbound-free fraction of drug. This allows for determination of *PPB* when extracting the same concentration of drug from a matrix with and without plasma-binding components, such as plasma and PBS, respectively.
- Herein, we applied SPME for determination of *PPB* and compared the generated results to the well-established RED approach for three drug substances, metoprolol, propranolol and diclofenac.
- Concordance between the results generated was observed, with SPME offering additional advantages such as speed and a simpler analytical workflow.
- This work supports the use of SPME as an approach to determination of *PPB*, however, further validation of the approach with a wider range of drug molecules will be of benefit.

Disclaimer

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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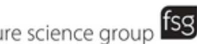

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