Abstract

Background

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.

Results/Methodology

Solid phase microextraction (SPME) was investigated for measurement of *PPB* from biological matrices and compared to 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 3 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.

Introduction

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 (approximately 16 hours) 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]. Rapid equilibrium dialysis (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]. Further, 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 which 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 non-exhaustive 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 fibre 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:

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

PPB can also be expressed as fraction unbound (f_u), reflecting the drug concentration which 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 characterise the distribution of small molecules in the plasma compartment during drug development, whilst 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 fibre phases have been applied to determination of drug binding to macromolecule, including mixed mode [18], polyacrylate [17], polydimethylsiloxane [19], and polypyrrole [16]. Often, these fibres are produced in-house and are customisable 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 fibre-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 fibre phase. Certain analytes may possess low affinity for the SPME fibre phase, giving poorer analytical sensitivity as a result. Charged and/or polar molecules are of concern, as they possess a lower affinity for the fibre 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 fibres.

Experimental

Chemicals and 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 (Ontario, 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 (Bellefonte, PA, USA); each fibre 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, Hull, 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. Phosphate buffered saline (PBS) tablets, dimethylformamide (DMF) and formic acid (reagent grade ≥ 95%) were purchased from Sigma-Aldrich (Dorset, UK). Methanol, acetonitrile, propranolol and water were of HPLC gradient grade and obtained from Fischer Scientific Ltd (Loughborough, UK).

Preparation of Standard Stocks, Working Solutions and Test Samples

Primary stock solutions for each test compound (metoprolol, propranolol and diclofenac) and their stable label isotopes utilised as internal standards (IS) were prepared in DMF (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. Internal standard working solutions for each analyte were prepared from the primary stock solution to give a final concentration of 100 ng/mL in acetonitrile.

RED and SPME Procedure for Analysis of Plasma Protein Binding and Subsequent Data Transformation

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

PBS solution was prepared by dissolving one PBS tablet into 200 mL of deionised water (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). SPME fibres were preconditioned with methanol for 15 minutes 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. Non-matrix volumes used to spike the samples were < 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 fibres (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 fibres 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 internal standard for 15 min. All extracts were subsequently analysed 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;

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

A single-use RED plate preloaded with 48 equilibrium dialysis membrane inserts was utilised 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 dialysed 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, Netherlands), and an equal volume of dialysed blank plasma was added to the PBS aliquot and 25 µL of dialysed 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 100ng/mL of internal standard. 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;

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

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 (Table S4), and were calculated using the following equation;

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

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 x 2.1 mm i.d., 1.7 µm particle-size (Waters, MA, USA) kept at 50^{o}C and a gradient elution applied employing the mobile phases; deionised 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^{TM}). Details of method calibration ranges for all analytes are given in the supplementary material (Table S1 – S3).

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^{o}C and an ion spray voltage of 5.5 kV. The analysis was performed using multiple reaction monitoring (MRM) 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).

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-$^{13}C_6$	302.0	220.0	93	12	49	30

Results and Discussion

The suitability of the LC-MS method to quantify the three analytes was assessed, with a calibration line and accompanying set of 6 QC (quality control) samples analysed 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 fibres 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 Table -2- 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.

Table -2- Comparison of f_u and % *PPB* values for metoprolol, propranolol and diclofenac across a concentration range of 10-500 ng/mL obtained using rapid equilibrium dialysis (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***
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.

** $\% \, Difference = \frac{\%PPB_{RED} - \%PPB_{SPME}}{\%PPB_{RED}} \; x \; 100$ (variance not reported as < 0.1 % in all cases)

The calculated bound percentage (*PPB*) (and unbound fractions, f_u by SPME correlated well with bound values determined by the RED device, which indicates 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 SPME was <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 literature can be explained by inter-animal variations in plasma protein content or due to typical analytical experimental errors.

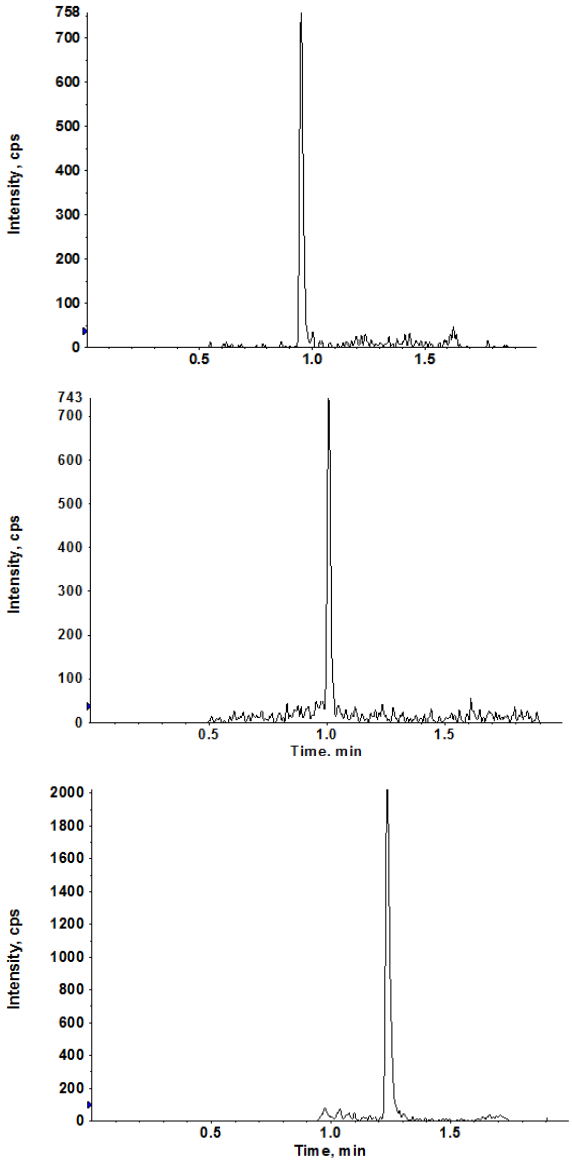
A paired t-test was conducted to compare the *PPB* values obtained using RED for all three analytes with *PPB* values measured using SPME. There was no significant difference in the values for RED (Mean = 73.8, Variance = 948.7) and SPME (Mean = 72.5, Variance = 977.7) conditions; t (crit) = 2.11, p = 0.05. This suggests that data obtained using SPME is 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 fibres used and the inter-fibre 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 fibres to the study of tramadol binding affinity to bovine serum albumin (BSA), 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 Supplementary Information.



Chromatogram of metoprolol LLQ (10 ng/mL) extracted from rat plasma by SPME, and desorbed into 600 µL of acetonitrile containing metoprolol-d_7, analysed using LC-MS/MS.

Chromatogram of propranolol LLQ (10 ng/mL) extracted from rat plasma by SPME, and desorbed into 600 µL of acetonitrile containing propranolol-d_7, analysed using LC-MS/MS.

Chromatogram of diclofenac LLQ (10 ng/mL) extracted from rat plasma by SPME, and desorbed into 600 µL of acetonitrile containing diclofenac-$^{13}C_6$, analysed using LC-MS/MS.

Figure -1- Example chromatograms of the LLQ (10 ng/mL) extracted from plasma using SPME and fibre desorbed into acetonitrile containing the internal standard.

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.

Table -3- Literature values for molecular weight, logP, pKa, and physiological charge of the three molecules of interest within this study [21,22]

	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

The use of a C18 fibre, 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 fibre and entering the LC-MS instrumentation as a result. Previous studies have made use of a number of SPME fibre phases for *PPB* assessment [18], however, this currently necessitates the use of prototype fibres, or the use of in-house derived fibres. Both options may not be suitable for wider application within the pharmaceutical industry. The performance of this study with a commercially available C18 fibre 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 fibre 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 fibre 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 fibre, 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 vs PBS (protein free vs protein containing). LC-MS as a technique can suffer from matrix effects, a phenomenon whereby non-analyte 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 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 utilised 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 to 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 through-put 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 to 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 to 6-8 h using RED. SPME also offers the future possibility of automation which will enhance throughput and increase the speed of sample processing.

Future Perspectives

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 which 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 which encompass varying charge states, protein binding values, logP, and pKa.

Summary Points

- *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 RED, however, this can require long analyte equilibration times (> 6h), 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 non-exhaustive extraction technique which 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.

References of Interest

*Buscher B, Laakso S, Mascher H, *et al.* Bioanalysis for plasma protein binding studies in drug discovery and drug development: Views and recommendations of the European Bioanalysis Forum. *Bioanalysis*. 6(5), 673–682 (2014).

This reference sets out approaches and sentiments towards *PPB* determination within the scope of the EBF and bioanalytical industry. This may be of interest to researchers working within the areas of DMPK and *PPB* testing.

*Waters NJ, Jones R, Williams G, Sohal B. Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. *Journal of Pharmaceutical Sciences*. 97(10), 4586–4595 (2008).

This reference describes the use of the RED device, the gold standard technique, for *PPB* assessment,

*Musteata F, Pawliszyn J, Qian M, … JW-J of, 2006 undefined. Determination of drug plasma protein binding by solid phase microextraction. *Elsevier* [Internet]. . Available from:

This reference describes an approach to *PPB* determination by SPME. This may be of interest to researchers looking to begin using SPME for *PPB* determination.

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