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Rapid Equilibrium Dialysis (RED): an *In-vitro* High-Throughput Screening Technique for Plasma Protein Binding using Human and Rat Plasma

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Abstract

Determining the extent to which a molecule binds to plasma proteins is a critical phase of drug development because the amount of plasma-bound drug influences compound dosing, efficacy, clearance rate and potential for drug interactions. Determination of the free (%Fu) and bound (%Bound) fractions of a test article in plasma is therefore a critical parameter, which is routinely determined in the process of drug discovery and development. This determination is enabled by equilibrium dialysis, an accepted and standard method for reliable estimation of the non-bound drug fraction in plasma. Although it is the preferred method, equilibrium dialysis has historically been labor-intensive, time-consuming, cost-prohibitive and difficult to automate. A Rapid Equilibrium Dialysis (RED) drug-protein binding assay using LC-MS/MS was developed using a novel technique that resulted in significantly improved assay precision and offers a speed advantage. A panel of compounds covering a range of expected protein binding was tested in plasma of human and rat species. A sensitive and selective method using quadruple tandem mass spectrophotometer interfaced with electro spray ionization was developed for the quantification of unbound drug in pretreated plasma. The mobile phases used were 0.1% Formic acid in Acetonitrile: 0.1% Formic acid in Water with gradient HPLC method. The unbound fraction of drugs was detected by mass spectrometer operated in ESI mode. In addition, data for a set of ten compounds was compared with literature values. With the described method, it is possible to screen a relatively large number of compounds for PPB in a drug discovery environment.

Keywords: Equilibrium dialysis; High-through-put screening; Pharmacokinetics; Protein binding; Rapid equilibrium dialysis

Introduction

The technologies such as high-throughput screening, combinatorial chemistry and genomics, generating greater numbers of hits, together with increasing external pressures for the pharmaceutical industry to bring safer, novel therapeutic entities to the clinic has focused efforts on higher throughput Absorption, Distribution, Metabolism, And Excretion – Toxicity [ADMET] screens in order to optimize drug-like properties earlier and more effectively. One such key parameter, plasma protein binding (PPB), impacts on both the pharmacokinetics and pharmacodynamics of a compound, as well as the safety margin [1,2].

The pharmacokinetic and pharmacodynamic properties of drugs are largely a function of the reversible binding of drugs to plasma or serum proteins. Generally, only the unbound drug is available for diffusion or transport across cell membranes and for interaction with pharmacological targets (e.g. receptor, ion channel, transporter and enzymes) [3]. As a result, the extent of plasma protein binding of a drug influences the drug's action as well as its distribution and elimination.

The extent of binding to plasma influences the way in which a drug distributes into tissues in the body. If a compound is highly bound, then it is retained in the plasma, which results in a low volume of distribution. This may impact on the therapeutic effects of the compound by limiting the amount of free compound which is available to act at the target molecule. Extensive plasma protein binding also limits the amount of free compound available to be metabolized which can, in turn, reduce the clearance of the compound.

The drugs are inert in bound form and are not metabolized or excreted and hence pharmacologically inactive. Only the free forms

are pharmacologically active. Determination of the free and bound fractions of a test article in plasma is a critical parameter routinely determined during the drug discovery process to understand a test article's availability for interaction with its biological target.

Equilibrium dialysis is typically regarded as the gold standard approach to measuring PPB, avoiding the nonspecific binding and large plasma volumes that encounter alternative techniques such as ultra centrifugation and ultra filtration [4]. However, this can be time consuming, labor-intensive methodology in terms of preparation time and equilibrium time. Standard equilibrium dialysis requires a large amount of drug, which precluded its use during early drug development, when compound is limited. Therefore, rapid equilibrium dialysis method was developed using RED device.

The rapid equilibrium dialysis (RED) device has recently been introduced by pierce Biotechnology (Thermo-Fisher Scientific, Waltham, MA) offering a novel apparatus design with a Teflon base plate and disposable dialysis cells. Each dialysis cell has an increased surface area-to-volume ratio relative to standard equilibrium dialysis

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approaches, offering the possibility of reduced equilibration times and higher assay throughput [5].

Herein, we have developed a high throughput plasma protein binding assay in human and rat plasma using RED devices. The assay format was specifically evaluated by testing ten known compounds covering a range of plasma binding properties from low to high binding.

Materials and Method

Chemicals and reagents

The standard compounds were selected according to their variance in binding to plasma proteins. Propranolol [6], Verapamil Hydrochloride and Ketoconazole were selected as high protein binding drugs where as Carbamazepine, Lidocaine, Albendazole and Erythromycin were of moderate binding along with Ofloxacin, Ranitidine and Atenolol as low protein binding drugs. All these drugs were purchased from Sigma Aldrich. Human plasma was obtained from Red Cross blood bank and Rat plasma was obtained from L.M. College of Pharmacy, Ahmedabad. Phosphate Buffered Saline (PBS; pH 7.4) was obtained from Sigma Aldrich. HPLC grade water was procured from Milli-Q water system (Millipore, USA). All the other chemicals used were of analytical grade and proven purity.

Instrumentation

Analysis was performed on LC MS/MS (Agilent triple quadrupole 6410). Software for controlling the system was Mass Hunter Qualitative Analysis Software. Multichannel pipettes and single channel pipettes were of Eppendorf. Equilibrium dialysis was performed using RED Device, Pierce Biotechnology, Thermo Scientific (Figure 1). The RED device consists of Reusable Base Plate made of Teflon and the Inserts. Each Insert is comprised of two side-by-side chambers separated by an O-ring- sealed vertical cylinder of dialysis membrane (Molecular Weight Cut Off -8,000 KD). Assay incubation was done in the Orbital shaker. (Kisker Biotech GmbH & Co. KG; UK).

Preparation of standard stock and working standard solutions

The standard stock solutions of the compounds were prepared separately in Dimethyl sulfoxide [DMSO] at a concentration of 10 mM. From 10 mM stock concentration, working solution of 200 μ M concentration was prepared in DMSO.

Sample preparation: 10 μ L of 200 μ M working solution of drugs were spiked separately into 190 μ L of human and rat plasma. Hence, the final concentration of the drugs was 10 μ M.

Plasma Protein binding using RED Device

The Plasma protein binding assay was performed according to the manufacturer of the RED device (Thermo fisher) [7,8]. The RED Device inserts were placed in Teflon coated base plate [9]. 200 μ L of spiked plasma was added to one of the chambers of RED device insert indicated by red ring while 350 μ L of PBS buffer (pH 7.4) was added to the other chamber. The unit was covered with Immunoware sealing tape and was incubated at 37 $^{\circ}$ C while shaking at 100 rpm for 4 hours. Equal volumes of post dialysis samples were removed from both the plasma and the buffer chambers in separate microcentrifuge tubes and equal volumes of fresh buffer and plasma were added to the tubes, respectively. The plasma and buffer mixtures were precipitated using chilled acetonitrile. After vortexing, the mixtures were centrifuged at 10,000 rpm at 4 $^{\circ}$ C for 10 minutes. The supernatant was collected and transferred to the HPLC vials for LC MS/MS analysis.

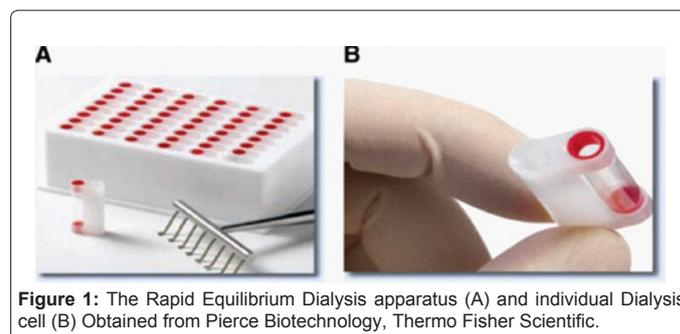


Figure 1: The Rapid Equilibrium Dialysis apparatus (A) and individual Dialysis cell (B) Obtained from Pierce Biotechnology, Thermo Fisher Scientific.

Compound	Rapid Equilibrium Dialysis, % Bound (Experimental)	% CV*	Rapid Equilibrium Dialysis % Bound (Literature)	% CV*
Propranolol	79	1.3	87	2.1
Verapamil	90	0.5	90	0.3
Ketoconazole	99	1.0	99	0.6
Carbamazepine	80	1.0	76	3.2
Lidocaine	67	0.2	70	1.6
Erythromycin	79	0.1	85	0.9
Albendazole	80	1.0	70	1.3
Ranitidine	26	2.0	15	3.1
Ofloxacin	41	3.0	32	2.5
Atenolol	11	2.5	16	3.6

*Data represents $n=3$ replicates

Table 1: Comparison of Human Plasma Protein Binding Values from RED with literature data.

LC MS/MS analysis

The LC MS/MS was done using Agilent triple quadrupole 6410. The gradient chromatographic condition was employed using a Waters XBridge TM C18 analytical column on Agilent Technologies 1200 series HPLC system. The mobile phases used for analysis were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The samples were analyzed in the positive ion mode, with an Electro Spray source (Table 1). Data collection and processing were performed with Agilent Mass Hunter Qualitative Software.

The values of percent bound for each drug were found from literature and these literature values were compared with experimental percent bound values.

The percentage of the test compound bound was calculated by the following formula:-

$$\% \text{ Free} = (\text{Concentration in buffer chamber} / \text{Concentration in plasma chamber}) \times 100$$

$$\% \text{ Bound} = 100 - \% \text{ Free}$$

Results

Comparative analysis of the percent bound by red device using human and rat plasma

Here, we have screened ten standard compounds for the plasma protein binding assay. These compounds were selected such that

they are having different plasma protein binding capacity. Standard compounds were tested at concentration of 10 μM. At pharmacological drug concentration of 10 μM, the percent bound (%) for highly bound compounds like Propranolol, Verapamil and Ketoconazole were 79, 90, and 99 respectively. For moderate binding drugs like Carbamazepine, Lidocaine and Erythromycin were 80, 67 and 79 respectively and for low binding drug like Ranitidine, Ofloxacin and Atenolol was 26, 41, and 11 for human plasma. These data are in good agreement with values found from literature for percent bound (Table 2). The determined values of the percent bound for all drugs were slightly different from literature values due to the differences in methods utilized or in the use of different plasma pools.

Discussion

Our validation studies using the RED device for the measurement of PPB shows that assay format can provide robust and accurate data with shorter preparation and dialysis times. It has the potentials to high-throughput methodology, being amenable to automation. Negligible volume shifts were observed; negating the need to assess volume shifts and aids its use as a high-throughput screen. A rigorous evaluation of the assay was made possible using human plasma, evidently derived from a heterogeneous population and influenced by a number of exogenous factors including diet, genetics and environment.

The nature of the dialysis experiment means nonspecific binding events occur on both the sample and dialysate sides to a similar extent, and thus the impact on PPB is expected to be negligible. Apart from plasma protein binding other potential applications include assessment of tissue and organelle binding in studies of drug distribution, as well as aiding understanding of disparity in potency screens.

In summary, the RED device provides robust and accurate PPB data in a flexible Equilibrium dialysis format with a shorter preparation and dialysis time. It requires negligible apparatus pretreatment, is easy to perform and amenable as a high-throughput method through robotic automation.

The data indicate that at relevant drug concentration, the percent bound of each compound tested in this study is in good agreement with literature values. The binding affinity values (% bound) of the

Compound	Rapid Equilibrium Dialysis, % Bound (Human Plasma)	% CV*	Rapid Equilibrium Dialysis % Bound (Rat Plasma)	% CV*
Propranolol	79	1.3	78	2.3
Verapamil	90	0.5	90	1.3
Ketoconazole	99	1.0	99	1.5
Carbamazepine	80	1.0	83	2.1
Lidocaine	67	0.2	63	3.0
Erythromycin	79	0.1	84	1.5
Albendazole	80	1.0	90	4.0
Ranitidine	26	2.0	32	5.2
Ofloxacin	41	3.0	24	4.4
Atenolol	11	2.5	29	6.0

*Data represents n= replicates

Table 2: Comparison of Human and Rat Plasma Protein Binding Values for Set of Standard Compounds.

LC parameter	Description
HPLC	Agilent 1200
Software Version	Mass Hunter Version B.01.04
Sample Preparation	Plasma Protein precipitation
Sample Matrix	Plasma + buffer
Sample Volume	100 μL
Column	X Bridge C18 5 μM (4.6 X 50 mm)
Mobile Phase	(a) 0.1% formic acid in Water (b) 0.1% formic acid in Acetonitrile
Flow rate (mL/min)	0.8 mL/min
Column Temperature (°C)	25
Injection Volume (μL)	10
Run time (min)	20
Flow	Gradient
QQQ Mass Spectrometer parameter	Description
Ion Source	ESI (Electrospray)
Polarity	Positive
Delta EMV	0
Start mass	100
End mass	1000
Scan mode	EIC (Extracted Ion Chromatogram)
Scan time (Dwell)	500
Fragmentation Voltage	135
Gas Temperature (°C)	300
Nebulizer (PSI)	45
Capillary Voltage	4000
Chamber Current (μA)	0.34
Capillary Current (nA)	13
MS1 Heater (°C)	100
MS2 Heater (°C)	100
Turbo1 Speed (%)	100
Turbo 1 Power (watt)	104.2

Table 3: Optimized LC-MS/MS parameters.

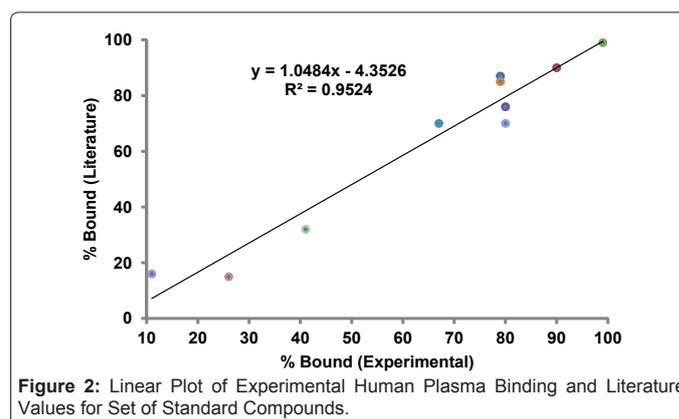


Figure 2: Linear Plot of Experimental Human Plasma Binding and Literature Values for Set of Standard Compounds.

compounds are classified into three categories, The compounds which have binding affinity greater than 90% are called highly bound and the compounds which fall between 90-50% are moderate bound and the compounds which have binding affinity less than 50% are classified as low plasma protein binding compounds [10].

The design of the RED device successfully overcomes several limitations of currently available equilibrium dialysis equipment. The RED device is easy to use and clean, and is automation friendly. Plasma

fraction bound values obtained using the RED device apparatus are comparable to literature values. The high throughput format provides an opportunity to conduct a wide range of analyses that have been frequently omitted in the past because they were too time consuming. These studies include plasma protein binding at pharmacological relative concentration, comparing plasma from multiple species, drug binding to components of plasma.

In this present study we have developed plasma protein binding assay in two species (Human and Rat) using RED devices. Under optimized assay conditions, the binding of the known ten drugs with human and rat plasma in the assay are in good agreement with those obtained in the literature (Table 3). A comparison of the plasma protein binding by plotting percent bound values from the experimental and literature allows forecasting capacity of the assay. It shows good correlation of r^2 of 0.952 (Figure 2).

In conclusion, the data obtained using RED device clearly indicates that this method provides an accurate estimation of the percent bound fraction. The 96-well design of RED device makes it compatible with the High-throughput screening [HTS] format, including bioanalytical mass spectroscopy.

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