

Overview

- Protein binding using rapid equilibrium dialysis (RED device, Linden Bioscience, MA)
- True equilibrium dialysis method, equilibrium reached within 4 hrs
- Simple, versatile, incubation in a 96-well format
- Detailed case study using acetaminophen for comparing binding results
- Peak Area (PA), PA ratio, & Standard Calibrators (STDs) used to estimate percent bound
- **Acetaminophen-d4** used as internal standard (IS)
- Detailed study into impact of IS selection
- Hydroxybutiratoil, Dextromethorphan, and Levofloxacin used as generic IS
- Incubation buffer effects – type of buffer and volume
- Monitoring non-specific binding
- Data obtained from both in-house and reference compounds support the use of the RED device in development when compared to literature/historical in-house data

Introduction

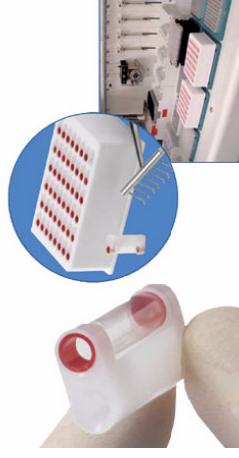
After administration, the ability of the drug to reach its intended therapeutic target is often affected by plasma protein binding (PPB). Screening for binding to plasma proteins occurs early in the discovery phase by ultrafiltration and is further investigated at later stages of development by equilibrium dialysis. Equilibrium dialysis is the accepted method for reliably estimating protein binding. However, it is labor intensive with limited throughput, and not easily automated. Recently, a novel equilibrium dialysis model utilizing a 96-well plate footprint, the Rapid Equilibrium Dialysis device (RED), has been described. In this study, the RED device is assessed as a high throughput alternative model for PPB.

Method

The RED device can accommodate 48 inserts in a 96-well plate format. Each insert consists of two chambers separated by a semi-permeable membrane. Plasma containing drug is added to one chamber while buffer is added to the second. To determine non-specific binding, a buffer sample (containing drug) is added to both chambers of the insert. The Teflon coated base plate is incubated at ~37°C while shaking at 100 rpm for 4 hours. Afterwards, an aliquot is removed from each chamber and equal amounts of fresh plasma and buffer are added to respective incubated aliquots. The plasma/buffer mixtures are precipitated using an organic/aqueous solution with 0.1% formic acid and centrifugation, the supernatant is transferred to a clean 96-well plate for LC/MS analysis using an AB Sciex 2000 or 3000 triple quadrupole equipped with an ESI interface and API source.

Rapid Equilibrium Dialysis (RED) Device

- Disposable tube inserts with two chambers – Red (plasma) and White (buffer)
- Teflon coated base plate – 96-well format
- High membrane to surface volume ratio – reduces dialysis time
- Automation friendly
- MWCO = 6,000 - 8,000



RED Procedure

- Pre-soak base/inserts
- Prepare plasma/buffer samples (Blank, STDs & QCs)
- Build plate – 200 µL plasma/ 350 µL buffer
- Incubate at 37°C at 100 rpm for approximately 4 hrs
- Remove 50 µL from both buffer & plasma chamber
- Add 50 µL buffer/plasma to respective aliquots
- Add 200 µL precipitation solution (IS cocktail)
- Mix/Centrifuge/Transfer (Tomtec)/Analyze
- PB Calculation:

$$\% \text{ Free Drug} = \frac{\text{Peak Area Ratio of Buffer Sample/IS}}{\text{Peak Area Ratio of Plasma Sample/IS}} \times 100\%$$

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

Typical Sample Plate Well Map

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
B	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
C	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
D	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
E	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
F	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
G	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
H	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma

Buffer Samples for NSB, Blank Plasma & Reference Compounds

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
B	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
C	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
D	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
E	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
F	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
G	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma
H	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma	Human Plasma

Results

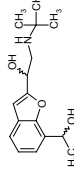
Initial Data – Questionable Results

Does Internal Standard Selection Matter?

Reference Compound Protein Binding Comparison

Compound	% Bound-Literature	% Bound-Lavandisole	% Bound-Dextromethorphan	% Bound-Hydroxybutiratoil
Gabapentin	5.5%	5.5%	5.5%	NA
Metronidazole	8.5%	8.5%	8.5%	NA
Fluconazole	10-12%	20.9%	2.85±2.3%	16.1±3.5%
Topiramate	13-17%	1.70%	44.0%	NA
Ranitidine	12-18%	13.0%	72.0%	30.37±4%
Propranolol	11-21%	33.0%	2.90%	NA
Procainamide	13-17%	32.4%	90.5%	NA
Levofloxacin	24-38%	65.7%	80.5%	NA
Lidocaine	85-95%	77.7%	93.5%	NA
Propranolol	81-93%	80.2%	92.6±8.1%	99.91±7%
Testosterone	85-95%	NA	NA	96.4%
Imipramine	88.7-91.5%	74.5%	92.5%	84.9%
Risperidone	89%	61.5%	97.5%	96.3%
Clozapine	94-97%	95%	98.3%	NA
Oxazepam	94-97%	98.0%	98.3%	NA
Warfarin	98-100%	99.3%	99.7%	99.1%

Does a Buffer Effect Exist?



Compound	% Bound-Literature	% Bound-Duibecco's PBS	% Bound-0.9% NaCl	% Bound-Sigma PBS
Fluconazole	10-12%	16.1%	-2.39%	3.00%
Ranitidine	12-18%	27.8%	41.1%	13.6%
Propranolol	81-93%	90.0%	54.7%	80.2%
Warfarin	98-100%	99.1%	99.0%	99.3%

All subsequent studies conducted with PBS, pH 7.4

Summarized PB Results

Compound	% Bound-Literature	% Bound-RED Device
Gabapentin	< 3%	5.60%
Metronidazole	8-14%	0-14.3%
Fluconazole	10-12%	0-29.4%
Topiramate	13-17%	1.70%
Ranitidine	12-18%	13.6%
Procainamide	11-21%	0-5.1%
Acetaminophen	< 20%	13.3%
Baclofen	20-42	12.4%
Levofloxacin	24-38%	32.2%
Lidocaine	65-75%	77.7%
Propranolol	81-93%	80.2%
Testosterone	85-95%	96.4%
Imipramine	88.7-91.5%	74.9%
Risperidone	89%	91.4%
Clozapine	95%	95.3%
Oxazepam	94-97%	96.0%
Warfarin	98-100%	99.3%

Protein Binding Determination in Human Plasma at ~1 µg/mL

Compound	% Bound-Literature	% Bound-Duibecco's PBS	% Bound-0.9% NaCl	% Bound-Sigma PBS
Fluconazole	10-12%	16.1%	-2.39%	3.00%
Ranitidine	12-18%	27.8%	41.1%	13.6%
Propranolol	81-93%	90.0%	54.7%	80.2%
Warfarin	98-100%	99.1%	99.0%	99.3%

Further Investigation: Acetaminophen Case Study

Peak Area, PA Ratio and Calculated Concentration

Acetaminophen 1840 ng/mL	Acetaminophen 1840 ng/mL	Acetaminophen 1840 ng/mL	Acetaminophen 1840 ng/mL
Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1 655	1 655	1 655	1 655
2 704	2 704	2 704	2 704
3 857	3 857	3 857	3 857
4 715	4 715	4 715	4 715
Mean 682	Mean 682	Mean 682	Mean 682
Stdev 29.2	Stdev 29.2	Stdev 29.2	Stdev 29.2
CV 4.3	CV 4.3	CV 4.3	CV 4.3
% Bound 4	% Bound 4	% Bound 4	% Bound 4

Does the Volume of Buffer Affect Binding?

Acetaminophen 1840 ng/mL	Acetaminophen 1840 ng/mL	Acetaminophen 1840 ng/mL
Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1 655	1 655	1 655
2 704	2 704	2 704
3 857	3 857	3 857
4 715	4 715	4 715
Mean 682	Mean 682	Mean 682
Stdev 29.2	Stdev 29.2	Stdev 29.2
CV 4.3	CV 4.3	CV 4.3
% Bound 4	% Bound 4	% Bound 4

Non-specific binding

- Simple test
- Drug prepared in buffer
- Equal amount added to each chamber (Red/White)
- Both chambers analyzed nominal
- This example displayed no issues related to NSB

Acetaminophen 1840 ng/mL	Acetaminophen 1840 ng/mL
Peak Area Ratio	Peak Area Ratio
1 1640	1 1640
2 1680	2 1680
3 1750	3 1750
4 1678	4 1678
Mean 1687	Mean 1687
Stdev 48.7	Stdev 48.7
CV 2.86	CV 2.86
% Bound 4	% Bound 4

Conclusions

- The RED device is a novel, reliable, quick, user-friendly alternative model for plasma protein binding
- The simple procedure, setup and 96-well footprint is easy to automate
- Internal standard selection can have a large impact – labeled or close analogs are recommended
- The type of incubation buffer has a significant effect
- The amount of buffer for incubation is not significant – using the recommended volume is easier during aliquot
- PB calculation using peak area ratio is reliable although more data needs to be accumulated to fully support replacing the use of Standard Calibrators
- Non-specific binding can be easily monitored

Future Direction/ Upcoming Projects

- Continue adding in-house compounds and references
- Support Early Development:
 - Compound analysis using stable labeled IS or analog
 - Standard Calibrators & QCs
 - Mouse, Rat, Rabbit, Dog, Monkey & Human
 - Two concentrations (n=4) based on PK C_{max}
- Addition of buffer samples to account for non-specific binding
- Reference compounds (low, medium & high) included in each analysis
- Continue to evaluate the need for STDs & QCs vs. using Peak Area ratios
- Investigate equilibrium over select timepoints

Acknowledgements

- Tai-nang Huang - Linden Bioscience, Woburn, MA
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References

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- Rowland, M, Tozer, T, eds. Clinical Pharmacokinetics: Concepts and Applications, Third edition, Philadelphia: Lippincott Williams & Wilkins, 1995.

