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In Vitro Plasma Protein Binding Assessment for Highly Protein-Bound Compounds Under the ICH M12 Guideline

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What Is Plasma Protein Binding and Why Does It Matter to Drug-Drug Interactions?

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Plasma protein binding refers to the reversible interaction between drug molecules and proteins in the blood, primarily albumin and α1-acid glycoprotein. The unbound (free) fraction of a drug (fu) is the portion not bound to these proteins. Only free drugs can cross cell membranes, interact with therapeutic targets, and undergo metabolism or excretion.

Since PPB affects a drug's distribution and clearance, especially hepatic clearance, accurately measuring fu is critical for predicting drug-drug interactions involving metabolizing enzymes (e.g., CYP450) and transporters (e.g., OATP1B1). Historically, conservative defaults (e.g., assuming fu = 0.01 for highly bound drugs) were used in DDI assessments, but recent guidelines like ICH M12 now emphasize experimentally determined fu values for more accurate predictions.

Agenda

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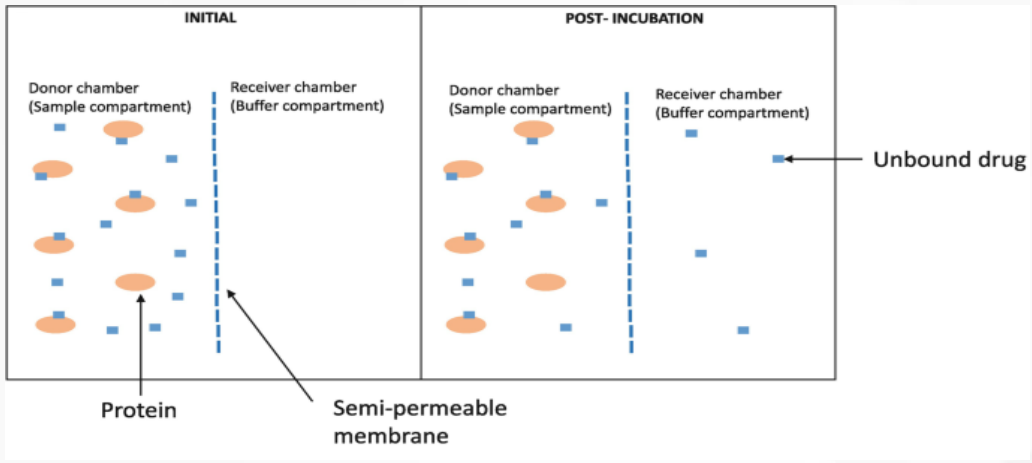
- Common Methods for Plasma Protein Binding (PPB) Determination
- ICH M12 Recommendation
- Analytical Challenges and Solutions in PPB for Highly Protein-Bound Compounds
- Best Practices for PPB Assay of Emerging Modalities

Equilibrium Dialysis



Advantage

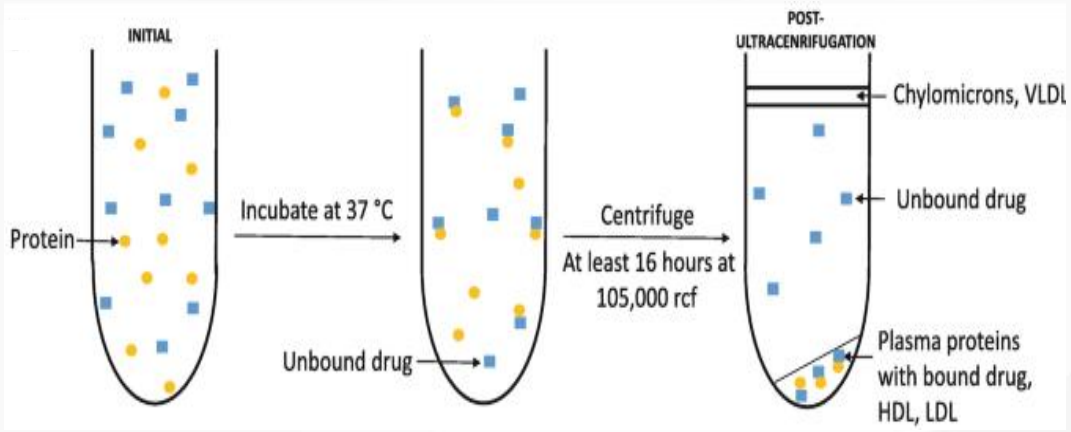
- Low nonspecific adsorption of equilibrium dialysis; materials, minimal impact on fu measurement;
- Temperature and pH can be controlled;
- Rapid equilibrium dialysis can use a 96-well plate, enabling high throughput;
- Requires only small sample volumes (<500µL);

Disadvantage

- Time-consuming: conventional equilibrium dialysis requires 3–48 hours; rapid equilibrium dialysis requires 2–6 hours
- Not suitable for compounds with poor stability
- Not suitable for compounds with poor solubility in buffer

Limaye, P.B., Parikh, K. (2024). Distribution - In Vitro Test: Protein Binding. In: Hock, F.J., Pugsley, M.K. (eds) Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays. Springer, Cham. https://doi.org/10.1007/978-3-030-73317-9_101-1

Ultracentrifugation



Advantages

- Suitable for highly hydrophobic compounds;
- High accuracy;
- No nonspecific adsorption to semipermeable membranes or devices;
- Absence of fluid shift, plasma dilution, and membrane leakage

Disadvantages

- Co-precipitation of free and bound drug (MW > 300 Da);
- Temperature can be controlled, but pH is difficult to control;
- No high-throughput capability, Time-consuming with limited sample numbers;
- Possible drug insolubility in the supernatant

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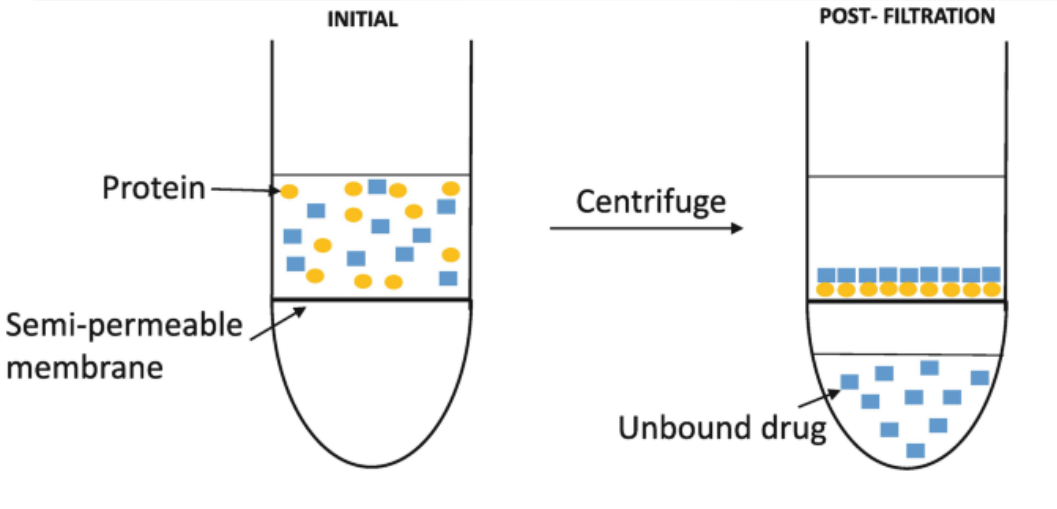
Ultrafiltration



Advantages

- High accuracy;
- High throughput (96-well plate);
- Relatively fast and easy to perform;
- Suitable for unstable compound.

Disadvantages

- Requires external force (pressure) and can be affected by consumables, e.g., protein leakage;
- Nonspecific binding may affect the unbound fraction96-well plates may suffer from “edge effects”;
- Possible drug insolubility in the ultrafiltrate;
- Not suitable for highly hydrophobic compounds

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How to select PPB techniques?

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Compound characteristics

Table 1. Plasma protein binding techniques selected based on compound characteristics.

Technique	Highly lipophilic	Highly hydrophilic	Biologicals	Labile	Sticky
Answers to survey II: **in vitro *plasma protein binding***					
Ultrafiltration	++	+++++		+++++	+
Equilibrium dialysis	++++	+++++			+
Ultracentrifugation	++		+	+	+++
Transil™ partitioning	+				+
Gel filtration	+				+
Answers to survey III: **in vivo *plasma protein binding***					
Ultrafiltration		+		+++	
Equilibrium dialysis	++	++			
Ultracentrifugation					+
Transil partitioning	+				+

Answers were obtained from the survey regarding in vitro *plasma protein binding studies (upper table) and* in vivo *plasma protein binding studies (lower table) in drug-development phase.*
The number of symbols indicate if the technique is used by many companies (greater number of symbols) or by just a few companies (smaller number of symbols).

Buscher, B., Laakso, S., Mascher, H., Pusecker, K., Doig, M., Dillen, L., … Timmerman, P. (2014). 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. https://doi.org/10.4155/bio.13.338

How to select PPB techniques?

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Anticipated protein binding

Table 2. Plasma protein binding techniques selected based on anticipated protein binding: very high protein binding (>98%), high protein binding (85–98%) and medium to low protein binding (<85%).

Technique	Very high (>98%)	High (85–98%)	Medium to low (<85%)
Answers to survey II: **in vitro *plasma protein binding***			
Ultrafiltration			++++
Equilibrium dialysis	+++	+++++	++
Ultracentrifugation	+	+	
Transil™ partitioning	+		
Gel filtration	+		
Answers to survey III: **in vivo *plasma protein binding***			
Ultrafiltration			+
Equilibrium dialysis	+	++	+
Ultracentrifugation	+	+	
Transil partitioning	+		

Buscher, B., Laakso, S., Mascher, H., Pusecker, K., Doig, M., Dillen, L., … Timmerman, P. (2014). 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. https://doi.org/10.4155/bio.13.338

Which type of biological analysis method to select?

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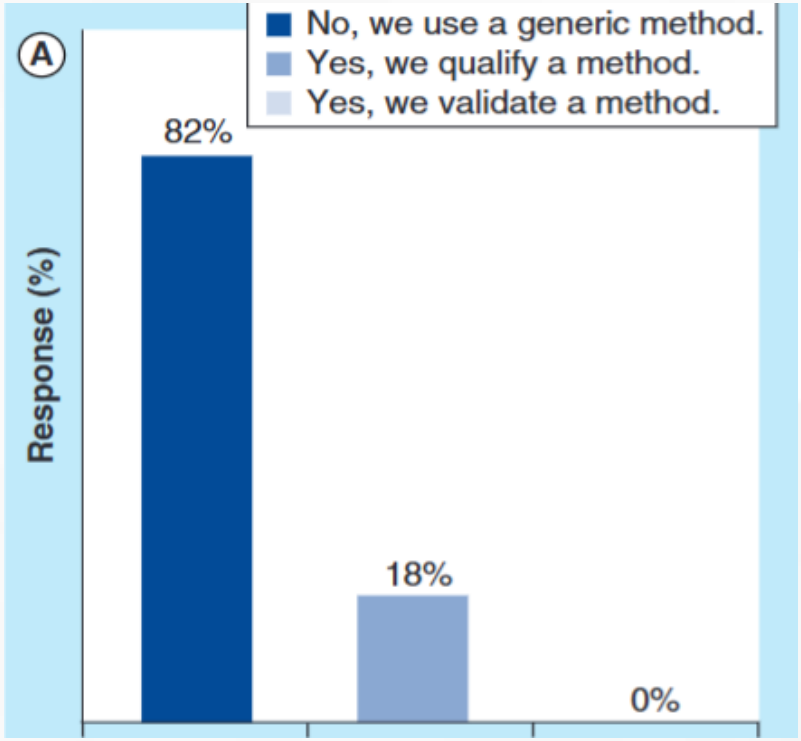
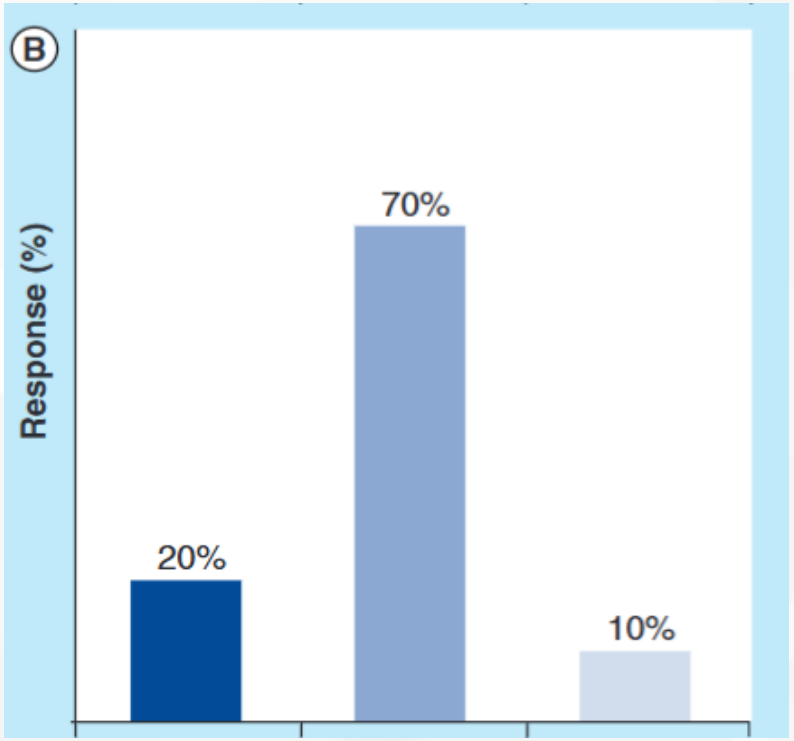
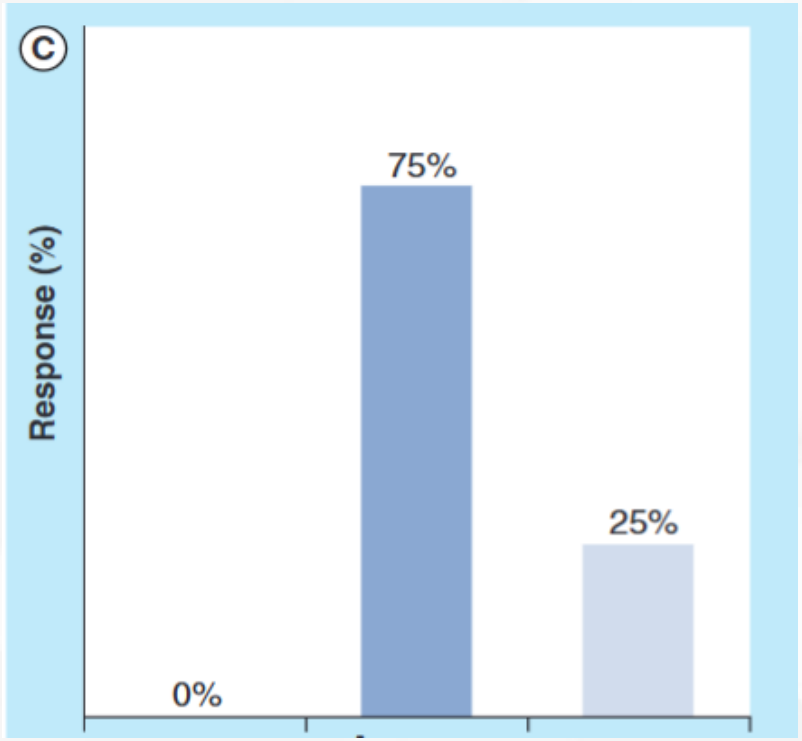
PPB in drug-discovery phase



***in vitro* PPB in drug-development phase**



***in vivo* PPB in drug-development phase**



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EBF recommendation

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Drug Discovery Stage

- No study plan;
- No need to do prestudy qualification;
- No need for positive control compound;.
- Use one concentration (in duplicate);
- Use generic analytical method (based on principles of screening or qualified assay) or limited prestudy method qualification (calibration samples).
- In-study qualification: limited calibration curve samples included with a priori defined broadened acceptance criteria (25% on calibration accuracy); QC samples optional.
- Appropriate IS recommended;
- Raw data available. Short summary report if requested.

Drug Development Stage (*In Vivo* and *In Vitro* Protein Binding Studies)

- Study plan recommended (No need to claim GLP statement);
- Preliminary experiments can be conducted before the actual PPB study;
- Positive controls compound optional;
- Use at least three drug concentration levels (at least three replicates at each level);
- Use qualified assay with prestudy or in-study method qualification.
- In-study and a prior defined acceptance criteria on calibration and QC (broadened to 20%, and 25% at LLOQ).
- Use of stable isotope IS recommended;
- Study report includes all experimental details and results.

Buscher, B., Laakso, S., Mascher, H., Pusecker, K., Doig, M., Dillen, L., … Timmerman, P. (2014). 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. https://doi.org/10.4155/bio.13.338

EBF: European Bioanalysis Forum

ICH M12 Guideline: 7.2 Protein Binding

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7.2 Protein Binding

A key parameter of interest in predicting the risk of DDIs for an investigational drug as a precipitant is plasma protein binding. Historically, considering uncertainties in protein binding measurements for highly bound drugs, regulatory agencies have recommended the $f_{u,p}$ (fraction unbound in plasma) be set at 0.01 (i.e., 1%). This conservative approach was as adopted to protect against false negative DDI predictions. Recent advances have resulted in the development of methodologies that allow accurate and precise measurement of protein binding for highly protein bound drugs. When choosing a protein binding assay, it is important to ensure the suitability of a given assay methodology for the investigational drug. The next step is to demonstrate the protein binding assay is precise and accurate. Such a demonstration should include validation data of the protein binding assay qualified with appropriate positive controls (i.e., range of compounds with high binding to relevant plasma proteins). The bioanalytical methods used in these experiments should have appropriate precision and accuracy at the required sensitivity range (i.e., Calibration standards and Quality Controls at 15%; Lower Limit of Quantification at 20%). Protein binding assays that have demonstrated these features can be used to determine the protein binding of an investigational drug (21). When novel and emerging protein binding assay methodologies are being established, the assay method should be qualified against an orthogonal method that is previously established and accepted for a range of highly bound compounds (22).

Of note, the protein binding experiments for the investigational drug should always include a positive control for which the assay was previously qualified to ensure in-study performance. The positive control should be demonstrated to be within 3-fold of the mean $f_{u,p}$ value that was previously reported with the qualification of the protein binding assay method.

ICH M12 Guideline on drug interaction studies, 2024

In Vivo and In Vitro Protein Binding Studies

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EBF Recommendation

- Study plan recommended (No need to claim GLP statement);
- Preliminary experiments can be conducted before the actual PPB study;
- **Positive controls compound optional**;
- Use at least three drug concentration levels (at least three replicates at each level);
- Use qualified assay with prestudy or in-study method qualification.
- **In-study and a prior defined acceptance criteria on calibration and QC (broadened to 20%, and 25% at LLOQ).**
- Use of stable isotope IS recommended;
- Study report includes all experimental details and results.

ICH M12 Recommendation

- **Positive controls compound required ;**
- **Calibration standards and Quality Controls at 15%; Lower Limit of Quantification at 20%**
- **Use qualified assay with appropriate positive controls**
- The positive control should be demonstrated to be within 3-fold of the mean fu,p value that was previously reported with the qualification of the protein binding assay method.

Challenges and Solutions for Bioanalysis

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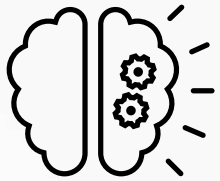

Challenges	Solutions
Low recovery	Optimization of sample extraction
High assay variability	Use of a more sensitive mass spectrometer
Large concentration differences between total and unbound fractions	Use of isotope-labeled internal standards
Matrix effects across different biological matrices and species	

Use of Isotope-labeled IS

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By using isotope-labeled internal standards, the impact of matrix effects from different species can be minimized, allowing a single calibration curve to be used for all samples.

TBA

	Human			Monkey			Dog			Rat			Mouse		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Theor. Conc.	1.5	100	400	1.5	100	400	1.5	100	400	1.5	100	400	1.5	100	400
Mea. Conc.	1.427	105.369	473.582	1.493	100.084	419.583	2.512	167.952	826.177	2.005	126.26	546.86	2.446	134.718	575.487
%Theoretical	95.1%	105.4%	118.4%	99.5%	100.1%	104.9%	167.5%	168.0%	206.5%	133.7%	126.3%	136.7%	163.1%	134.7%	143.9%
%Bias	-5%	5%	18%	0%	0%	5%	67%	68%	107%	34%	26%	37%	63%	35%	44%
n	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Isotope-labeled IS

	Human			Monkey			Dog			Rat			Mouse		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Theor. Conc.	1.5	100	400	1.5	100	400	1.5	100	400	1.5	100	400	1.5	100	400
Mean	1.6	103.9	445.4	1.6	102.0	423.9	1.6	102.3	432.2	1.6	101.4	436.6	1.6	100.6	432.6
S.D.	0.06	4.14	17.09	0.06	6.25	18.13	0.13	2.92	8.04	0.05	3.02	14.67	0.08	2.23	6.87
%CV	3.60%	3.99%	3.84%	3.59%	6.13%	4.28%	7.79%	2.85%	1.86%	3.52%	2.98%	3.36%	5.11%	2.22%	1.59%
%Theoretical	103.7%	103.9%	111.3%	109.1%	102.0%	106.0%	109.6%	102.3%	108.0%	103.9%	101.4%	109.2%	108.2%	100.6%	108.1%
%Bias	4%	4%	11%	9%	2%	6%	10%	2%	8%	4%	1%	9%	8%	1%	8%
n	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6

Sample Preparation Optimization

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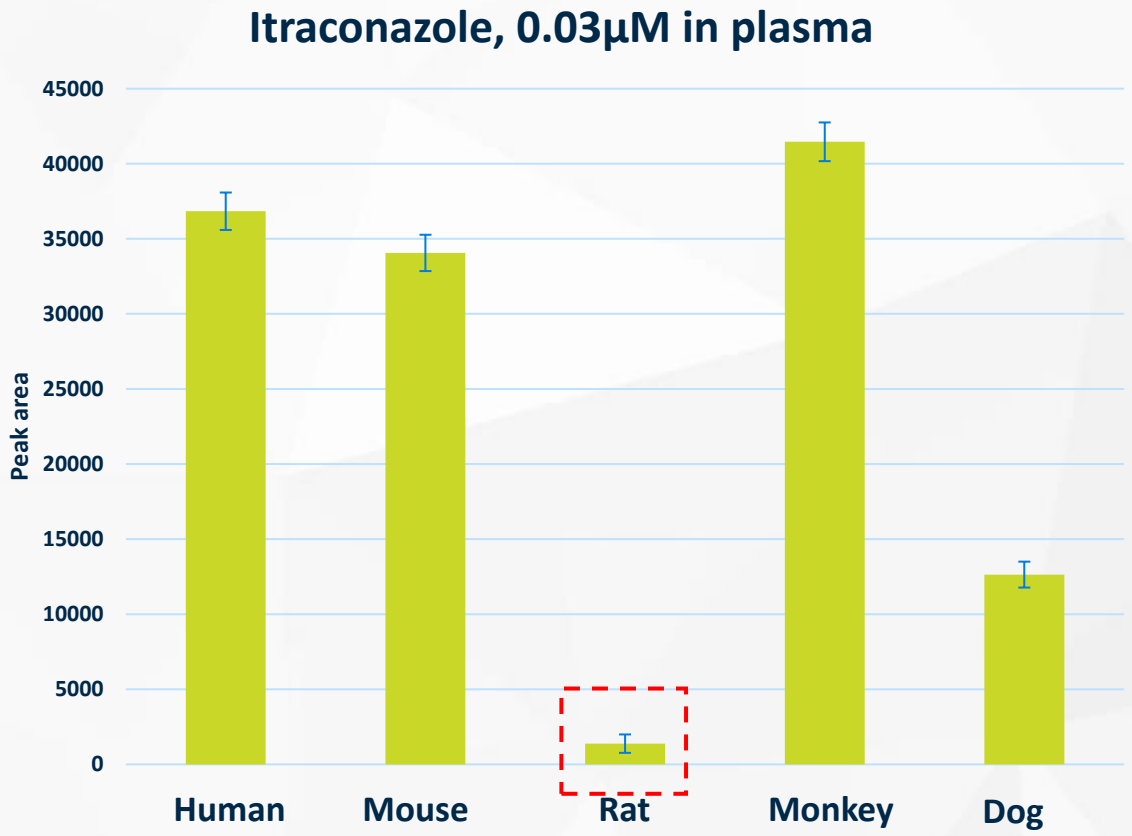
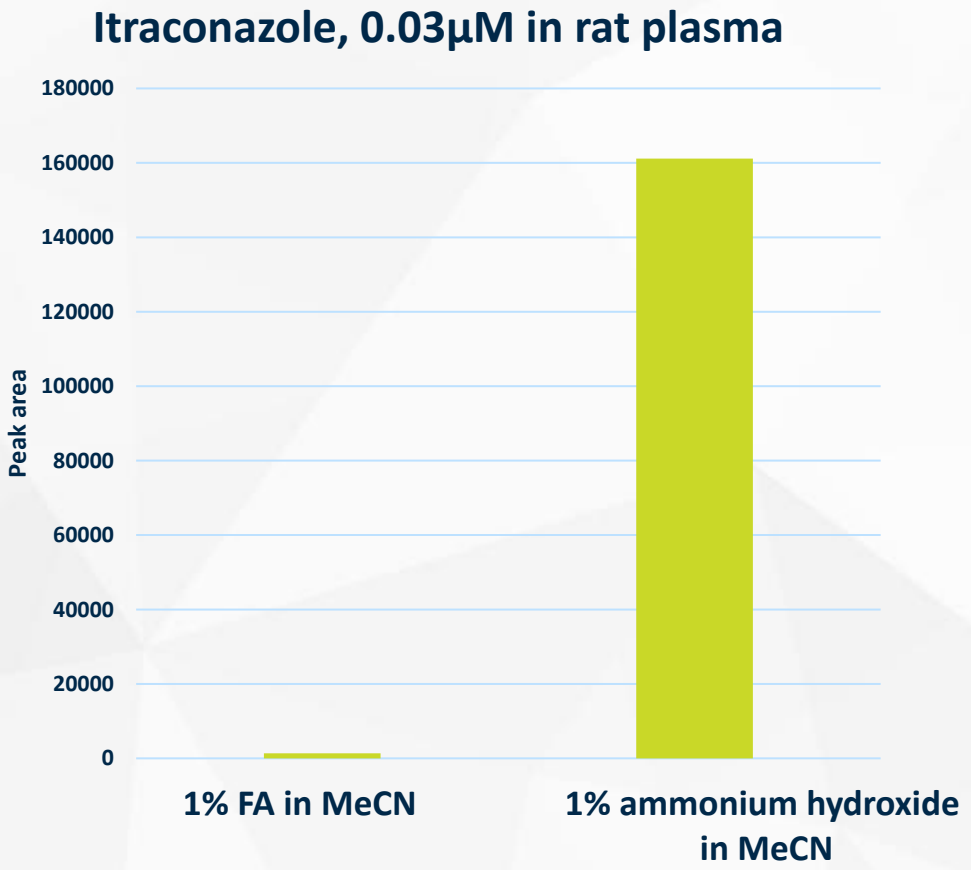
By adjusting the pH of the precipitating agent, the recovery can be improved.





LC-MS Method Optimization

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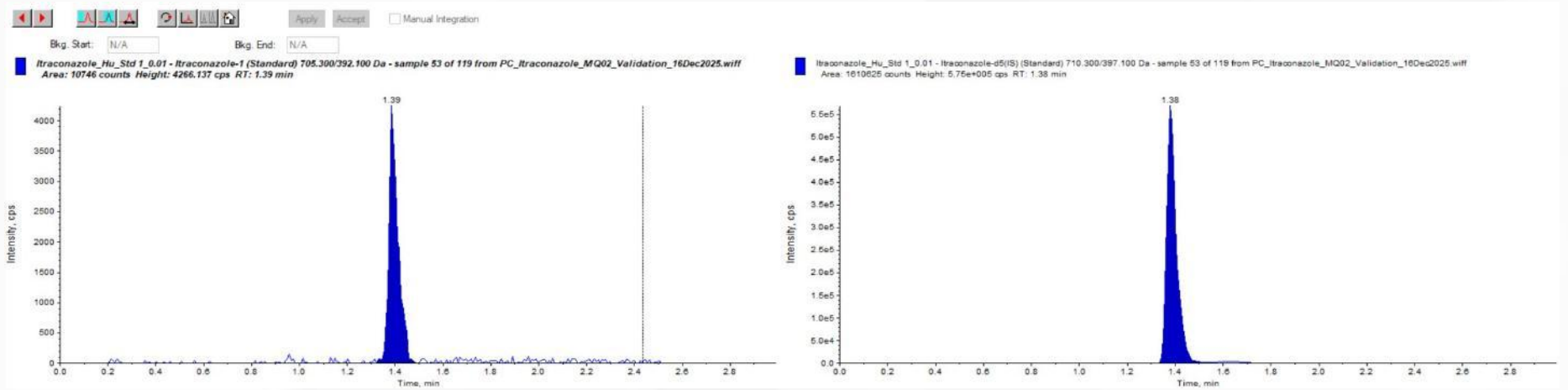
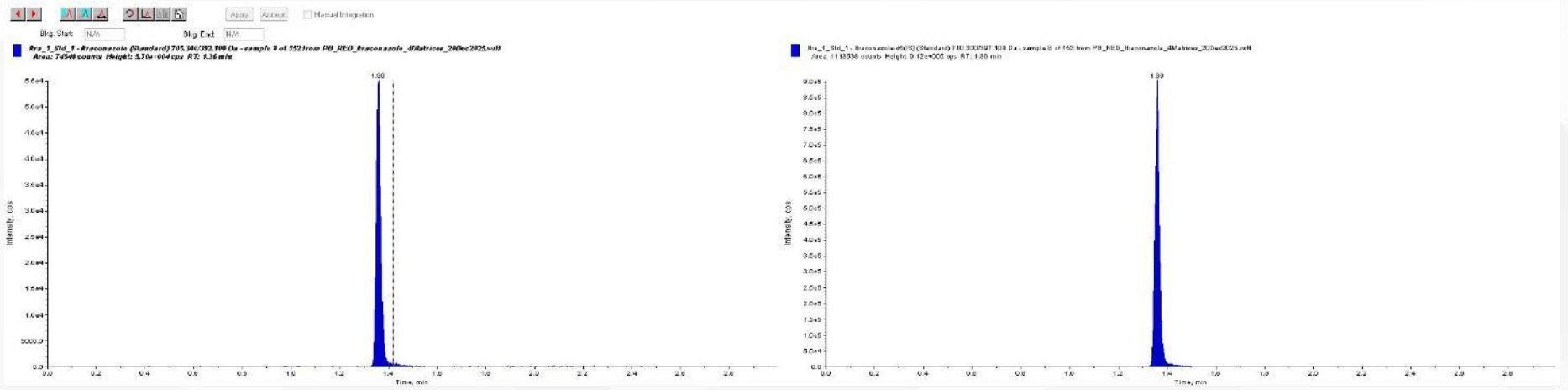
By using a more advanced mass spectrometer, the analytical sensitivity can be improved.

MS 5500+
Itraconazole
0.01µM



MS 6500+
Itraconazole
0.01µM



LC-MS Method Qualification

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Itraconazole (Positive Control)

Sample ID	Rep.	Calibration Conc. (uM)	Conc. Found (uM)	%(Found - Added)/Added
STD 1*	1	0.001	0.000676	-32.4%
STD 2	1	0.002	0.0018	-10.0%
STD 3	1	0.005	0.00457	-8.6%
STD 4	1	0.01	0.0106	6.0%
STD 5	1	0.02	0.0199	-0.5%
STD 6	1	0.1	0.0886	-11.4%
STD 7	1	0.5	0.503	0.6%
STD 8	1	2	1.94	-3.0%
STD 9	1	4	3.89	-2.8%
STD 1	2	0.001	0.00104	4.0%
STD 2*	2	0.002	0.00246	23.0%
STD 3	2	0.005	0.00548	9.6%
STD 4	2	0.01	0.00924	-7.6%
STD 5	2	0.02	0.0227	13.5%
STD 6	2	0.1	0.104	4.0%
STD 7	2	0.5	0.486	-2.8%
STD 8	2	2	2.13	6.5%
STD 9	2	4	4.05	1.3%

* excluded

	Human			Monkey			Dog		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Theor. Conc.	0.003	0.030	3	0.003	0.030	3	0.003	0.030	3
Mean	0.00321	0.0328	3.25	0.00290	0.0312	2.86	0.00314	0.0325	2.86
S.D.	0.00026	0.00320	0.131	0.00022	0.00174	0.164	0.00028	0.00187	0.066
%CV	7.97%	9.78%	4.02%	7.42%	5.56%	5.73%	8.77%	5.75%	2.32%
%Theoretical	107.1%	109.2%	108.4%	96.8%	104.1%	95.2%	104.8%	108.2%	95.4%
%Bias	7.1%	9.2%	8.4%	-3.2%	4.1%	-4.8%	4.8%	8.2%	-4.6%
n	6	6	6	6	6	6	6	6	6

	Mouse			Rat		
	LQC	MQC	HQC	LQC	MQC	HQC
Theor. Conc.	0.003	0.030	3	0.003	0.030	3
Mean	0.00336	0.0337	3.26	0.00271	0.0312	2.74
S.D.	0.00012	0.00136	0.103	0.00020	0.00165	0.116
%CV	3.54%	4.03%	3.15%	7.50%	5.28%	4.22%
%Theoretical	111.9%	112.3%	108.6%	90.2%	104.0%	91.4%
%Bias	11.9%	12.3%	8.6%	-9.8%	4.0%	-8.6%
n	6	6	6	6	6	6

PPB for Itraconazole (Positive Control)

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Itraconazole was spiked into mouse, rat, dog, monkey and human plasma to achieve the final concentration at 3µM. Triplicate (n=3) aliquots of the spiked matrix will be added to the red chambers of the RED device inserts, and phosphate buffered saline will be added into the white chambers of the inserts. The RED device plate will be sealed and incubated for 24 hours at 37°C with shaking. The control listed above will also be tested as the positive control for the assay.

After incubation, samples from both the red and white chambers will be analyzed by qualified LC-MS/MS. The percentages of free and bound fractions will be determined using the concentration of samples.

Compound	Mouse Plasma		Rat Plasma		Dog Plasma		Monkey Plasma		Human Plasma	
	Percent Free Fraction	SD	Percent Free Fraction	SD	Percent Free Fraction	SD	Percent Free Fraction	SD	Percent Free Fraction	SD
Itraconazole	0.192	0.039	0.275	0.049	0.311	0.099	0.467	0.037	0.528	0.16
Itraconazole Historic data	0.130	0.061	0.208	0.073	0.181	0.044			0.153	0.068
Reference									0.07-0.22	

Di, Li et al. Industry Perspective on Contemporary Protein-Binding Methodologies: Considerations for Regulatory Drug-Drug Interaction and Related Guidelines on Highly Bound Drugs. Journal of Pharmaceutical Sciences 106 12 (2017): 3442-3452

PPB for Oligonucleotide Therapeutics

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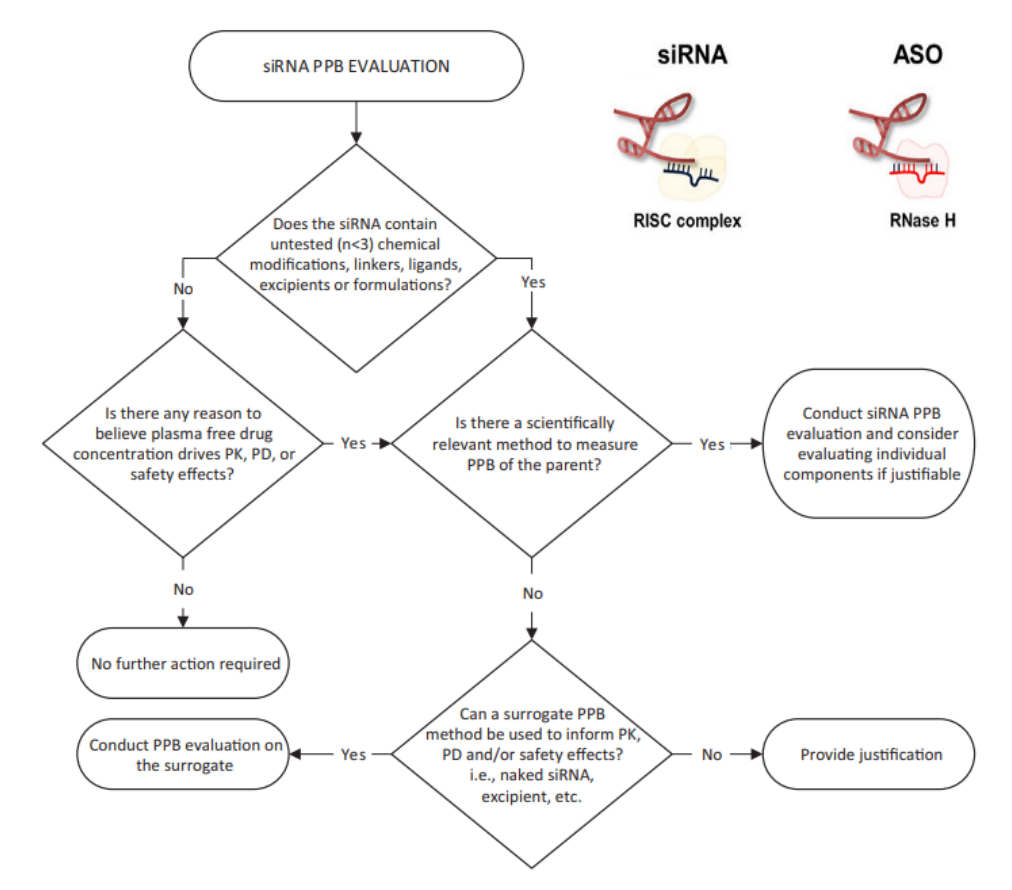
- A relatively high plasma protein binding was observed, with similar binding across species, and the binding decreased as the concentration increased;
- PPB of ASO is commonly assessed using ultrafiltration or ultracentrifugation. PPB of siRNA typically uses ultrafiltration or electrophoretic mobility shift assay (EMSA);
- Requires special sample preparation and LC-MS methods;
- If higher sensitivity detection is needed, MSD can be considered.
- It is recommended to include siRNA PPB data in regulatory submissions when the molecule contains new chemical modifications, linkers, ligands, excipients, or formulations not previously evaluated in approved clinical drugs, or when plasma free-drug levels may influence PK, PD, or safety.



Humphreys SC, Davis JA, Iqbal S, et al. Considerations and recommendations for assessment of plasma protein binding and drug-drug interactions for siRNA therapeutics. Nucleic Acids Research. 2022 Jun;50(11):6020-6037. DOI: 10.1093/nar/gkac456. PMID: 35687098; PMCID: PMC9226521.

PPB for Antibody-Drug Conjugate (ADC)

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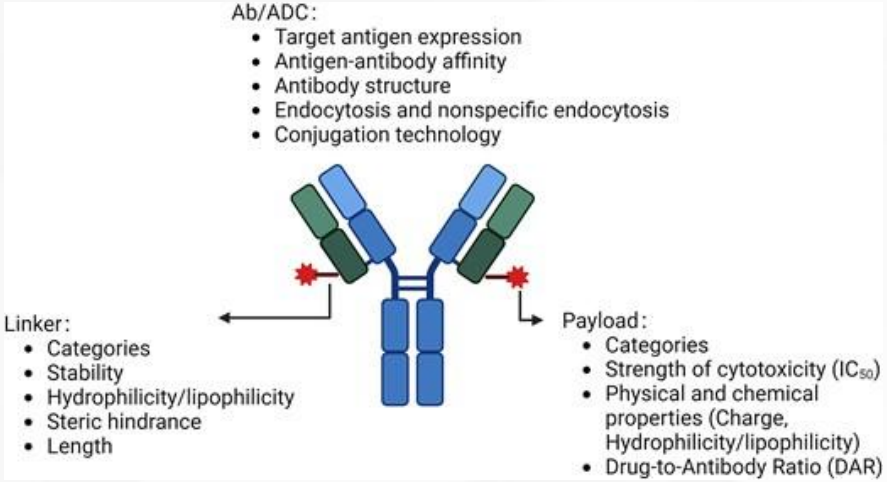
- The plasma protein binding of ADC payloads can vary significantly across different species, including humans.
- The payload concentration in the plasma after ADC administration is generally low, so PPB should be carried out at a low concentration of payload to mimic *in vivo* conditions.
- If payload cannot be detected by conventional mass spectrometry due to the sensitivity limitation of the detection method, radiolabeled payloads can be used to carry out PPB assay.

Plasma Protein Binding Value of MMAE in Mouse, Rat, Cynomolgus, and Human

Species	Concentration		
	1 nmol/L	10 nmol/L	100 nmol/L
Mouse	18.8%	19.6%	28.5%
Rat	72.9%	73.5%	72.0%
Cynomolgus	17.1%	17.8%	18.9%
Human	67.9%	77.5%	82.2%

http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/125399Orig1s000PharmR.pdf



Yumei Cheng, Jing Lu, Chonghao Zhang, Weiyuan Yan, Panpan Zhu, Qiuping Qin, Likun Gong, Overview of antibody-drug conjugates nonclinical and clinical toxicities and related contributing factors, Antibody Therapeutics, Volume 8, Issue 2, April 2025, Pages 124–144, https://doi.org/10.1093/abt/tbaf004

Summary

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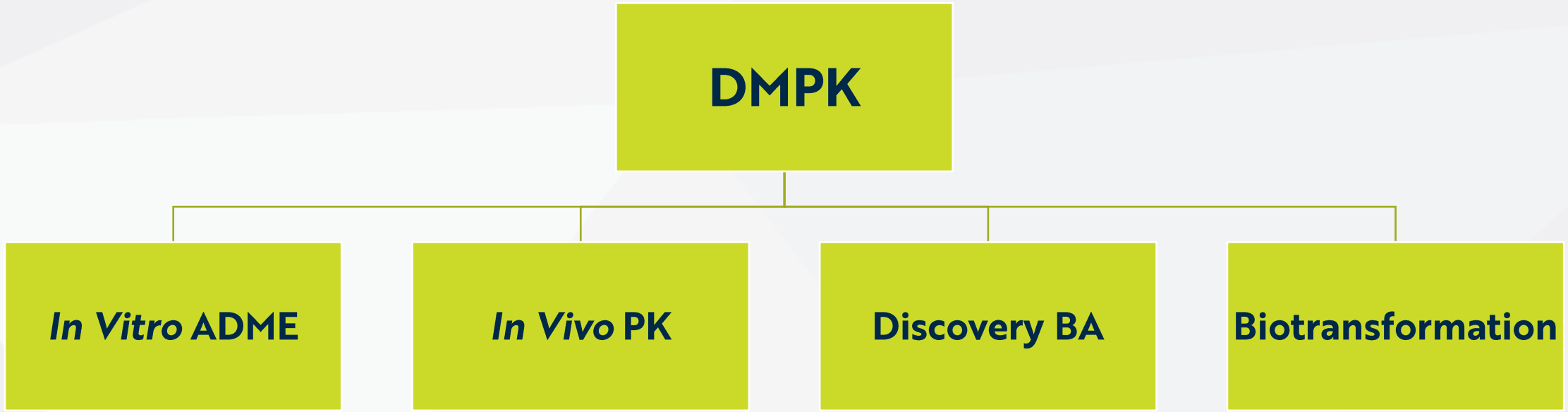
- High-quality, sensitive bioanalytical methods can successfully support PPB assessment for highly protein-bound compounds.

- Warfarin and itraconazole served as positive controls validating the PPB assay. This work provides practical guidance for aligning PPB assessment with evolving regulatory expectations under ICH M12.

- Emerging therapeutic modalities require adapted strategies and careful experimental design.

Integrated DMPK service



- Metabolic Stability
- Plasma and Tissue Protein Binding
- CYP/UGT Interaction Assessment
- Permeability
- FDA Full Panel Transporters

- Mouse and Rat PD/PK/TK
- Formulation Screening
- Vehicle Comparison Selection
- Cassette PK Screenings
- MTD
- AAALAC and OLAW Assurance

- Sample and Large Molecules Bioanalysis
- LC-MS & LC-HRMS
- ELISA & MSD
- qPCR
- PK Calculation

- Metabolite Identification
- Metabolite Profiling
- Structure Elucidation
- Metabolic Pathway Clarification
- MIST Assessment

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Five state-of-the-art labs

Four Continents. One Partner.

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