Apr 01, 2025

Plasma Protein Binding Equilibrium Dialysis for Human, Rat and Mouse Plasma

DOI
https://dx.doi.org/10.17504/protocols.io.dm6gp9yb5vzp/v1

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Protocol Citation: Yurii Kheylik, Nadiia Diyuk, Yaroslav Lesyk 2025. Plasma Protein Binding Equilibrium Dialysis for Human, Rat and Mouse Plasma. **protocols.io** https://dx.doi.org/10.17504/protocols.io.dm6gp9yb5vzp/v1

Protocol status: Working
We use this protocol and it's working

Created: December 18, 2024

Last Modified: April 01, 2025

Protocol Integer ID: 116461

Keywords: Plasma Protein Binding, Equilibrium dialysis, Human plasma, Mouse plasma, Rat plasma, ADMET, DMPK, Fraction unbound, PPB, plasma protein binding equilibrium dialysis for human, plasma protein binding equilibrium dialysis, drug fraction unbound in plasma, plasma protein binding, drug fraction unbound, protein binding, plasma protein, mouse plasma fraction unbound, lipoprotein partitioning, vivo doses in laboratory animal, vivo dose, estimations of therapeutic indice, drug interaction, free fatty acid concentration, protein, concentration, translating concentration

Disclaimer

Abstract

Fraction unbound (fu) is a critical parameter that needs to be measured accurately because it has significant impacts on the predictions of drug-drug interactions, estimations of therapeutic indices, and developments of PK/PD relationships. Moreover, Plasma Protein Binding (PPB) is critical when translating concentrations in in-vitro models to in-vivo doses in laboratory animals or humans. Plasma protein binding or the drug fraction unbound in plasma (fu,p) is known to be affected by protein, drug, free fatty acid concentrations, lipoprotein partitioning, temperature, pH, and the presence or absence of other.

Materials

Equipment:

- HPLC system with mass spectrometric detector API4000/API5000.
- Incubator Series II Water Jacketed CO_2 incubator.
- Multi-channel automatic pipettes: 2-125 µL, 1-30 µL, 15-1250 µL, E1-ClipTip.
- Reusable dialyzer HTD96b.
- Dialysis membrane strips (HTDialysis LLC, USA; cat. no. 1101).
- Disposable tips for Thermo automatic pipettes Scientific Cliptip.
- Polypropylene tubes in a 96-well rack, 0.75 ml.
- Silicone 96-well micromats with a round bottom, pre-perforated.

Reagents:

- 100 mM (1x) Potassium phosphate buffer (PPB), pH 7.4.
- Human, rat, or mouse plasma.
- Compounds stock solutions: prepare 20 mM stocks of reference and test compounds in DMSO. Then, dissolve DMSO stock in acetonitrile (ACN) to 100 µM, and mix by vortexing.
- Verapamil is used as a reference compound in the assay with human, rat, and mouse plasma.
- Quenching solution with internal standards. Dissolve appropriate for LC-MS/MS analysis internal standards in ACN.

Safety warnings

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Samples Preparation

6h 5m

1 Preparation of Phosphate-buffered saline (PBS).

1.1
- PBS [M] 10 millimolar (mM) (1X), pH pH 7.4 .
- To prepare 10X ([M] 100 millimolar (mM)), the buffer packet is first dissolved in 50 mL of MilliQ water.
- Filter the solution with a syringe filter, store at 4 °C no more than 4 weeks.
- Before the test, dilute the 10X solution to the working concentration of 1X (10 mM), bring the pH to pH 7.4 .
- 1M PBS store at 4 °C no more than 7 days.

2 Preparation of stock solution.

2.1 ACN stock, [M] 100 micromolar (µM) . Add 995 µL of acetonitrile and 5 µL of [M] 20 millimolar (mM) DMSO stock substance to a 1.4 mL microtube, mix by pipetting. Store no more than two weeks at 4 °C .

3 Preparation of plasma.

3.1 The plasma is slowly thawed and its pH is measured. If necessary, adjust the pH to pH 7.4 using a solution of hydrochloric acid (acid concentration should not be more than 1M).

4 Preparation of dialysis membrane.

4.1 Before the test, cut off the required amount of the membrane strip, cut it lengthwise so that after soaking, they are divided into two single strips (Fig. 1). Place the membrane in MilliQ water or PBS for 01:00:00 . Divide the soaked membrane into two single strips.

1h

Note

WARNING! Make sure only a single sheet of dialysis membrane is placed between Teflon bars.

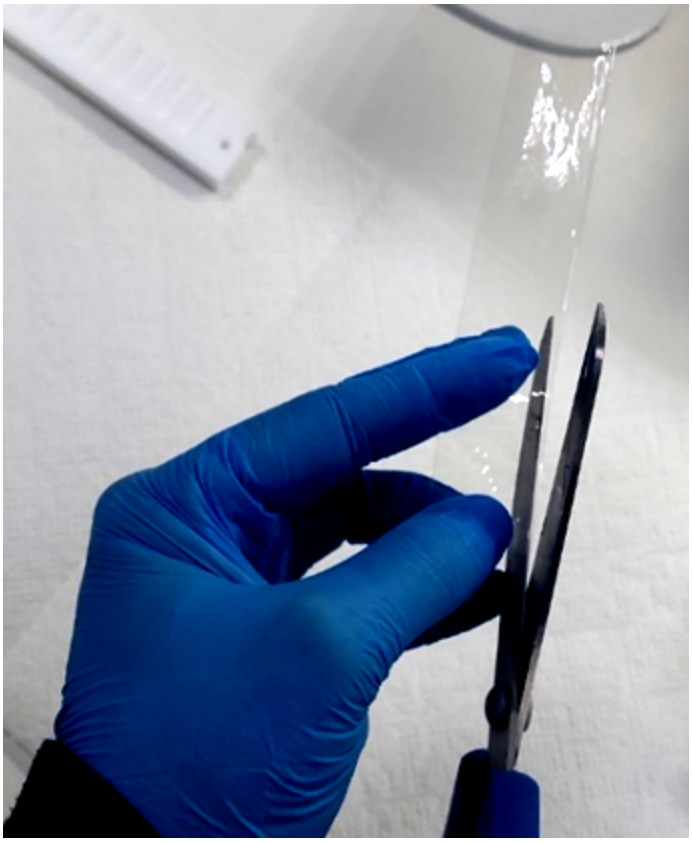

Fig. 1. Scheme of cutting the dialysis membrane before soaking.

5 Assembling the Teflon Bars with Dialysis Membrane

5.1
- Lay the first Teflon bar flat on the bench (Fig. 2), insert the two stainless steel connecting rods so they are perpendicular to the Teflon bar.
- Place the membrane on the Teflon bar.
- Ensure that the membrane is approximately 2mm below the top edge of the bar and the lower membrane edge overlaps the bottom of all wells (Fig. 2a).

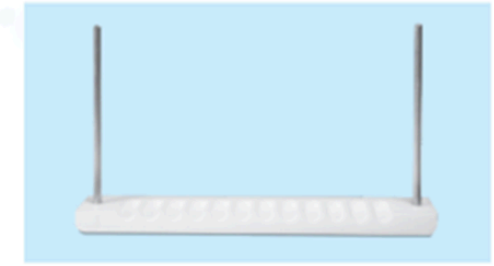

Fig.2 Teflon bar with steel connections

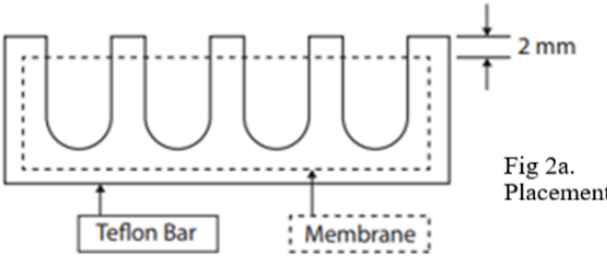

Fig 2a.
Placement of membrane on Teflon bar

6 Assembly of Dialysis Machine

6.1
- Take the machine and make sure that it is in the open position (Fig. 3).
- Insert the Teflon block into the machine and the pressure plate between the Teflon block and the pressure shaft.
- Tighten the assembled block, evenly rotating the cam levers with both hands (Fig. 3b).

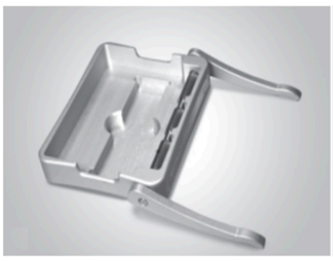

Fig.3 Open position

Fig.3a Insert the teflon block and pressure plate

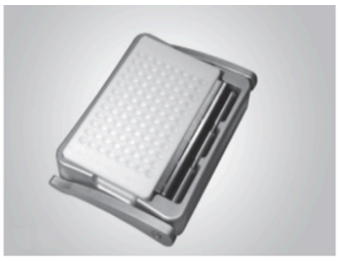

Fig.3b Tighten the assembled block

7 Carrying Out the Test

7.1
- Prepare [M] 1 micromolar (µM) model solutions of substances in plasma.

- Add 3 µL ACN substance in 297 µL of plasma (Fig. 4).
- Mix by pipetting 4-5 times, pouring down the wall of the test tube.

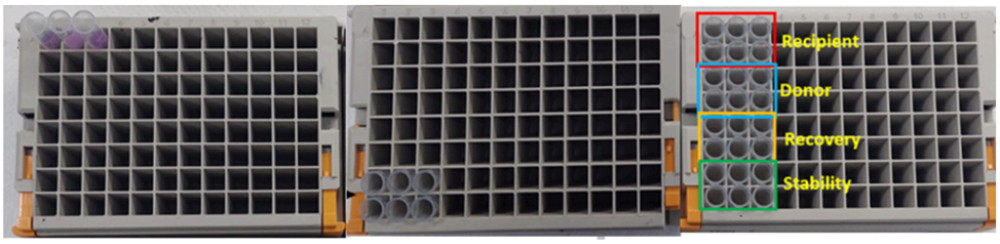

Fig. 4 Scheme of arrangement of microtubes in the plates

7.2

- Add 125 µL of solutions of substances in plasma to the donor cells of the machine (Fig. 5).
- Add 50 µL each of model solution and PBS in the 'Stability' microtubes of the final 96-well plate.
- Quench the solution in microtubes 'Stability' with 400 µL IS solution, mix on a vortex and store at 4 °C until analysis.

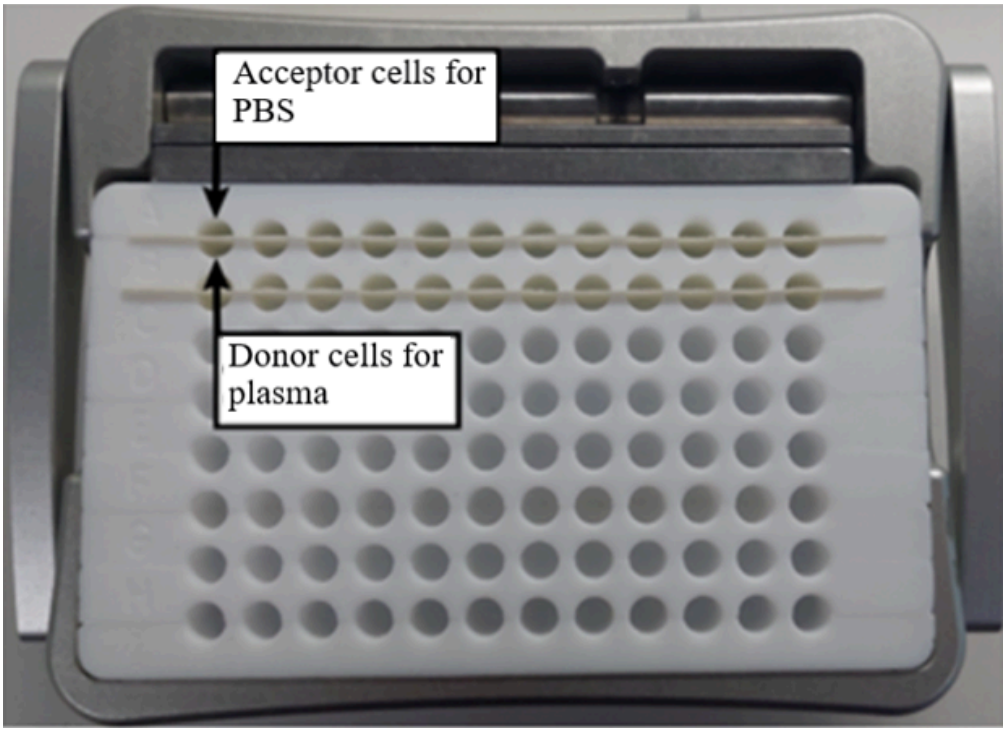

Fig 5. Schematic of a collective dialysis device

7.3 The dialysis device and model solutions in a CO_2 incubator and at 250 rpm and 37 °C for 05:00:00 .

5h

Note

WARNING! Most compounds reach equilibrium in less than 4-5 hours at 37 °C shaking at 250 rpm. For confidence, you can conduct the following kinetic experiment: compound spike into buffer and dialyze against buffer to evaluate the equilibrium time required prior to initiating any binding experiments.

8 After the Incubation

8.1
- To balance the matrix effect, add 50 µL blank plasma to the recipient tubes and vice versa, in the donor add recovery 50 µL of buffer.
- After that, transfer 50 µL of the acceptor solution to recipient tubes, 50 µL donor solution into donor tubes, and 50 µL of model solution to recovery tubes.
- All samples should be quenched immediately, by adding 400 µL of IS solution in recipient, donor, and recovery tubes.
- To check specificity and selectivity, should make a blank solution from the blank plasma.

8.2 And finally, close the test tubes with rubber caps, mix with a vortex and centrifuge for 6000 rpm, 00:05:00 . Supernatants are analyzed using the HPLC system coupled with tandem mass spectrometer.

5m

Data Analysis

9 The percentage of substances bound to plasma proteins recovery and stability are calculated according to the following equations.

$$Protein\ binding, f_b = \left(1 - \frac{area\ ratio\ in\ buffer}{area\ ratio\ in\ plasma}\right) \cdot 100\%$$

$$Recovery = \left(\frac{area\ ratio\ in\ buffer + area\ ratio\ in\ plasma}{area\ ratio\ in\ recovery\ sample}\right) \cdot 100\%$$

$$Stability = \left(\frac{area\ ratio\ in\ recovery\ sample}{area\ ratio\ in\ stability\ sample}\right) \cdot 100\%$$

10 If diluted plasma was used in the test, it is converted to 100% plasma according to the formula:

$$Undiluted\ protein\ binding = \left(1 - \frac{1/D}{((1/(1 - \frac{Protein\_binding}{100}) - 1) + 1/D}\right) \cdot 100\%$$

where D is the dilution factor .

11 The percentage of unbound fraction fu is calculated by the formula:

$$Fraction\ unbound, f_u = \left(\frac{area\ ratio\ in\ buffer}{area\ ratio\ in\ plasma}\right) \cdot 100\%$$

or

$$Fraction\ unbound, f_u = 100 - f_b$$

12 For quality control in the test should be used positive control compounds with well-known PPB characteristics (e.g., warfarin for high-binding compounds and atenolol for low-binding compounds) should be included in the study to ensure validity and reproducibility of results. Acceptance criteria for positive control compound results should be based on the literature and historical data obtained in your laboratory.