Protein Binding Assays

The Physiological Metabolism and the Distribution/Elimination Models use protein binding data as an input.

Selecting a Protein Binding Assay

Two assay procedures are provided in this chapter. Use the protein binding assay appropriate to your needs, based on the following considerations.

Ultrafiltration Protein Binding

The ultrafiltration protein binding assay (page 127) may result in high non-specific binding, but the addition of binding inhibitors as indicated in the protocol can help to alleviate this drawback.

Pros:	Cons:
• Simple procedure	• High non-specific binding to filter membrane • 4 compounds/ day

Equilibrium Dialysis Protein Binding

The equilibrium dialysis protein binding assay (page 132) is appropriate for compounds which show high non-specific binding (NSB>50%).

Pros:	Cons:
• Accuracy • Low non-specific binding to filter membrane • 8 compounds/ day	• Poor stability • Laborious, time consuming

Ultrafiltration Protein Binding Assay

Goal

To measure protein binding (PB) % of test compounds in human and rat (if needed) serum. Approximately 10 µL of 10 mM test compound in DMSO will be used to determine the % value at single concentration, 10 µM.

Set-up

Instruments

- Ultrafiltration Unit (Millipore, Ultrafree-MC): Regenerated cellulose membrane: MWCO 10K (UFC3LGC00)
- Eppendorf centrifuge
- 96-well plate
- LC/UV or LC/MS

Reagents

- Human serum: 6 donors (3 males + 3 females)
- Phosphate buffered saline (PBS)
- 5% tween 80 in PBS
- 5% benzalkonium chloride in PBS

Preparation of reagents

- pH 7.4 phosphate buffered saline (50 mM PBS): 2.622 g NaH_2PO_4 H_2O + 11.5 g Na_2HPO_4 + 10.85 g NaCl for 2 L
- Human serum: Separate from whole blood or purchase from BioWhittaker (14-402E)
- Pre-treating solutions:
  - 1st run: 5% tween 80 (TW80) in PBS: 5 ml TW 80 (Sigma Product # P-1754) + 95 ml PBS
  - 2nd run: 5% benzalkonium chloride (BAK) in PBS: 5 ml BAK (Sigma Product # B-6275) + 95 ml PBS
  - **Note**: if NSB >50% at first run (ex., for basic compounds), use 5% BAK in PBS as a pre-treatment

Suggested Reference

For details, see the paper, “Modulation of Nonspecific Binding in Ultrafiltration Protein Binding Studies,” Kyoung-Jin Lee, Rachel Mower, Tom Hollenbeck, Jesus Castelo, Nikole Johnson, Perry Gordon, Patrick J. Sinko, Kevin Holme, and Young-Hee Lee, Pharm. Res. 20: 1015-1021 (2003).

- Internal standard (IS) solution: 1µM IS in 1:1 mixture of water: MeOH (1 L glass bottle, stored at 4°C)
- Standard dilution solution: 1% MeOH in PBS

- Washing solution: 1:1 mixture of water: MeOH (2 L glass bottle)

WORKING SOLUTIONS

- Stock solution: 10 mM in DMSO at -20°C
- Prepare working solutions using 10 mM stock solution:
    - 10µM working solution in serum (0.1% DMSO): 1.5µL stock (10mM) + 1500 µL human serum
    - 10µM working solution in PBS (0.1% DMSO): 1.5µL stock (10mM) + 1500 µL PBS
    - 10µM standard solution in 1% MeOH in PBS (0.1% DMSO): 1.5 µL stock (10 mM) + 1500 µL 1% MeOH in PBS
- Calibration for PBS donor and filtrate, and serum filtrate: Prepare 10, 5, 2, 1, 0.5, 0.2, 0.1, 0 µM calibrators using 10 µM standard solution with 1% MeOH in PBS

Protocol summary

- Human and rat serum: 400 µL for single unit
- Single concentration: 10 µM in serum (w 0.1% DMSO)/ n=3
- NSB measurement: 10 µM in PBS (w 0.1% DMSO)
- PB calculation: Use NSB and serum filtrate measured (use 10 µM nominal value for serum donor conc)
- Individual filter tube format
- Pre-treatment of filter (NSB)
- Sample handling and bioanalysis: 96 well format
- Amount required: Approximately 10 µL of 10 mM stock solution
- Serum required: Approximately 2 ml for each compound (3 ml if negative control tested)
- Throughput: 4 compounds / 1 FTE/ day

Experimental Procedures

1. Array and label each reservoir tube (Table 49).

2. Connect upper cup to each disposable tube. Add 25 µL pre-treating solution to the upper cup and wet for 5 min. Centrifuge at 3000 g for 10 min. Add 200 µL PBS to each upper cup and equilibrate 30 min. Remove upper solution by gentle tapping.

3. Reconnect each cup to respective labeled reservoir tube.

4. Transfer 400 µL PBS dosing solution (10 µM, n=3) and serum dosing solution (10 µM, n=3) to upper cup, and equilibrate approximately 1 hr at room temperature.

5. After 1 hr equilibration, take 50 µL PBS sample from the top cup and transfer it to RESPECTIVE 96-well analytical plate (Table 50).

6. Centrifuge serum samples at 3,000 g/ room temp for 18 min or until filtrate collects about 60 µL (Eppendorf Centrifuge).

7. Centrifuge PBS samples at 3,000 g/ room temp for 3 min or until filtrate collects about 60 µL (Eppendorf Centrifuge). If 5% BAK is used for pre-treatment, centrifuge 2.5 min or until filtrate collects about 60 µL.

8. Transfer 50 µL of calibration solutions (0-10µM) to analytical plate.

9. After centrifuge, discard the top filter cups, and take 50 µL of each filtrate and transfer it to analytical plate.

10. Add 50 µL IS solution to all calibration/ filtrate/ donor wells to analytical plate.

11. Add 100 µL washing solution to designated wells of analytical plate.

12. Seal the analytical plate with adhesive sealing film and store samples with label at -80°C for LC-UV or LC-MS analysis.

13. Analyze 20 µL aliquots of the individual permeability samples and the standards using a suitable analytical instrument.

Table 49. Labeling Reservoir Tube

P1-1	H1-1		P2-1	H2-1		P3-1	H3-1		P4-1	H4-1	
P1-2	H1-2		P2-2	H2-2		P3-2	H3-2		P4-2	H4-2	
P1-3	H1-3		P2-3	H2-3		P3-3	H3-3		P4-3	H4-3	
Comp 1			Comp 2			Comp 3			Comp 4		
PBS	10μM Human		PBS	10μM Human		PBS	10μM Human		PBS	10μM Human	

Table 50. Final Analytical Plate for Compound 1

0	washing	washing										
0.1μM	P1-1	H1-1										Filtrate
0.2μM	P1-2	H1-2										
0.5μM	P1-3	H1-3										
1μM	washing											
2μM	P1-1											Donor
5μM	P1-2											
10μM	P1-3											
Calibra-tion	PBS	Human										

PROTEIN BINDING CALCULATION

1. Starting drug conc at serum (C_T): nominal drug conc at 10 μM

2. NSB = (C_{BT}-C_{Bf})/C_{BT}, where NSB: nonspecific binding to membrane filter at pH 7.4 PBS, C_{BT}: drug conc in the PBS before centrifuge and C_{Bf} : drug conc in the PBS filtrate after centrifuge

3. $f_u = C_f/(1\text{-NSB})/C_T$, where f_u: free fraction, C_f: drug conc in the serum filtrate after centrifuge, and C_T: nominal drug conc, 10 μM

4. Protein binding (PB) % = 100*(1- f_u)

Equilibrium Dialysis Protein Binding Assay

Goal

To measure protein binding (PB) % of test compounds, especially for the compounds which show high non-specific binding (NSB>50%) at UF method under pre-treatment of either tween 80 or BAK. Approximately 10 µL of 10 mM test compound in DMSO will be used to determine the PB value at a single concentration, 10 µM.

Set-up

Reagents and instruments

- Amika dialyzer: MB 74-2301, equilibrium dialyzer-96 plate, MWCO 10k/ MB 74-2302 plate rotator
- Human Serum: Biowhitaker 14-402E, Rat serum: Harlan BT 4501
- See UF method

Dosing solutions

Dosing Solutions (C_{T1}) in human serum, rat serum and PBS in 96 deep well (Table 51):

- 10 µM: 2 µL of 10 mM stock + 1 ml of human serum → 20 µM/ Dilute 20 µM to 10 µM with human serum (500 µL of 20 µM + 500 µL of human serum, prepare 2 tubes)
- 10 µM: 2 µL of 10 mM stock + 1 ml of rat serum → 20 µM/ Dilute 20 µM to 10 µM with rat Serum (500 µL of 20 µM + 500 µL of rat serum, prepare 2 tubes)
- 10 µM: 2 µL of 10 mM stock + 1 ml of PBS → 20 µM/ Dilute 20 µM to 10 µM with PBS (500 µL of 20 µM + 500 µL of PBS, prepare 2 tubes)

Table 51. Dosing Solution Plate Format and Preparation

	Human Serum	Human serum	Rat Serum	Rat Serum	PBS	PBS	7	8	9	10	11	12
Comp 1	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 2	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 3	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 4	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 5	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 6	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 7	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						
Comp 8	10 µM	10 µM	10 µM	10 µM	10 µM	10 µM						

CALIBRATION PLATE FORMAT

Calibration plate format and preparation in 96 shallow well (Table 52):

- Prepare 10, 5, 2, 1, 0.5, 0.2, 0.1, 0 µM calibrators using 10 µM dosing solution in human serum and blank human serum.

Table 52. Calibration Plate Format and Preparation

	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	9	10	11	12
		20 µL of 1 µM	20 µL of 2 µM	20 µL of 5 µM	20 µL of 10 µM	40 µL of 10 µM	100 µL of 10 µM	300 µL of 10 µM in HS				Source
Comp 1	180 µL	180 µL	180 µL	180 µL	180 µL	160 µL	100 µL	0 µL				Blk human serum
Comp 2												
Comp 3												
Comp 4												
Comp 5												
Comp 6												
Comp 7												
Comp 8												

Protocol summary

- Miniaturized and HTS protein binding assay

- 96 well format Equilibrium Dialyzer
- Human (and/or rat) serum: 200 µL for single unit
- Single concentration: 10 µM in serum (w 0.1% DMSO)/ n=4
- NSB measurement: 10 µM in PBS (w 0.1% DMSO)
- Equilibrium: 4 hrs at 37°C/ 25 rpm
- Use one calibration for free drug in receiver (by adding serum)/total drug in donor (by adding buffer) side concentrations
- Amount required: Approximately 10 µL of 10 mM stock solution
- Serum required: Approximately 4 ml for each compound
- Throughput: 8 compounds/1 FTE/day

Experimental procedures

1. Transfer 200 µL of human serum, rat serum, and PBS dosing solutions to upper 96-well dialysis plate (Table 53), and cover with silicon mat. Use 8-multi channel pipette for transferring.
2. Transfer 200 µL of drug free PBS to bottom 96-well dialysis plate, and cover with silicon mat. Use 8-multi channel pipette for transferring.
3. Incubate the dialysis plate for 4 hrs at 37°C incubator with rotation at 25 rpm. Place a beaker of water in the incubator.

Table 53. Donor Plate Map (Serum Side): 200 µL of Dosing Solutions

	Human Serum				Rat Serum				PBS			
	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12
Comp 1	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 2	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 3	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 4	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 5	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 6	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 7	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 8	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln

Sample handling and extraction procedures

1. 3 different samples and 1 calibration will be transferred and extracted: starting dosing solution (C_{T1}), donor drug concentration at equilibrium (C_{T2}), and receiver drug concentration at equilibrium (C_{f2}).

2. Transfer 50 µL of sera and buffer dosing solutions (C_{T1}) to dosing solution sample plate (Table 54) from dosing solution plate (Table 51).

3. Transfer 50 µL of calibrator solutions to a 96 well plate for extraction (Table 55).

4. Add 50 µL of PBS or human serum as indicated in Table 54 and Table 55.

5. Add 200 µL of Water:MeOH mixture (1:1) to columns 9 to 12 in standard plate (Table 55) to compensate weight difference of standard plate for centrifugation.

6. After incubation (4 hr), transfer 50 µL of receiver samples (Buffer side) to C_{f2} Receiver sample plate (Buffer side, Table 56)/ keep the dialysis plate at 37°C warm pad while sample handling. Add 50 µL of human or rat serum as indicated.

7. Transfer 50 µL from the donor samples (Serum side) to C_{T2} Donor sample plate (Serum side, Table 57)/ keep the dialysis plate at 37°C warm pad while sample handling. Add 50 µL of PBS or human serum as indicated.

8. Add 100 µL of Water:MeCN (1:1, 0.1% $ZnSO_4$, 1 µg/ml of IS) to plates of Table 54, Table 55, Table 56, and Table 57, and place them on a rotator for 5 min.

9. Store the four plates in -80°C freezer for 30 min.

10. Thaw the four plates at room temperature for 10 min.

11. Centrifuge at 2000 g and 4°C for 1 hr. Keep the centrifuged plates on ice during sample transferring.

Table 54. Extraction of Starting Dosing Solutions (total 200 µL): 50 µL of dosing sol'n + 50 µL of PBS or human serum + 100 µL of Water:MeCN (1:1, 0.1% $ZnSO_4$, 1µg/ml of IS)

	Human Serum				Rat Serum				PBS			
	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12
Comp 1	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 2	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 3	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 4	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 5	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 6	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 7	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
Comp 8	HS Dsln	HS Dsln	HS Dsln	HS Dsln	RS Dsln	RS Dsln	RS Dsln	RS Dsln	PBS Dsln	PBS Dsln	PBS Dsln	PBS Dsln
	50 µL PBS								50 µL Human Serum			
	100 µL Extraction solution											

Table 55. Extraction of Calibration Solutions (total 200 ul):
50 µL of calibration sol'n + 50 µL of PBS + 100 µL of Water:MeCN (1:1, 0.1% $ZnSO_4$, 1 µg/ml of IS)

	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Water:MeOH (1:1)			
Comp 1	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 2	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 3	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 4	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 5	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 6	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 7	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
Comp 8	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	200 µL	200 µL	200 µL	200 µL
	50 µL PBS											
	100 µL Extraction solution											

Table 56. Extraction of Receiver Samples from Buffer Side (total 200µL) :
50 µL of rcvr sample + 50 µL of serum + 100 µL of Water:MeCN (1:1, 0.1% $ZnSO_4$, 1 µg/ml of IS)

	PBS				PBS				PBS			
	Col 12	Col 11	Col 10	Col 9	Col 8	Col 7	Col 6	Col 5	Col 4	Col 3	Col 2	Col 1
Comp 1	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 2	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 3	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 4	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 5	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 6	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 7	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Comp 8	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
	50 µL Human serum				50 µL of Rat serum				50 µL of Human serum			
	100 µL Extraction solution											

Table 57. Extraction of Donor Samples from Serum Side (total 200µL) : 50 µL of dnr sample + 50 µL of serum or PBS + 100 µL of Water:MeCN (1:1, 0.1% $ZnSO_4$, 1 µg/ml of IS)

	Human Serum				Rat Serum				PBS			
	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12
Comp 1	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 2	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 3	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 4	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 5	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 6	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 7	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Comp 8	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
	50 µL PBS				50 µL PBS				50 µL Human serum			
	100 µL Extraction solution											

Preparation

Preparation of bioanalytical plate for each compound (Table 58). Keep the centrifuged plates on ice during sample transferring.

1. Transfer 100 µL of supernatant of calibration solutions to row 1 and add 100 µL of “wash” solution to R1C9.
2. Transfer 100 µL of “wash” solution to row 2 and row 6.
3. Transfer 100 µL of supernatant of receiver samples to row 3.
4. Transfer 100 µL of supernatant of donor samples to row 4.
5. Transfer 100 µL of supernatant of dosing solution samples to row 5.
6. Seal the analytical plate with adhesive sealing film and store samples with label at -80°C for LC-UV or LC-MS analysis.
7. Analyze 20 µL aliquots of the individual permeability samples and the standards using a suitable analytical instrument.

8. Injection sequence: Inject R1C1 to R1C9/ Inject R2C1 to R6C1/ Inject R2C2 to R6C2…

Table 58. Bioanalytical Map: Transfer 100 µL of Supernatant/ Wash (100 µL of Water: MeOH

	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12
Row 1	Blank	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	wash			
Row 2	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash
Row 3	HS rcvr	HS rcvr	HS rcvr	HS rcvr	RS rcvr	RS rcvr	RS rcvr	RS rcvr	PBS rcvr	PBS rcvr	PBS rcvr	PBS rcvr
Row 4	HS dnr	HS dnr	HS dnr	HS dnr	RS dnr	RS dnr	RS dnr	RS dnr	PBS dnr	PBS dnr	PBS dnr	PBS dnr
Row 5	HS dsln	HS dsln	HS dsln	HS dsln	RS dsln	RS dsln	RS dsln	RS dsln	PBS dsln	PBS dsln	PBS dsln	PBS dsln
Row 6	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash	wash
Row 7												
Row 8												

Calculation

- Measured concentrations:
  - Starting dosing drug concentrations (C_{T1})
  - Donor drug concentration at equilibrium (C_{T2})
  - Receiver drug concentration at equilibrium (C_{f2})
- Parameters:
  - NSB = (C_{T1}- C_{T2} - C_{f2})/ $C_{T1,}$ where NSB: nonspecific binding at PBS buffer
  - Free fraction = C_{f2}/C_{T2}
  - PB % = 100*(C_{T2} - C_{f2})/ C_{T2}

Comparison of UF and ED Data Used in pkEXPRESS

A comparison between the UF and ED methods is shown below for a set of 18 compounds that have high non-specific binding characteristics. If the non-specific binding for a compound is >50% using UF with both tween 80 and BAK pre-treatments, it is recommended that the ED method be used for determination of the protein binding for the compound.

Table 59. Comparison of PB (%) and fraction unbound between UF and ED (18 compounds)

	PB (%): Mean		fu: Mean	
	UF	ED	UF	ED
14C-Antipyrine	11.55	13.67	0.885	0.863
14C-Caffeine	26.12	20.05	0.739	0.800
14C-Fluorocytosine	-2.39	-4.26	1.024	1.043
14C-Theophylline	41.02	46.54	0.590	0.535
14C-Ibuprofen	99.45	93.19	0.005	0.068
3H-Ketoprofen	98.98	99.27	0.010	0.007
3H-Propranolol	63.90	71.14	0.361	0.289
3H-Etoposide	89.44	92.44	0.106	0.076
3H-Hydrocortisone	63.93	64.67	0.361	0.353
3H-Vinblastine	85.66	82.89	0.143	0.171
Diltiazem	79.23	56.53	0.208	0.435
Chlorpheniramine	69.01	53.00	0.310	0.470
Promethazine	98.26	92.88	0.017	0.071
Clonidine	23.31	15.67	0.767	0.843
Lorazepam	85.52	79.32	0.145	0.207
Verapamil	92.20	77.24	0.078	0.228
Imipramine	82.56	80.21	0.174	0.198
Diclofenac	99.56	99.89	0.004	0.001

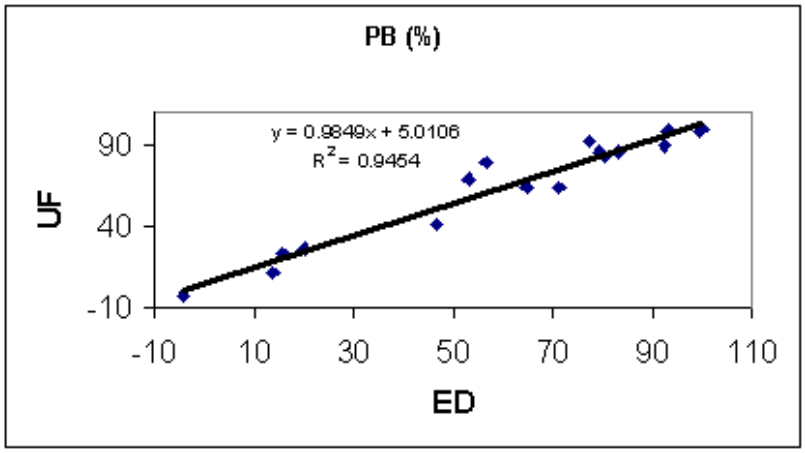

Figure 41. Correlation of PB (%) and fraction unbound between UF and ED (18 compounds)