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Bovine Serum Albumin in Caco-2 Permeability Testing and Effect on P_{app} Values Determined with Highly Protein Bound Compounds

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Introduction

Determination of *in vitro* apparent permeability (P_{app}) in Caco-2 cell monolayers is a routine aspect of many drug discovery programs. For many compounds, however, non-specific binding to the cells and/or filter plate assembly, possibly combined with limited solubility, results in substantially reduced compound recovery from the test system (<<50%), and/or the lack of quantifiable concentrations in the receiver sample. To mitigate non-specific binding and improve solubility, the assay buffer is frequently supplemented with protein (e.g., bovine or human serum albumin, fetal bovine serum, calf serum, etc.), however, detailed protocols vary (protein added to both compartments, to the receiver compartment only, or to the basolateral compartment only) and limited systematic data is available comparing the impact of those varying approaches on P_{app}. The present study determined the impact of adding 2% BSA to the assay buffer on P_{app} values for a set of compounds covering a wide range of plasma protein binding.

Methods

Bidirectional permeability in Caco-2 cell monolayers was determined for a set of 16 compounds covering a range of reported plasma protein binding and human intestinal absorption. Cell monolayers were set up in Falcon® 24-Multiwell 1 µm filter inserts and grown to confluence for 21 to 25 days. Monolayer integrity was confirmed by TEER and lucifer yellow A-B flux testing.

The assays were performed using four conditions: (1) protein-free assay buffer (HBSS with 10 mM HEPES, pH 7.4), (2) buffer supplemented with 2% bovine serum albumin in both compartments, (3) buffer with 2% BSA in the receiver compartment only, (4) buffer with 2% BSA in the basolateral compartment only. Compounds were assayed at 10 µM and incubated for 90 minutes at 37ºC. Donor (0 and 90 min) and receiver samples (90 minutes) were quantified by LC/MS/MS or liquid scintillation counting, and P_{app} values and efflux ratios were calculated and compared between conditions.

In addition, compound binding to human plasma and to 2% BSA in assay buffer was determined using the RED Device (Thermo Fisher Scientific) at 500 rpm for three hours at 37ºC.

Results

The degree of protein binding in assay buffer with 2% BSA was somewhat lower then that in human plasma (**Figure 3**).

The impact of 2% BSA in the Caco-2 assay buffer on A-to-B and B-to-A P_{app} varied widely for different compounds and conditions tested (**Table 1**).

P_{app} values for certain highly protein bound (>99% plasma protein binding) compounds (e.g., naproxen, warfarin) known to exhibit good apparent permeability in Caco-2 cells decreased substantially (>10-fold) in presence of BSA in both donor and receiver compartments, while effects on P_{app} values for compounds with moderate (verapamil, propranolol) or low (metoprolol, labetalol) protein binding were less pronounced (2-fold or less) (**Table 1**, **Figure 2**). As a result of the drastic change in P_{app} value, highly permeable compounds with high protein binding may be misclassified as low permeability compounds leading to underestimated intestinal absorption (**Figure 1**).

Altered P_{app} values in turn resulted in changes in efflux ratios which were most pronounced for highly protein bound efflux substrates (saquinavir, furosemide, sulfasalazine) (**Table 2**). In particular, presence of BSA in the basolateral compartment led to a decrease in efflux ratios, which may mask efflux, while presence of BSA in the receiver compartment only did not appear to affect the identification of efflux substrates.

Conclusions

Addition of protein to the assay buffer is a useful and frequently employed approach to mitigate non-specific binding, improve solubility, and/or better mimic physiological conditions in Caco-2 permeability screening assays. However, caution should be exercised when interpreting the resulting data as P_{app} values may be substantially altered depending on compound properties and the particular assay conditions used.

In particular when protein is added to the donor compartment (a useful approach to improve compound recovery), compounds exhibiting high plasma protein binding (99% or greater) may show substantially decreased (>10-fold) P_{app} values resulting in misclassification of high permeability compounds as low or moderate permeability.

Efflux ratios for highly protein bound efflux substrates were also altered. In particular, the presence of BSA in the basolateral compartment led to a decrease in efflux ratios.

References

1. Li C, et al., J. Biomol. Screen. 12:1084 (2007).
2. Usanski. HH, et al., J. Phamacol. Exp. Ther. 314:391 (2005).
3. DrugBank 3.0, http://www.drugbank.ca/, Knox C, et al., Nucleic Acids Res 39:D1035-4 (2011).

Table 1. Protein Binding, Absorption, and Effect of 2% BSA on the Apparent Permeability of the Test Compounds

	Compound	Protein Binding: Human Plasma	Protein Binding: 2% BSA in Buffer	Human[1, 2,3] Intestinal Absorption	A-to-B P_{app} [10^{-6} cm/s]: Assay Buffer without BSA	A-to-B P_{app} [10^{-6} cm/s]: 2% BSA in AP and BL	A-to-B P_{app} [10^{-6} cm/s]: 2% BSA in receiver (BL)	B-to-A P_{app} [10^{-6} cm/s]: Assay Buffer without BSA	B-to-A P_{app} [10^{-6} cm/s]: 2% BSA in AP and BL	B-to-A P_{app} [10^{-6} cm/s]: 2% BSA in receiver (AP)	B-to-A P_{app} [10^{-6} cm/s]: 2% BSA in donor (BL)
Complete Absorption Moderate to High Permeability	Naproxen	100%	96%	100%	24	1.0	43	19	0.90	30	0.08
	Warfarin	99%	94%	98%	20	1.5	25	17	1.6	24	0.87
	Verapamil	95%	65%	100%	26	12	21	11	16	27	5.6
	Propranolol	89%	61%	90%	25	12	29	14	13	30	5.7
	Quinidine	92%	64%	80%	9.4	8.8	15	16	17	33	7.4
	Carbamazepine	81%	54%	100%	28	19	30	24	19	33	12
	Metoprolol	45%	38%	95%	16	18	16	18	18	20	20
	Labetalol	70%	50%	90%	5.7	6.5	6.6	9.1	8.0	15	8.4
Incomplete Absorption Low Permeability	Terfenadine	100%	83%	70%	1.6	2.0	3.3	1.9	1.5	3.7	0.53
	Digoxin	70%	60%	70%	1.2	1.4	0.82	14	7.8	9.6	10
	Saquinavir	100%	96%	12%	0.71	0.23	1.2	8.0	2.8	22	1.2
	Fexofenadine	88%	78%	33%	0.13	0.10	0.11	1.6	0.69	2.4	0.83
	Ranitidine	65%	59%	50%	0.32	0.46	0.43	1.2	1.3	2.2	2.2
	Mannitol	0%	0%	18%	0.26	0.35	0.44	0.33	0.25	0.27	0.31
	Furosemide	98%	87%	55%	0.11	<0.04	0.14	6.5	0.77	9.2	0.80
	Sulfasalazine	100%	94%	13%	0.06	0.04	0.08	7.6	0.45	10	0.57

Table 2. Effect of 2% BSA on the Efflux Ratios of the Test Compounds

Compound	Efflux Ratio: Assay Buffer without BSA	Efflux Ratio: 2% BSA in AP and BL	Efflux Ratio: 2% BSA in receiver (AP or BL)	Efflux Ratio: 2% BSA in BL
Naproxen	0.80	0.93	0.70	0.001
Warfarin	0.89	1.1	1.0	0.025
Verapamil	0.43	1.3	1.3	0.17
Propranolol	0.56	1.1	1.1	0.17
Quinidine	1.7	2.2	2.2	0.62
Carbamazepine	0.83	0.98	1.1	0.35
Metoprolol	1.1	0.99	1.2	1.2
Labetalol	1.6	1.2	2.3	1.5
Terfenadine	1.2	0.73	1.1	0.08
Mannitol	1.3	0.72	0.61	0.86
Digoxin	12	5.8	12	12
Saquinavir	11	12	18	1.8
Fexofenadine	12	6.8	21	6.0
Ranitidine	3.7	2.8	5.1	5.2
Furosemide	62	19	66	5.4
Sulfasalazine	119	11	126	6.1

Figure 1. Effect of 2% BSA on the Relationship between Apparent Permeability and Human Absorption

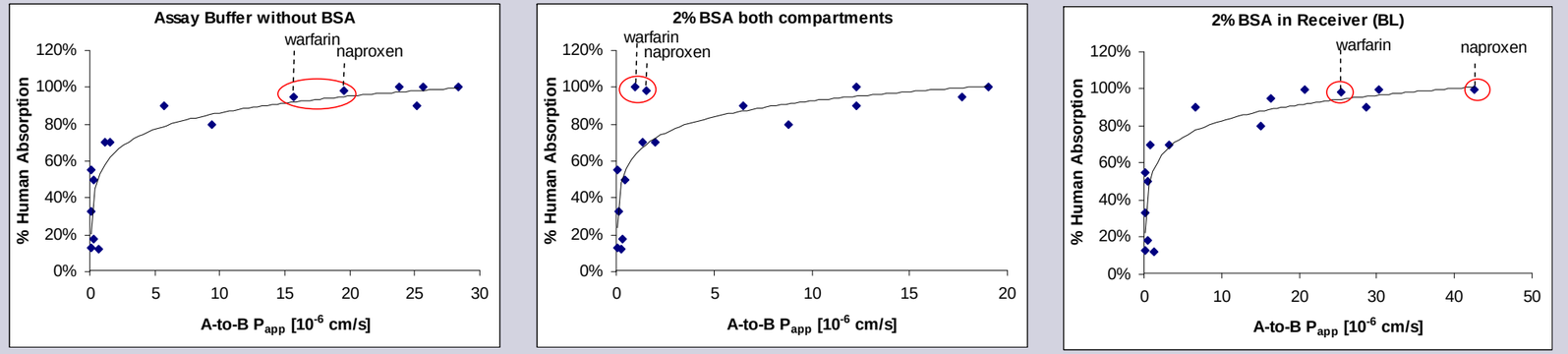


Figure 2. Effect of 2% BSA on A-to-B P_{app} for Moderate/High P_{app} Compounds

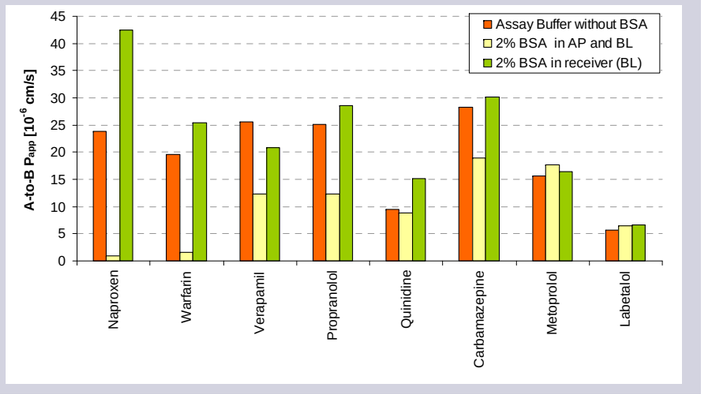
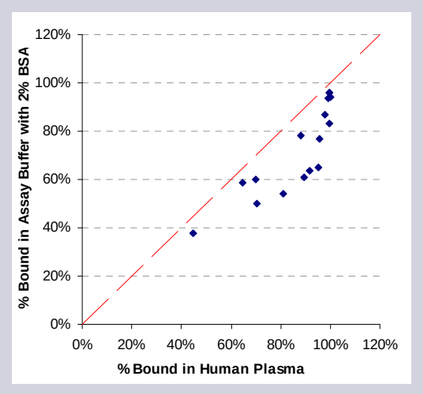


Figure 3. Protein Binding in Human Plasma and Assay Buffer with 2% BSA



All data are means of duplicate determinations

CLS-DL-GT-PST001