

Technical Application Bulletin	
TAB Number:	TAB Biol.001v1
Product:	B-CLEAR [®]
Subject:	B-CLEAR [®] : Modifications for Protein in Dose Solution
Reference:	B-CLEAR [®] Operator's Manual
Date:	03 January, 2008
Purpose:	To describe the use of protein in B-CLEAR [®] uptake/efflux studies

Introduction

Candidate compounds are often extensively protein-bound, which can be important in determining fundamental pharmacokinetic characteristics such as clearance and distribution volume. The solubility and stability of candidate compounds can be improved when they bind to soluble proteins. In addition, compounds with high protein binding often exhibit increased potential for non-specific binding to components of *in vitro* assays. High levels of non-specific binding can confound the interpretation of data from *in vitro* assays. Use of protein in these assays often can decrease the extent of non-specific binding and therefore provide more relevant data.

Use of protein in B-CLEAR[®] can allow compounds with a wider range of physicochemical characteristics to be evaluated. The B-CLEAR[®] system was used to evaluate the effects of physiological concentrations of protein (bovine serum albumin, BSA) on the hepatic accumulation, biliary excretion index (BEI), and intrinsic clearance (Cl_{biliary}) of compounds with a range of protein binding.

Method Overview

Similar to the standard B-CLEAR[®] protocol, the modified B-CLEAR[®] methodology for using BSA requires running parallel experiments with the Test Compound in BSA-containing media to determine the amount of Test Compound excreted into the bile. In one set of experiments, hepatocytes are incubated in Plus (calcium-containing) buffer; the buffer is then removed and the hepatocytes incubated with dose solution containing Test Compound and BSA. Subsequently, hepatocytes are washed, lysed, and the lysate is analyzed for Test Compound. Quantification of this lysate gives the total amount of Test Compound in both the hepatocytes and bile (the total amount of Test Compound taken up AND excreted). In a parallel experiment, hepatocytes are incubated in Minus (calcium-free) buffer prior to incubation with the dose solution. Incubation in Minus buffer opens the tight junctions and prevents the Test Compound from accumulating in the bile pockets. The hepatocytes are washed, lysed, and the lysate is analyzed for Test Compound. Quantification of this lysate provides the total amount of Test Compound in the hepatocytes. The amount of Test Compound excreted into the bile pockets is determined by

subtracting the amount of Test Compound in the Plus buffer incubation lysate (hepatocytes + bile pockets) from the amount of Test Compound in the Minus buffer lysate (hepatocytes). For a detailed, step-by-step procedure on performing the B-CLEAR[®] assay using BSA, see “Application Methodology.”

Results

The effect of BSA on the accumulation, BEI, intrinsic $Cl_{biliary}$, and unbound intrinsic $Cl_{biliary}$ of taurocholate and digoxin was determined in B-CLEAR[®]-RT (rat); the results are presented in Tables 1 and 2. Compounds were selected based on their degree of binding to BSA (taurocholate = 85.8 ± 2.01 % bound, digoxin = 14.8 ± 1.97 % bound). All compounds were evaluated at a dose concentration of 1 and 10 μ M in the absence and presence of BSA.

Table 1. Accumulation, BEI, Intrinsic $Cl_{biliary}$ and Unbound Intrinsic $Cl_{biliary}$ of taurocholate in B-CLEAR[®]-RT.

Taurocholate		Accumulation (pmol/mg protein)		BEI (%)	Intrinsic $Cl_{biliary}$ (ml/min/kg)	Unbound Intrinsic $Cl_{biliary}$ (ml/min/kg)
		cells + bile	Cells			
- BSA	1 μ M	46.5 \pm 16.5	7.24 \pm 3.06	82.7 \pm 11.6	32.5 \pm 16.5	32.5 \pm 16.5
	10 μ M	329 \pm 75.2	41.5 \pm 20.7	87.4 \pm 6.72	22.9 \pm 5.40	22.9 \pm 5.40
+ BSA	1 μ M	8.33 \pm 2.43	0.747 \pm 0.204	90.4 \pm 3.80	5.73 \pm 2.06	40.4 \pm 14.5
	10 μ M	71.1 \pm 13.9	8.52 \pm 4.01	88.1 \pm 4.77	4.98 \pm 1.03	35.1 \pm 7.26

Hepatic accumulation of taurocholate increased in response to the incubation concentration both in the absence and presence of BSA. The BEI (the percent of compound taken up by the hepatocytes that is excreted into the bile) for taurocholate was unaffected by adding BSA to the system. However, the accumulation and intrinsic $Cl_{biliary}$ decreased approximately 80%, consistent with the extent of taurocholate binding to BSA (85.8 ± 2.01 %). As expected, the addition of BSA resulted in a substantial decrease in the intrinsic $Cl_{biliary}$; however, the unbound intrinsic $Cl_{biliary}$ was not altered, consistent with the conventional assumption that only unbound compound is available for uptake and clearance in hepatocytes.

Table 2. Accumulation, BEI, Intrinsic $Cl_{biliary}$ and Unbound Intrinsic $Cl_{biliary}$ of digoxin in B-CLEAR[®]-RT.

Digoxin		Accumulation (pmol/mg protein)		BEI (%)	Intrinsic $Cl_{biliary}$ (ml/min/kg)	Unbound Intrinsic $Cl_{biliary}$ (ml/min/kg)
		cells + bile	Cells			
- BSA	1 μ M	27.4 \pm 8.34	14.3 \pm 5.16	50.1 \pm 6.09	10.4 \pm 2.74	10.4 \pm 2.74
	10 μ M	203 \pm 50.9	107 \pm 39.2	48.5 \pm 8.49	7.68 \pm 1.30	7.68 \pm 1.30
+ BSA	1 μ M	19.8 \pm 5.94	9.91 \pm 4.30	51.5 \pm 9.61	7.88 \pm 1.56	9.25 \pm 1.83
	10 μ M	145 \pm 65.5	66.2 \pm 39.7	57.0 \pm 10.0	6.31 \pm 2.14	7.40 \pm 2.51

Digoxin accumulation increased in proportion to the incubation concentration both in the absence and presence of BSA. The BEI for digoxin was unaffected by adding BSA to the

system, consistent with results for taurocholate. The unbound intrinsic Cl_{biliary} determined in the absence and presence of BSA was identical. This further supports the conventional assumption that the unbound compound is available for uptake and clearance in hepatocytes.

Conclusions

Adding BSA to the incubation media may provide some benefit in analyzing compounds with high non-specific binding and/or solubility issues that could not be studied under standard B-CLEAR[®] assay conditions. The extent of protein binding directly affects the hepatic accumulation of a test compound. The unbound concentration is the driving force for uptake into the hepatocyte. If the extent of protein binding is known (or can be determined), the unbound intrinsic Cl_{biliary} can be calculated which will allow comparison of the hepatobiliary disposition parameters determined in the absence and presence of protein.

The effect of BSA in the B-CLEAR[®] system on the unbound intrinsic Cl_{biliary} of compounds with protein binding values greater than 95% is under evaluation.