

PROTEIN BINDING BLOOD PARTITIONING

The extent of a drug's binding to plasma or serum proteins is a determinant of drug distribution and elimination. The unbound drug concentration is more closely related to the activity of a drug than total plasma/serum concentration because only unbound drug can pass through most cell membranes. Protein binding is also a factor in the calculation of *in vivo* hepatic clearance based on *in vitro* intrinsic clearance.

Knowledge of the partitioning behavior of a therapeutic compound into red blood cells is important to the interpretation and understanding of the compound's pharmacokinetic (PK) profile. A high partitioning ratio may also indicate accumulation of the compound in red blood cells, and potential hematotoxicity. Cerep offers specific high-throughput assays to measure these parameters to aid in the selection of drug candidates at an early stage in the drug discovery process.

WHAT IS PLASMA PROTEIN BINDING?

The binding of therapeutic compounds to plasma or serum proteins is a reversible, saturable process which can be an important factor in assessing the pharmacokinetic and pharmacodynamic profile of a drug. Protein binding, which occurs through a mix of electrostatic, van der Waals, and hydrogen bonding interactions, is determined by the physicochemical features of the compound and the protein to which it binds. Albumin is the most significant contributor to the binding of therapeutic drugs but alpha 1-glycoproteins and lipoproteins can also be involved.

WHY IS PROTEIN BINDING IMPORTANT?

The degree of protein binding of a therapeutic agent should be considered when interpreting the PK profile of a compound. High plasma protein binding will lead to more drug present in the central blood compartment and therefore a lower volume of distribution (although this relationship will not always hold true if the drug in question also shows a high degree of binding to tissue proteins). Low protein binding means more drug is free to partition into tissues and will therefore result in a high volume of distribution. Also, only free drug is available to exert a pharmacological effect but in most pharmacokinetic studies it is the total plasma concentration which is measured. Therefore, knowledge of the unbound fraction in blood is essential to the correlation of total plasma concentration with activity.

High protein binding of a compound can have important clinical effects with regard to drug toxicity. A small change in the extent of binding of a very highly bound drug will mean a very large change in drug concentration in plasma. Alterations in the degree of drug binding can result from drug interactions or disease state. For example, phenytoin is approximately 90% protein bound, but can be displaced by co-prescribed drugs. Valproic acid, in particular can increase the free phenytoin fraction 30 to 100%, which in turn can lead to toxicity.

WHAT ABOUT BLOOD PARTITIONING?

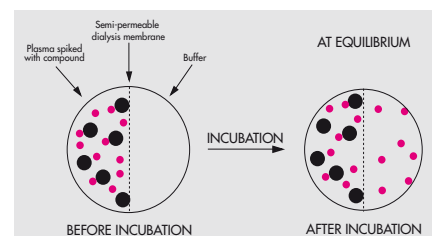
Red blood cell (RBC) partitioning can be considered to be a specific case of protein binding. Certain therapeutic compounds have a high degree of affinity for the red blood cell fraction of whole blood and so have large RBC-to-plasma concentration ratios. In the design of pharmacokinetic studies, compounds with high partition ratios will likely require assaying whole blood rather than plasma to accurately measure drug concentrations *in vivo*. Also, when assessing the pharmacokinetic profile of a drug, if it is necessary to calculate the clearance of a drug as a function of blood flow to a particular organ, the total blood concentration and therefore the RBC-to-plasma partition ratio must be known. Finally identification of compounds with high RBC-to-plasma ratios is useful at the lead optimization stage as an indicator of potential problems with red blood cell accumulation and hematotoxicity, particularly, for compounds which are intended for long-term use.

MEASUREMENT OF PROTEIN BINDING

The two techniques commonly used to measure plasma or serum protein binding *in vitro* are ultrafiltration and equilibrium dialysis. While ultrafiltration has the advantage of a short assay time, the main problem with this technique is non-specific binding of test compounds to the filtration apparatus. The use of equilibrium dialysis can significantly reduce this problem when the assay is performed in a teflon-coated equilibration block. Although the equilibration method requires a longer assay time, the assay can be run in a 96-well high-throughput format which allows for the determination of protein binding of many compounds simultaneously.

HOW DOES EQUILIBRIUM DIALYSIS WORK?

A solution of plasma or serum spiked with the test compound and a solution of buffer are placed in opposite sides of a compartment separated by a dialysis membrane. The dialysis membrane allows small molecules to move freely between compartments but prevents the movement of larger proteins (above a specific molecular weight cutoff). After a sufficient time period of equilibration, the free drug fraction in both compartments reaches equilibrium. At this point both compartments are sampled and analyzed to determine the amount of drug on each side. The amount measured in the plasma compartment includes both free and bound drug, while that on the buffer side represents free drug only.



PROTEIN BINDING - BLOOD PARTITIONING ► APPLICATION NOTE

The analyte peak areas obtained from HPLC-MS/MS analysis of each sample are used to calculate protein binding according to the following formulas:

$$\text{Protein binding (\%)} = \frac{(\text{Area}_{pe} - \text{Area}_{be}) \times \frac{V_{pe}}{V_{pi}}}{[(\text{Area}_{pe} - \text{Area}_{be}) \times \frac{V_{pe}}{V_{pi}}] + \text{Area}_{be}} \times 100$$

$$\text{Recovery (\%)} = \frac{(V_{pe} \text{Area}_{pe} + V_{be} \text{Area}_{be})}{V_{pi} \text{Area}_{pi}} \times 100$$

Where

- Area_{pe} = Peak area of analyte in plasma at equilibrium
- Area_{be} = Peak area of analyte in buffer at equilibrium
- V_{pe} = Volume of plasma at equilibrium
- V_{pi} = Initial volume of plasma
- V_{pe}/V_{pi} = Experimentally determined at Cerep as constant value of 1.13
- V_{be} = Volume of buffer at equilibrium
- Area_{pi} = Peak area of analyte in plasma initially

■ CEREP'S PROTEIN BINDING ASSAY

The samples are prepared as follows:

The dialysate compartment is loaded with 0.05 M phosphate buffer and the sample side is loaded with plasma spiked with the test compound at a concentration of 10 μM .

After loading, samples are covered and incubated at 37°C for a time sufficient for equilibrium to be reached (overnight 18 \pm 2h). At this time each compartment is sampled, diluted with acetonitrile/buffer and centrifuged. The supernatants are analyzed by HPLC-MS/MS.

Also, included in the standard assay is a recovery determination. An aliquot of the spiked plasma is sampled at the start of the assay and processed for HPLC-MS/MS analysis in the same manner as the assay samples. The sum of the mass of the test compound in the test buffer and plasma samples compared to the mass in the plasma at the start of the assay is used to calculate percent recovery. Low recovery indicates degradation or non-specific binding of the compound during this assay.

For compounds with limited stability in whole plasma, an alternative means to measure percent protein binding by equilibrium dialysis is by incubation with solutions of albumin and/or alpha-acid glycoprotein. These proteins are the main contributors to the binding of therapeutic drugs, and the percentage of compound bound to the individual proteins is a good indicator of binding in whole plasma.

► SPECIES AVAILABLE

Different species of plasma or serum are available including human, monkey, dog, rat and mouse. Please inquire for other species.

► QUALITY CONTROL

Acebutolol, quinidine and warfarin are tested in each assay as reference compounds. These compounds yield protein binding values that represent low, medium, and high binding to human plasma proteins, respectively.

■ HOW DOES BLOOD PARTITIONING WORK?

Aliquots of fresh whole blood and reference plasma are spiked with the test compound and incubated for an hour at 37°C. At the end of the incubation period, plasma is separated from whole blood by centrifugation and all plasma samples are diluted and analyzed by HPLC-MS/MS. The analyte peak areas obtained from HPLC-MS/MS analysis of each sample are used to calculate the RBC-to-plasma ratio ($K_{RBC/PL}$) as follows:

$$K_{RBC/PL} = \frac{1}{H} \times \left(\frac{I_{REF\ PL}}{I_{PL}} - 1 \right) + 1$$

Where:

- H is the hematocrit (percent of total blood cells in whole blood sample, v/v),
- $I_{REF\ PL}$ is the peak area of the analyte in the reference plasma,
- I_{PL} is the peak area of the analyte in plasma separated from whole blood.

■ CEREP'S BLOOD PARTITIONING ASSAY

The samples are prepared as follows:

Heparinized whole blood is obtained fresh and stored at 4°C prior to use. Reference plasma and whole blood are spiked with a test compound at 5 μM with a final DMSO concentration of 0.5%.

Samples are incubated, with shaking, for 60 minutes at 37°C. The blood samples are spun to collect the plasma fraction. Aliquots are sampled and acetonitrile is immediately added. The samples are mixed, centrifuged, and the supernatants analyzed by HPLC-MS/MS.

► SPECIES AVAILABLE

Different species of blood are available including human, monkey, dog, rat and mouse. Please inquire for other species.

► QUALITY CONTROL

The reference used in the blood partitioning assay is methazolamide which is known to accumulate in red blood cells ($K_{RBC/PL} > 5$).

■ ASSAY SELECTION GUIDE

Tissue distribution

The extent of binding of a drug to plasma proteins is a determinant of drug distribution in the body. A drug with extensive binding to plasma proteins generally exhibits a small distribution volume approaching the volume of blood. However, when a drug has high binding to tissue, the volume of distribution can be much greater than the actual volume of the body fluid.

- . Plasma/Serum protein binding
- . Blood partitioning
- . *In vivo* PK/tissue distribution in rats
- . *In vivo* PK/tissue distribution in mice



FRANCE

Le Bois l'Évêque
86600 CELLE L'ÉVESCAULT
tel. +33 (0)5 49 89 30 00

(Headquarters)
155 boulevard Haussmann
75008 PARIS
tel. +33 (0)1 45 64 44 60

USA

15318 N.E. 95th Street
REDMOND, WA 98052
tel. +1 (425) 895 8666

JAPAN

Namiki Shoji Co., Ltd.
Kenseishinjuku Bldg. 5-5-3
Shinjuku, Shinjuku-ku
TOKYO, 160-0022
tel. +81 (0)3 3354 4026
fax +81 (0)3 3352 2196

CHINA

Ai Di Sheng (Edison) Road 326,
302-1 room
Zhangjiang High-Tech Park
SHANGHAI
tel. +86 18702160370

■ QUESTIONS OR CONCERNS?

Please contact us: sales@cerep.com

sales@cerep.com
www.cerep.com