Plasma Protein Binding of Challenging Compounds

KEITH RICCARDI, SHANNON CAWLEY, PHILLIP D. YATES, CHENG CHANG, CARRIE FUNK, MARK NIOSI, JIAN LIN, LI DI

Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc., Groton, Connecticut 06340

Received 6 April 2015; revised 25 April 2015; accepted 27 April 2015

Published online 2 June 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24506

ABSTRACT: Accurately determining fraction unbound (f_u) with currently available methods has been challenging for certain compounds. Inaccurate f_u values can lead to the misinterpretation of important attributes of a drug candidate. Our analysis of over 2000 Pfizer drug discovery compounds showed no systematic bias in low or high f_u precision using the equilibrium dialysis method. However, the accuracy of the plasma protein binding (PPB) estimate for highly bound compounds may be poor, should equilibrium not be fully achieved in the equilibrium dialysis assay. Here, a dilution method and a presaturation method were applied to accelerate equilibration for a set of challenging compounds. These improved methods demonstrate the ability to provide an accurate measurement of PPB for highly bound compounds with f_u values much less than 1%. Therefore, we recommend that the actual experimental f_u value be used for the prediction of drug–drug interaction potential and for the characterization of important drug candidate properties. Our recommendation calls into question the need for current regulatory guidelines that restrict the reporting of f_u values below 1%. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2627–2636, 2015

Keywords: protein binding; equilibrium dialysis; dilution method; presaturation method; nonspecific binding; albumin; alpha-1-acid glycoprotein; drug interactions; in vitro models

INTRODUCTION

There are several misconceptions regarding plasma protein binding (PPB) in drug discovery.[1,2] On one hand, it is important to measure PPB accurately because it affects therapeutic index (TI). When PPB is different between the toxicological species and humans, it plays a role in deriving TI. Typically, a narrower TI is accepted by regulators with reference to no species difference in PPB to err on the conservative side. PPB can have an effect on clearance calculation and dose projection. Although fraction unbound (f_u) has no bearing on dose calculation in most cases assuming the unbound exposure driving the pharmacology,[1] binding could affect dose prediction when potency is measured in the presence of proteins. Unbound intrinsic clearance (after correction of binding) and unbound potency (e.g., unbound IC_{50}, after correction of binding) should be used to estimate dose. PPB also plays a key role in development of pharmacokinetics/pharmacodynamics relationships and prediction of drug–drug interaction (DDI) potential, potency/selectivity/toxicity (if they are measured in the presence of proteins), and so on. The wide impact of PPB on drug candidates is mainly driven by the lack of an easy way to directly measure free drug concentration, a value most relevant for pharmacological activity.[1,2] f_u is essential for obtaining free drug concentration from total drug concentration, a value that can be directly measured. Despite the critical nature of PPB, f_u itself has no relevance to efficacy in most cases and should not be optimized through structural modification or used to develop structure–PPB relationships.[1,3] f_u is conceptually different from free drug concentration and confusion is often generated when the two terms are used interchangeably.[1,3] These principles apply not only to PPB, but also binding to various tissues (e.g., liver, brain, pancreas, skeletal muscle, heart, kidney, and skin), liver microsomes, hepatocytes, or assay media containing proteins (e.g., bovine serum albumin, fetal bovine serum). f_u values, regardless of plasma or tissue, should not be optimized or used for structure-binding relationship development.

Numerous methods have been developed to measure PPB during various stages of drug discovery and development.[4–8] The equilibrium dialysis method is widely considered to be the gold standard for PPB determination as nonspecific binding (refer to binding to plastic wells and dialysis membrane) has a minimal impact on the results.[9] The assay is easy to perform, inexpensive, and amenable to a high-throughput format. The equilibrium dialysis method is widely used in the pharmaceutical industry to measure plasma f_u, tissue binding, and media binding for *in vitro* assays.[7,9–14] Although equilibrium dialysis is known to be reliable for most compounds across a range of diverse structures and physiochemical properties, an accurate f_u determination can be challenging for some compounds, for example, compounds that are highly bound, large molecular weight, highly lipophilic, insoluble, or have high nonspecific binding. f_u values for these compounds tend to be more variable from experiment to experiment and are more difficult to measure reliably. In addition to reduced confidence in the data, this can lead to greater uncertainty in predicting certain key drug candidate properties, such as TI and DDI. As a result, the regulatory agencies have published guidelines on the lower reportable limit of f_u for PPB. For example, the European Medical

Abbreviations used: AAG, alpha-1-acid glycoprotein; ACN, acetonitrile; DDI, drug–drug interaction; DMSO, dimethyl sulfoxide; EMA, European Medical Agency; f_u, fraction unbound; HTD 96, high-throughput dialysis device in a 96-well format; IS, internal standard; LC–MS/MS, high-performance liquid chromatography coupled with tandem mass spectrometry; MWCO, molecular weight cut-off; PBS, phosphate buffer saline; PPB, plasma protein binding; QC, quality control; RED, rapid equilibrium dialysis; RT, room temperature; TI, therapeutic index

Correspondence to: Li Di (Telephone: +860-715-6172; Fax: +860-441-6402; E-mail: li.di@pfizer.com)

This article contains supplementary material available from the authors upon request or via the Internet at http://wileylibrary.com.

Journal of Pharmaceutical Sciences, Vol. 104, 2627–2636 (2015)

Agency (EMA) DDI guideline recommends a lower limit of 1% for PPB f_u to aim at a conservative DDI prediction: "when an estimation of f_u is used, figures lower than 1% should not be used because of the uncertainties in the estimation. Thus, as an example, if the free fraction has been estimated to be 0.5% *in vitro* or *ex vivo*, a 1% free fraction should be used."[15] The US FDA DDI guidelines have similar recommendations.[16] Setting a lower f_u limit at 1% is somewhat arbitrary, does not necessarily reflect an assay's capability or performance, and it can result in the overprediction of DDI potential for highly bound compounds and result in unnecessary and expensive clinical studies. It is, therefore, important to understand the limitations of a PPB assay and to define expectations on reportable f_u values using scientific data.

In this study, the precision and accuracy of f_u for a set of highly bound and challenging compounds was evaluated. Experimental approaches to improve the accuracy of the PPB assay were examined to offer a rationale for the estimation of a high-confidence f_u determination in this challenging space.

MATERIALS AND METHODS

Materials

Plasma lots from five species (human, Wistar Han rat, CD-1 mouse, beagle dog, and cynomolgus monkey) were purchased from Bioreclamation, LLC (Hicksville, New York). Each plasma lot was mixed sex with at least six donors of half male and half female. Test compounds were obtained from Pfizer Global Material Management (Groton, Connecticut) or purchased from Sigma–Aldrich (St. Louis, Missouri). Itraconazole and its three metabolites[17] (hydroxy-itraconazole, keto-itraconazole, and N-desalkyl-itraconazole) were obtained from Toronto Research Chemicals (Ontario, Canada). The rapid equilibrium dialysis (RED) plates were purchased from Thermo Scientific (Rockford, Illinois) with a membrane molecular weight cut-off (MWCO) of 8K. The 96-well equilibrium dialysis (HTD 96, high-throughput dialysis device in a 96-well format) device and cellulose membranes with MWCO of 12–14 K were obtained from HTDialysis, LLC (Gales Ferry, Connecticut). Velocity V11 peelable seals were obtained from BD Falcon (Bedford, Massachusetts). Deep 96-well plates of 1.2 mL blocks were from Axygen Scientific Inc. (Union City, California) and tips of 96 blocks were obtained from Apricot Designs (Monrovia, California).

PPB with Equilibrium Dialysis

Plasma was thawed in a water bath at 37°C and the pH of plasma was adjusted to pH 7.4 with 1 N hydrochloric acid. dimethyl sulfoxide (DMSO) stock solutions of test compounds were prepared at 200 μM, added in 1:100 ratio to plasma (standard protocol) or diluted plasma as specified, and mixed thoroughly with a 96-well pipettor (Apricot Design PP550). The final compound concentration for the equilibrium dialysis experiments was 2 μM containing 1% DMSO. For diluted plasma, a percentage of plasma is added to phosphate buffer saline (PBS) to make up the desirable concentration of plasma. Volume shift was obtained by measuring the liquid volumes in the donor and receiver wells at different time points under the incubation conditions using volumetric pipette.

PPB with RED Device

Plasma samples spiked with test compounds (2 μM, 220 μL) were added to the donor wells and 350 μL PBS (without calcium or magnesium; Lonza, Walkersville, Maryland) was added to the receiver wells of the RED device (receiver material is made of high-density polypropylene). Quadruplicates were run for each test compound. Before and after incubation, an aliquot of 15 μL of plasma spiked with 2 μM test compound was added to a 96-deep well plate containing 45 μL of PBS and 200 μL of cold acetonitrile (ACN) with mass spectrometry internal standard (IS), CP-628374[18] or equivalent. These samples were used for recovery and stability evaluation. The RED sample blocks were covered with Breathe Easy™ gas permeable membranes (Sigma–Aldrich) and placed on an orbital shaker (450 rpm; VWR Scientific Products, Radnor, Pennsylvania) in a humidified (75% RH) incubator at 37°C with 5% CO_2/95% O_2 for 4 h (standard protocol) or a specified time. At the end of the incubation, 15 μL of plasma samples from the donor wells were taken and added to a 96-deep well plate containing 45 μL of PBS and 200 μL of cold ACN with IS. Aliquots of 45 μL dialyzed PBS were taken from the receiver wells and added to 15 μL of blank plasma and 200 μL of cold ACN with IS in a 96-deep well plate. The plates were sealed (Nunc aluminum sealing tape; Thermo Scientific) and mixed with a vortex mixer (Multi-Tube Vortexer; VWR Scientific Products) for 3 min, then centrifuged (Eppendorf, Hauppauge, New York) at 1900 g, RT (room temperature) for 5 min. The supernatant was transferred to a new deep well plate, dried down, reconstituted, and subsequently analyzed using high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Sertraline was used as a quality control (QC) sample on every plate in quadruplicates.

Presaturation Method with RED

The detailed experimental protocol of the presaturation method was reported previously.[19] PBS buffer solutions of test compounds containing 0.5% DMSO were prepared with the receiver concentration at approximately onefold, twofold, and 10-fold of the estimated f_u values based on the initial data from the equilibrium dialysis assay using the standard protocol. In the preincubation steps to saturate the nonspecific binding, the solutions were added to both the donor (250 μL) and the receiver (380 μL) wells of the RED device with Teflon receiver block (different than the standard protocol, in which the receiver material is high-density polypropylene), incubated on an orbital shaker (450 rpm) in a humidified (75% RH) incubator at 37°C for 30 min and removed at the end of preincubation. The procedure was repeated twice with the same solutions and, in the last preincubation, the solutions were allowed to soak the device overnight. At the end of the three preincubations, the solutions were removed and equilibrium dialysis experiments were set up as discussed above for the standard RED assay with the following changes: (1) the receivers were filled with PBS solutions of test compounds at concentrations approximately onefold, twofold, and 10-fold of the estimated f_u values; (2) 0.5% DMSO in both acceptor and donor wells (similar to the standard protocol, which starts at 1% DMSO); (3) equilibrium dialysis incubation time was 18 h rather than 4 h in the standard protocol; and (4) the Teflon receiver block was used instead of the polypropylene receiver block.