Understanding and Reducing the Experimental Variability of *In Vitro* Plasma Protein Binding Measurements

HAIPING WANG,[1] MATT ZRADA,[2] KEN ANDERSON,[2] RAVI KATWARU,[1] PAUL HARRADINE,[1] BERNARD CHOI,[1] VINCE TONG,[3] NATASA PAJKOVIC,[3] RALPH MAZENKO,[2] KATHY COX,[1] LUCINDA H. COHEN[1]

[1]Department of Pharmacodynamics, Pharmacokinetics and Drug Metabolism, Merck Research Laboratories, Rahway, New Jersey
[2]Department of Pharmacodynamics, Pharmacokinetics and Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania
[3]Department of Pharmacodynamics, Pharmacokinetics and Drug Metabolism, Merck Research Laboratories, Boston, Massachusetts

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ABSTRACT: The experimental measurement of plasma protein binding is a useful *in vitro* Absorption Distribution Metabolism and Excretion(ADME) assay currently conducted in both screening and definitive early development candidate modes. The fraction unbound is utilized to calculate important pharmacokinetic (PK) parameters such as unbound clearance and unbound volume of distribution in animals that can be used to make human PK and dose predictions and estimate clinically relevant drug–drug interaction potential. Although these types of assays have been executed for decades, a rigorous statistical analysis of sources of variability has not been conducted because of the tedious nature of the manual experiment. Automated conduct of the incubations using a 96-well equilibrium dialysis device as well as high-throughput liquid chromatography–mass spectrometry quantitation has now made this level of rigor accessible and useful. Sources of variability were assessed including well position, day-to-day, and site-to-site reproducibility. Optimal pH conditions were determined using a design of experiments method interrogating buffer strength, CO_2% and device preparation conditions. Variability was minimized by implementing an in-well control that is concurrently analyzed with new chemical entity analytes. Data acceptance criteria have been set for both the in-well control and the range of analyte variability, with a sliding scale tied to analyte-binding characteristics. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

Beginning in 2007, standardized workflows and procedures have been adopted across all Merck bioanalytical laboratories; site-specific protocols have been replaced with a single analytical procedure for each assay. This new model has increased efficiency, and more importantly, flexibility by allowing work to be shared across all of the laboratories in the network regardless of the origination of the request. Standardized workflows provide the framework for an efficient and lean operational model, where work can be easily shifted from laboratory to laboratory. Highly detailed protocols with clearly defined assay parameters are needed. In a discovery environment, a fit-for-purpose strategy often determines experimental design and analytical acceptance criteria.[1] Many factors are taken into account, including sample throughput, resource allocation, and most importantly, how the data are used. Acceptance criteria provide a measure of acceptable analytical error, generally determined by the level of precision and accuracy of calibration standards and/or quality control samples.[2] It is important, however, to understand that certain assays are more sensitive to environmental conditions and, in those cases, interlaboratory variability needs to be carefully assessed, minimized, and controlled.[3]

The determination of the unbound concentration of drugs in plasma, plasma protein binding (PPB), is an experiment that is highly sensitive to assay conditions and presents a serious challenge to bioanalytical laboratories.[4] The measurement of PPB is an important *in vitro* Absorption Distribution Metabolism and Excretion (ADME) assay currently conducted at Merck in both screening and definitive early development candidate selection mode. The objective of the assay is to determine the extent of binding a drug candidate exhibits to plasma constituents, primarily plasma proteins.. Specifically, the unbound fraction of a compound ($f_{u,\ plasma}$) is calculated by taking the ratio of the measured unbound drug concentration over the measured total drug concentration, which may also be reported as a percentage. According to the unbound drug hypothesis, pharmacological activity is determined by the concentration of unbound drug at the site of the therapeutic target.[5] As a result, pharmacokinetic (PK) analyses, PK/pharmacodynamic (PK/PD) models, human PK, and drug–drug interaction predictions generally rely on unbound, rather than total drug concentration in their calculations.[6,7] As a compound may be differentiated and selected for further development based on parameters calculated using PPB data, it is therefore critical that the assay provides the appropriate amount of rigor (accuracy, precision, and reproducibility) so that valid data-driven decisions are made. Experimental variability (intraday and interday) should be determined and controlled, especially in the measurement of highly bound drugs where uncertainty in fraction unbound is highly sensitive to experimental error.[8]

Equilibrium dialysis is a traditional method with a history of widespread use and is our current method for measuring PPB in discovery and early development programs. The use of a commercially available device in a 96-well format facilitates

Correspondence to: Lucinda H. Cohen (Telephone: +732-594-3745; Fax: +732-594-0000; E-mail: lucinda_cohen@merck.com)

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robotic automation and increases sample throughput.[9] Because of the widespread use of equilibrium dialysis, common pitfalls and sources of assay variability are the subject of many papers in the literature. Insufficient equilibration,[9,10] volume shift,[11,12] lack of pH control,[13–16] and protein leakage[17] are known to contribute to assay variability and should be controlled during a PPB experiment.

In this study, we report how Six Sigma Methodology[18] was used to identify, reduce, and control variability of the PPB assay. Measurement systems analysis (MSA)[19] and design of experiment (DOE)[20] methods provided a statically rigorous framework for the evaluation of assay variability. MSA interrogates the interday and laboratory ruggedness and repdocibility of the assay, whereas DOE allows multiple factors, that is, sources of variability to be simultaneously and efficiently evaluated. Results of the MSA revealed that small differences in the procedures of different laboratories were contributing to interlaboratory variability; in some cases, experimental protocols were written with an acceptable range for a specific assay parameter instead of an absolute value. Results of the MSA, more importantly, identified the major source of variability of the assay. Lack of pH control was also identified as a source of variability and was investigated by the simultaneous optimization of three incubation parameters by DOE methodology. We established systematic acceptance criteria and the use of in-well controls to monitor assay performance to both increase the data quality and reduce the need to repeat experimental determinations.

EXPERIMENTAL

Chemicals and Reagents

Test compounds warfarin, clozapine, diltiazem, diclofenac, fluconazole, fluoxetine, and verapamil were obtained from Sigma–Aldrich (Madison, Wisconsin). HPLC grade water and all analytical organic solvents used were purchased from Fisher (Fair Lawn, New Jersey). Control sodium ethylenediaminetetraacetic acid human plasma was purchased from BioReclamation Inc. (Hicksville, New York). Chemicals (Na_2HPO_4, NaCl, and $NaH_2PO_4{\cdot}2H_2O$) used in buffer preparation were purchased from Thermo Fisher (Fairlawn, New Jersey). Carbon dioxide gas used during incubations was from Airgas (Meadville, Pennsylvania).

Buffer Preparation

Sodium phosphate (100 mM) and 150 mM NaCl buffer (phosphate buffer solution, PBS) were prepared by following method. A basic solution was made by dissolving 14.2 g/L Na_2HPO_4 and 8.77 g/L NaCl in deionized water. An acidic solution was made by dissolving 15.6 g/L $NaH_2PO_4{\cdot}2H_2O$ and 8.77 g/L NaCl in deionized water. The basic solution was then titrated with the acidic solution to pH 7.40.

Instrumentation

A Hamilton Star Plus liquid handling robot was used to prepare the plates for incubation, aliquotting of buffer and plasma samples after incubation, matrix matching of buffer and plasma, and standard curve preparation. Mass spectrometric analysis was performed on an Applied Biosystems Sciex API 4000 triple quadrupole mass spectrometer (Toronto, Canada) equipped with an ESI source. Analyst software (V. 1.5) was used for data acquisition and peak integration. Ultra-performance liquid chromatography (UPLC) was performed on a Thermo Fischer Scientific (Chelmsford, MA) Transcend dual inlet upltra pressure liquid chromatograph. Waters Acquity UPLC™ HSS T3 (2.1 × 50 mm^2, 1.8 μm) columns were obtained from Waters Corporation (Milford, Massachusetts). Deep 96-well collection plates were purchased from Analytical Sales and Services (Pompton Plains, New Jersey). Plasma pH was measured with a microcombination pH microelectrode (Microelectrode Inc., Bedford, New Hampshire). A Thermo Scientific CO_2 incubator with temperature control was utilized during equilibrium dialysis Statistical analysis was conducted using Minitab version 16.2.1. MSA results were evaluated using gage reproducibility and repeatability, analysis of variance (ANOVA), nonparametric Kruskal–Wallis, and Moods' median tests as well as general statistical tests. DOE methods utilized a two-level full factorial design with three factors, eight runs, and singlet replicates representing the mean of 10 measurements for each run. Main effects including estimated coefficients and two- and three-way interactions were analyzed. All derived terms were free of aliasing.

Dialysis Method

Plasma-spiking solution was prepared by transferring 5 μL of 2 mM dimethyl sulfoxide solution to 995 μL of the plasma to create an intermediate concentration of 10 μM. The intermediate solution was further diluted by taking 100 μL of the 10 μM solution and adding it to 300 μL of plasma yielding a 2.5-μM analyte concentration in plasma. Dialysis was performed using a reusable 96-well HT dialysis micro equilibrium device by HT Dialysis LLC (Groton, Connecticut) and dialysis membrane strips (molecular weight cutoff 12 – 14 kDa). Membrane strips were hydrated by soaking in ethanolic PBS. The dialysis block was prepared by adding 120 μL of the pH 7.4 100 mM PBS to one side of the membrane. Next, 120 μL of the analyte-spiked plasma was added to the other side of the dialysis membrane. After aliquotting into the Teflon block, samples were incubated at 37°C in a humidified incubator for 4 h with 5% CO_2. Following a 4-h incubation period, a 50-μL aliquot was removed from the plasma side of the equilibrium dialysis block and added to a 96-well plate. To this sample was added 50 μL PBS and 200 μL of solution containing 200 nM diclofenac, 200 nM labetalol, and 100 nM imipramine in acetonitrile (acetonitrile internal standard mix). Additionally, a 50-μL aliquot was removed from each well of the buffer side of the equilibrium dialysis block, and added to a 96-well plate. To this sample was added 50 μL plasma and 200 μL acetonitrile internal standard mix solution.

Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry

Ultra-performance liquid chromatography was performed on a Waters Acquity UPLC™ HSS T3 (2.1 × 50 mm^2, 1.8 μm) column. The samples were eluted from the column at 0.75 mL/min with a stepwise procedure. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. A gradient elution program was utilized where the solvent composition was held at 5% B for 0.25 min and then changed from 5% B to 95% B in 1.5 min. The mobile phase composition was then held at 95% B for an additional 0.4 min. The column was re-equilibrated at the original solvent composition