Impact of Recovery on Fraction Unbound Using Equilibrium Dialysis

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ABSTRACT: 'Historically, recovery had been used to evaluate the data quality of plasma protein binding or tissue binding obtained from equilibrium dialysis assays. Low recovery was often indicative of high nonspecific binding, instability, or low solubility. This study showed that, when equilibrium was fully established in the dialysis assay, low recovery due to nonspecific binding had no impact on the determination of fraction unbound. The conclusion was supported by the principles of the equilibrium dialysis assay, experimental data, and mathematic simulations. The results suggested that the use of recovery as an acceptance criterion for the equilibrium dialysis assay in drug discovery was too restrictive, and introduced the additional burden of repeating studies unnecessarily. © 2011 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:1327–1335, 2012

Keywords: plasma protein binding; brain homogenate binding; equilibrium dialysis; recovery; stability; nonspecific binding; simulation; ADME

INTRODUCTION

Determination of plasma protein binding and tissue binding is important in drug discovery and development. Binding experiments provide indirect measurements of free drug concentrations in systemic circulation and biophases wherein the therapeutic targets reside. Fraction unbound (f_u) is a critical parameter in estimating therapeutic index, developing pharmacokinetics–pharmacodynamics relationships, and projecting clinical doses because the free drug concentration at the site of action is responsible for the pharmacological activity based on the free drug hypothesis.[1] Binding studies are not only frequently applied to plasma and tissues (brain, liver, pancreas, heart, lung, and muscles)[2–6] but also to *in vitro* bioassay matrices (e.g., liver microsomal binding)[7,8] to predict *in vivo* outcomes from *in vitro* data and develop *in vitro–in vivo* correlation.

Many methodologies have been developed to measure plasma protein binding and tissue binding.[2,9,10] Among the most commonly applied binding methods are equilibrium dialysis,[11–14] ultrafiltration,[15,16] immobilized human serum albumin/alpha-1-acid glycoprotein protein columns,[17–19] and kinetic approaches.[20,21] High throughput 96-well formats are available for equilibrium dialysis and ultrafiltration methods to increase throughput. In spite of known limitations (unknown test compound concentration, volume shift, and Donnan effect),[2] Equilibrium dialysis is considered the most accurate method for measuring binding because of its physiological relevance and the fundamental design of the dialysis devices that minimize the impact of nonspecific binding. Nonspecific binding is defined in this paper as loss of compounds due to binding to the wall and/or membrane of the apparatus.

Recovery (also known as mass balance) is traditionally considered an important parameter in evaluating the quality of an assay in a closed system, such as plasma protein/tissue binding or monolayer transport (e.g., Caco-2, Madin–Darby canine kidney). Low recovery is often an indication of material lost during the assay due to high nonspecific binding, low solubility, or compound instability. Recovery is therefore commonly used as an acceptance criterion for these assays. Typically, experiments that fail because of low recovery are repeated in order to meet the necessary acceptance level (frequently set arbitrarily, e.g., acceptable recovery between 70% and 130%), a practice which consumes time and resources and generates a lot of frustration. In this study, we examine the impact

Abbreviations used: DPBS, Dulbecco's phosphate-buffered saline; EqD, standard equilibrium dialysis device HTD 96 by HTDialysis; f_u, fraction unbound; IS, internal standard; MWCO, molecular weight cutoff; RED, rapid equilibrium dialysis device by Thermo Scientific

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of recovery on the f_u measurement using equilibrium dialysis. The goal is to identify the important parameters that influence the assay quality and avoid using unnecessary acceptance criterion that has minimal impact on the assay results.

EXPERIMENTAL

Materials

Both human plasma and Wistar Han rat brain homogenate were mixed gender and ordered through Bioreclamation, LLC (Hicksville, New York). The brain homogenate was prepared by the vendor using 1 g of brain in 4 mL of Dulbecco's phosphate-buffered saline (DPBS) with high-speed tissue grinders (fivefold dilution). The homogenates were further processed in house using a glass Dounce homogenizer (Thermo Scientific, Waltham, Massachusetts) to reduce the size of the brain tissues. The plasma and brain homogenate were frozen at −80°C before use. No significant difference of binding characteristics were observed between fresh and freeze/thaw brain homogenates. Test compounds were obtained from Pfizer Global Material Management (Groton, Connecticut) or purchased from Sigma–Aldrich (St. Louis, Missouri). The equilibrium dialysis device (EqD) and cellulose membranes with molecular weight cutoff (MWCO) 12–14 kDa were obtained from HTDialysis, LLC (Gales Ferry, Connecticut) and the rapid equilibrium dialysis (RED) plates were purchased from Thermo Scientific (Rockford, Illinois) with a membrane MWCO of 8 kDa. Velocity V11 peelable seals were obtained from BD Falcon (Bedford, Massachusetts). Deep 96-well plates of 1.2 and 2.2 mL blocks were from Axygen Scientific Inc. (Union City, California) and tips of 96 blocks were obtained from Apricot Designs (Monrovia, California).

Equilibrium Dialysis for Plasma Protein Binding and Brain Homogenate Binding Study

Test compounds were dissolved in dimethyl sulfoxide (DMSO) to 10 mM and further diluted to 100 μM with DMSO for binding studies. The stock solutions (100 μM) were added (1:100; v/v) to the brain homogenates (fivefold diluted with DPBS) or plasma (no dilution) and mixed well with a 96-well pipettor from Soken SigmaPet or Apricot Design PP550. The final compound concentration for the equilibrium dialysis experiments was 1 μM with 1% DMSO. The stabilities of all samples during dialysis were evaluated in separate experiments in parallel with binding studies.

Binding Studies with EqD Device

The dialysis membranes were prepared prior to experiments. The cellulose membranes (MWCO 12–14 kDa) were immersed in deionized water for 15 min, followed by 15 min in 30% EtOH/deionized water, 1 min in deionized water, then at least 15 min or overnight in DPBS. The EqD device was assembled according to the manufacturer's instructions.[22] A 150 μL aliquot of brain homogenate or plasma spiked with 1 μM compound was added to one side of the chamber (donor) and 150 μL of DPBS was added to the other side of the dialysis membrane (receiver). Before and after incubation, an aliquot of 20 μL of brain homogenate or plasma spiked with 1 μM of compound was added into a 96-deep well plate containing 80 μL of DPBS and 200 μL of cold acetonitrile (ACN) with mass spectrometry (MS) internal standard (IS, CP-628374).[23] These samples were used for the recovery calculation and stability evaluation. The EqD device was covered with Breathe Easy gas permeable membranes obtained from Diversified Biotech (Dedham, Massachusetts). Compounds were assessed in triplicate using three EqD devices for each experiment (replicates were between devices rather than within a given device). EqD devices were placed on a shaking plate at 450 rpm and incubated for 6 h in a humidified incubator at 37°C with 5% CO_2. The sampling cleanup procedure was designed such that the sample composition was consistent for all the samples to eliminate any potential confounding issues from varying background or ionization efficiency during analysis with liquid chromatography (LC)–MS. At the end of the incubation, 20 μL of the brain homogenate or plasma samples from the donor wells were taken and added into a 96-deep well plate containing 80 μL of DPBS and 200 μL of cold ACN with IS (1.65 μg/mL). Aliquots of 80 μL of dialyzed DPBS were taken from the receiver wells and added to 20 μL of blank brain homogenate or plasma and 200 μL of cold ACN with IS in a 96-deep well plate. The plates were sealed and mixed with a vortex mixer (VWR, Radnor, Pennsylvania) for 3 min, then centrifuged at 1550g and 4°C (Eppendorf, Hauppauge, New York) for 5 min. The supernatant was transferred to a new deep well block, sealed and subsequently analyzed using LC–MS–MS.

Binding Studies with RED Device

All of the experimental conditions were identical as described above for the EqD assay with the following exceptions. Donor volume was 220 μL and receiver was 350 μL. The incubation time was 4 h. At the end of the incubation, 15 μL of the brain homogenate or plasma samples from the donor wells were taken and added into a 96-deep well plate containing 45 μL of DPBS and 180 μL of cold ACN with IS (1.65 μg/mL). Aliquots of 45 μL dialyzed DPBS were taken from the receiver wells and added to 15 μL of blank brain homogenate or plasma and 180 μL of cold ACN with IS in a 96-deep well plate. The plates were sealed and