Equilibrium Gel Filtration to Measure Plasma Protein Binding of Very Highly Bound Drugs

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ABSTRACT: For very highly bound drugs (fu < 2%), the determination of the unbound fraction in plasma (fu) and a reliable estimation of protein-binding differences across species, populations, or concentrations is challenging. The difficulty is not mostly assay sensitivity but rather experimental bias. In equilibrium gel filtration (EGF)—opposite to the commonly used methods—the amount bound at a set-free concentration is determined. Therefore, signals and differences are bigger for more highly protein-bound drugs. We describe here a new experimental set-up developed to investigate binding in plasma and compare results with those obtained with standard methods for nine Novartis compounds. The method was then applied for two drugs for which it was challenging to obtain precise data with standard methods: midostaurin and siponimod. Despite the very high binding (fu ≤ 0.1%), precise estimation of up to 10-fold species differences relevant for safety assessments was possible. Evidence for the correctness of the data by comparison with other pharmokinetics parameters is provided. Sensitivity to potential sources of experimental bias is compared with standard methods and advantages and disadvantages of the methods are discussed. In conclusion, EGF allows accurate determination of fu for very highly bound drugs and differentiation even above 99.9% of binding. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:752–759, 2014
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INTRODUCTION

The binding of drugs to plasma proteins influences their organ distribution as well as clearance processes.[1] When comparing systemic exposure between species in the context of safety assessments or for prediction of a human efficacious dose, species differences in plasma protein binding should be considered. Similarly, to assess the need for a dose adjustment in a special population (e.g., renal or hepatic impaired patients), not only differences in total exposure per dose, but also differences in plasma protein binding to the reference population are important. The reason is, as postulated in the free drug hypothesis, that target exposure to the free and not to the total drug concentration causes an effect. In general, the steady-state free plasma concentration is considered a surrogate for the free concentration in the tissue of interest.[2] Although plasma protein binding is an important parameter and differences between species and populations are critical to know for dose selection, the importance of plasma protein binding has also been overstated in some contexts. Changes in plasma protein binding, for example, because of displacement by other drugs (drug interaction) or disease progression mostly do not require a dose adjustment.[3] Typically corresponding changes in organ distribution and clearance keep the relevant free exposure largely unchanged, whereas the total exposure may change. Similarly, plasma protein binding should not be considered *per se* as a selection criterion in drug discovery, as differences in plasma protein binding mostly affect total systemic exposure, not the pharmacologically relevant free exposure.[2]

A variety of methods are available to measure plasma protein binding. Among them, the commonly used equilibrium dialysis (ED), ultrafiltration (UF), and ultracentrifugation (UC) are the subjects of many reviews.[4–7] Less frequently used methods such as solid-phase microextraction,[8] erythrocytes partitioning, distribution between plasma and solid-supported lipid membranes,[9] microdialysis,[10] high-performance frontal analysis,[11] high-performance affinity chromatography with albumin,[12] circular dichroism, and optical biosensors were also discussed. Although assessing plasma protein binding is conceptually easy, it becomes experimentally challenging, when the free fraction is very low (<2%), which might be the case for 20%–30% of drugs.[6] The challenge is not primarily the high sensitivity needed to measure low free concentrations, but to avoid the experimental bias the different methods used to separate bound from free drug can create.[13] Highly protein-bound drugs are typically hydrophobic and frequently bind to walls and membranes of an apparatus in an aqueous environment. That is not a problem in plasma, where binding is mostly to proteins, but makes separation through membranes difficult. Also, once the free fraction is separated from the plasma proteins, nonspecific binding to the wall may become relevant. When employing radiolabeled compounds and standard separation methods to determine protein binding, radiolabeled impurities with a low protein binding will enrich in the plasma water and can cause a strongly biased apparent fu for very highly bound drugs.[14,15]

We applied equilibrium gel filtration (EGF)[16,17] in a new set-up to determine species differences and fu's for very highly bound drugs. We routinely use this method for approximately 30% of compounds moving into clinical development, that are not accessible to UF (applicable for about half of the compounds based on control experiments testing free permeation and

Abbreviations used: AGP, alpha 1-acid glycoprotein; ED, equilibrium dialysis; EGF, equilibrium gel filtration; fu, unbound fraction in plasma; LSC, liquid scintillation counting; PBS, phosphate-buffered saline; UC, ultracentrifugation; UF, ultrafiltration.

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recovery) and ED (works for another 20% based on control experiments testing time to equilibrium and recovery). EGF has a principle difference to the standard methods ED, UF, and UC: the free and not the total drug concentration is the independent variable of the experiment. This is an advantage for very highly bound drugs, as any difference in binding will cause a change in the big signal of the amount bound, when binding at a chosen free concentration is compared.

MATERIALS AND METHODS

Compounds

All radiolabeled compounds were synthesized in the laboratories of Novartis Pharma. Stock solutions were prepared in ethanol.

Biological Material

Plasma was obtained from male animals: Hanover–Wistar rats, Beagle dogs, Göttingen minipigs, and Cynomolgus monkey. Human plasma was obtained from healthy male volunteers. Pooled plasma of at least three male individuals was obtained by centrifugation of fresh blood containing ethylenediaminetetraacetic acid and stored at −20°C until use. The pH of plasma samples was measured and adjusted to approximately 7.4 if relevantly different from 7.4.

Measurement of Radioactivity

The radioactivity concentrations were measured using a Packard Tricarb liquid scintillation counters (Packard Instrument Inc, Downers Grove, IL, USA). Data were converted from Bq/g to ng/mL assuming a density of 1.00 g/mL for all samples and using the specific radioactivity of the test compound.

Protein-Binding Measurements

Ultrafiltration

To assess the suitability of UF using the Centrifree® system (Amicon Inc., Beverly, Massachusetts; molecular cutoff of 30 kDa), the recovery and free permeation of compounds in phosphate-buffered saline (PBS) was investigated. If compounds showed good recovery and good free permeation through the membrane (at least 70%) when centrifuged in PBS, UF was used. The corresponding stock solutions were directly spiked (1:200, v/v) into plasma to get the intended final concentrations of 10–10,000 ng/mL. The spiked plasma was incubated at 37°C for 30 min under constant gentle agitation. Aliquots of the spiked plasma samples ($n = 3$) were transferred into prewarmed Centrifree® devices, which were centrifuged at 2000 *g* for approximately 10 min at 37°C. The radioactivity concentrations were determined in the ultrafiltrate (free concentration: C_f) and in the sample introduced into the reservoir before UF (total concentration: C_t).

Equilibrium Dialysis

The time needed to reach equilibrium between the two compartments of the ED device (Teflon 96 well ED block using 12–14 kDa dialysis membranes; HTDialysis, Gales Ferry, Connecticut), as well as recovery was determined in an initial experiment in PBS (points of time: 0.5, 1, 2, 4, 6, 8, and 24 h). If compounds showed a recovery of at least 80% and an equilibration time of less than 8 h ED was employed. The corresponding stock solutions were directly spiked (1:200, v/v) into plasma to get the intended final concentrations of 10–10,000 ng/mL. Aliquots of spiked plasma (0.15–0.2 mL) were dialyzed against identical volumes of PBS for the determined time period at 37°C ($n = 4$ for each concentration and species). After dialysis, radioactivity concentrations were determined in the buffer compartment (C_f) and in the plasma compartment (Ct).

Ultracentrifugation

Because of the poor recovery and poor free permeation in UF and ED, UC was applied to determine fu of Fingolimod. Diluted plasma (1:20 in PBS) was spiked with the stock solutions (1:200, v/v) resulting in final concentrations of 0.1–300 ng/*g* for Fingolimod. The spiked samples were incubated for 5 min at 37°C before centrifugation (16 h, 200,000*g*, 37°C). The concentration of the test compound was determined before centrifugation (Ct) and in the supernatant (plasma water layer under the lipoprotein layer, Cf) after centrifugation.

Equilibrium Gel Filtration

For the analysis of total binding two 5 mL HiTrap Desalting columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were used in series. The columns were equilibrated with PBS containing the radiolabeled compound under investigation at the chosen concentrations for 16–24 h. Equilibration was at 37°C and 0.2–1 mL/min, at least until a stable compound concentration eluted. Any concentration can be chosen that is detectable with the analytical method, but because this is the free concentration of the experiment, it should be low. For the characterization of a new compound (fu, species differences, and concentration dependency) typically three concentrations in the range of 0.1–50 ng/mL are selected. Starting with the lowest concentration, the same two columns are equilibrated consecutively to the different concentrations tested, usually with an overnight equilibration to a new higher concentration. At each compound concentration, plasma samples from different species are injected alternatingly to limit the impact of any inter-run variability on the assessment of species differences. For the validation experiments (Table 1), only one or two concentrations were chosen because concentration dependency was known. Fibrin in plasma was removed by centrifugation for 10 min at 10,000*g*. As needed, cleared plasma was diluted with PBS, frozen in aliquots, and thawed before injection. Plasma was injected at volumes/dilutions chosen to ensure that a binding equilibrium was achieved on the column (volumes typically corresponding to 12.5–100 μL of undiluted plasma, lower plasma volumes for compounds displaying higher binding). Plasma (30–200 μL) was injected into the equilibrated gel filtration column and run at 0.2–0.4 mL/min. The eluate was analyzed for total protein by UV absorption (280 nm) and for total radioactivity in collected fractions by liquid scintillation counting (LSC) in a Packard Tricarb liquid scintillation counter. Fractions were collected in tubes suitable for LSC to avoid transfer steps and potential losses because of adsorption. Drug concentrations were determined by LSC using the respective specific radioactivities. Quantification can also be performed by liquid chromatography-mass spectrometry (LC/MS); in principle only two samples per run need to be analyzed: in our setup, for example, the fraction 0–3 mL to provide the free concentration and 3–6 mL for the amount bound.