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Application of Cassette Ultracentrifugation Using Non-labeled Compounds and Liquid Chromatography-Tandem Mass Spectrometry Analysis for High-Throughput Protein Binding Determination

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ABSTRACT

Membrane-based devices typically used for serum protein binding determination are not fully applicable to highly lipophilic compounds because of nonspecific binding to the device membrane. Ultracentrifugation, however, completely eliminates the issue by using a membrane-free approach, although its wide application has been limited. This lack of utilization is mainly attributed to 2 factors: the high cost in acquiring and handling of radiolabeled compounds and low assay throughput owing to the difficulties in process automation. To overcome these challenges, we report a high-throughput workflow by cassette ultracentrifugation of nonradiolabeled compounds followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Twenty compounds with diverse physicochemical and protein binding properties were selected for the evaluation of the workflow. To streamline the working process, approaches of matrix balancing for all the samples for LC-MS/MS analysis and determining free fraction without analytical calibration curves were adopted. Both the discrete ultracentrifugation of individual compounds and cassette ultracentrifugation of all the test compounds followed by simultaneous LC-MS/MS analysis exhibited a linear correlation with literature values, demonstrating respectively the validity of the ultracentrifugation process and the cassette approach. The cassette ultracentrifugation using nonradiolabeled compounds followed by LC-MS/MS analysis has greatly facilitated its application for high-throughput protein binding screening in drug discovery.

Introduction

Protein binding is typically part of an ensemble of *in vitro* profiling assays for new chemical entities (NCEs) in drug discovery because of its profound impact on drug pharmacokinetics.[1,2] The significance of free fraction determination lies in the understanding that only unbound drug molecules can permeate through cell membranes to reach therapeutic targets to produce the expected pharmacologic actions.[3] Moreover, only the free drugs can penetrate into the clearance organs such as liver for metabolism and kidney for excretion.[4,5] The restrictive effect of high serum protein binding on blood-brain barrier penetration also makes the protein binding determination a key assessment for the blood-brain barrier penetration of drug molecules.[6,7]

Among various methods used for protein binding determination, equilibrium dialysis[8] and ultrafiltration[9] are most widely adopted. The working mechanism for both techniques is based on a semipermeable filter membrane to separate the free fraction of drug molecules from the protein bound portion. Ultrafiltration is simple in setup and operation generating biological samples for concentration determination in a much shorter time frame of under 30 min than other techniques, making ultrafiltration a suitable methodology for chemically or metabolically labile compounds.[10] Equilibrium dialysis is especially well received by organizations performing high-throughput protein binding screening owing to its easy automation by using plate-based devices such as HTDialysis (HTD)[11] and rapid equilibrium dialysis (RED)[12] via liquid handling tools. However, the use of dialysis membrane in both devices introduces nonspecific binding,[13,14] represented by poor recovery of test compounds after dialysis. The issue of absorption to the membrane is especially problematic for lipophilic compounds such as cyclosporine A[15] and some platinum derivative drugs.[16] In ultrafiltration,

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the extent of nonspecific binding can be determined by centrifuging the ultrafiltrate spiked with a known concentration of test compound and then measuring the compound loss to correct the free fraction data obtained for the compound. However, the results are considered less reliable when the nonspecific binding is >20% of the spiked compound concentration.[17] These challenges have made membrane-based devices an unsuitable technique for protein binding determination for some compounds. In our laboratory, it has been also observed that permeation of lipophilic compounds through the dialysis membrane could be obstructed resulting in an artificially lower free fraction determined, even though the extent of nonspecific binding is negligible as indicated by a full recovery of compounds after equilibrium dialysis. Although the mechanism for the hindered permeation is not completely understood, the biased free fraction determined could mislead the prediction of *in vivo* therapeutic index and interpretation of pharmacologic effect.

As an alternative to the membrane-based devices for protein binding determination, ultracentrifugation uses a membrane-free approach, separating compound-spiked plasma into phases via a differential sedimentation of plasma components based on their molecular weight. The 3 main phases from the separation are generally categorized as top layer containing chylomicrons and very-low-density lipoprotein, middle aqueous protein-free layer containing unbound drug molecules, and bottom serum gradient and protein sediment containing bound drug molecules. After ultracentrifugation, the fraction of free drug in the middle aqueous layer relative to the starting concentration in serum is determined for protein binding assessment. Although not as widely adopted as equilibrium dialysis, ultracentrifugation has demonstrated its utility for protein binding evaluation over the years, displaying good correlation in data generated with those from equilibrium dialysis and ultrafiltration,[18] as well as good correlation with *in vivo* data.[19] Ultracentrifugation has been particularly used for lipoprotein bound compounds, which tend to be more lipophilic and susceptible to nonspecific binding in the membrane-based apparatus.[20,21] In addition to eradicating potential absorption to the dialysis membrane, ultracentrifugation also has the added capability of determining binding to specific proteins of interest, for instance, distinguishing binding to lipoproteins and albumin via quantitation of bound drugs in the respective binding proteins, which are separated into individual phases through ultracentrifugation.[16] This unique capability suits the need for protein binding characterization versus binding screening. The data obtained on binding to specific proteins via ultracentrifugation are not achievable through equilibrium dialysis or ultrafiltration because the protein-containing phase is a homogeneous mixture of all the proteins rather than a molecular weight–based protein gradient as in the ultracentrifugation.

There are several factors that have limited the wide application of ultracentrifugation. First, ultracentrifugation conventionally uses radiolabeled compounds, and radioactivity is measured for quantitation and thereafter free fractions.[14,18,22,23] The utilization of radiometric reading, though perceived to provide the most accurate measurement of testing compounds, limits extensive application of the method in drug discovery when radiolabeling is not readily available, let alone the complicated and costly handling and disposal of radiolabeled compounds. Second, unlike plate-based equilibrium dialysis devices such as HTD and RED which are designed to give access to multichannel liquid handling tools for parallel sample preparation and incubation, centrifuge tube–based ultracentrifugation is confined to manual handling. Finally, limited by the number of tubes that can be accommodated in a centrifuge device, ultracentrifugation yields a much lower throughput than desired, making it far less attractive for high-throughput applications in drug discovery.

In this study, we evaluated the feasibility of ultracentrifugation using nonradiolabeled compounds, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis for protein binding determination as an alternative to equilibrium dialysis. In addition, we evaluated the approach of cassette ultracentrifugation for high-throughput screening application. Twenty marketed compounds with diverse molecular weight, physicochemical properties, and serum free fraction were selected for the evaluation of the approach (Table 1). Preassay compound pooling for protein binding determination has been previously evaluated for both ultrafiltration[24] and equilibrium dialysis,[25,26] demonstrating acceptable correlation of free fractions determined between the discrete and pooled processes. To our knowledge, this is the first study assessing a cassette approach for ultracentrifugation. To evaluate the validity of LC-MS/MS as the analytical readout, free fraction values derived from side-by-side ultracentrifugation of radiolabeled compounds followed by liquid scintillation counting and their nonradiolabeled counterparts followed by LC-MS/MS analysis were compared. The applicability of ultracentrifugation to protein binding determination was evaluated by comparing free fractions obtained for the selected compounds from the discrete ultracentrifugation with literature values generated by equilibrium dialysis or ultrafiltration. The cassette approach was assessed by evaluating the correlation of free fractions generated with literature values as well. The applicability of ultracentrifugation for protein binding determination, especially for compounds not amenable to membrane-based devices, as well as ultracentrifugation in cassette mode for high-throughput protein binding screening in drug discovery are discussed.

Experimental

Chemicals and Reagents

HPLC grade water and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ). Formic acid and dimethyl sulfoxide (DMSO) were from EMD Chemicals (Gibbstown, NJ). All the test compounds were purchased from Sigma (San Jose, CA). Human serum was purchased from Gemini Bio-Products (West Sacramento, CA).

Serum Shift Assay

The effect of human serum and its components on the antiviral activity of the compounds was determined by infecting MT-2 cells

Table 1
Physicochemical Property of Selected Compounds

Compound Name	Molecular Weight	LogP[a]	Pka[a] (Strongest Acidic Site)
Atenolol	266	0.57	14.08
Carbamazepine	236	2.10	15.96
Chlopromazine	318	5.18	–
Chlorpheniramine	274	3.74	–
Desipramine	266	4.02	–
Dexamethasone	392	1.93	12.42
Digoxin	780	1.04	7.15
Diltiazem	414	3.09	12.86
Erythromycin	733	2.37	12.44
Haloperidol	375	3.70	13.96
Imipramine	280	4.53	–
Ketoconazole	530	4.30	–
Minaprine	298	2.15	19.25
Nicardipine	479	4.34	–
Phenytoin	252	2.26	9.47
Propranolol	259	3.03	14.09
Ritonavir	720	4.24	13.68
Timolol	316	1.44	14.08
Verapamil	454	5.23	–
Warfarin	308	2.41	6.33

[a] Data from Drug Bank, calculated values of ALOGPS and ChemAxon.