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Compilation of 222 drugs' plasma protein binding data and guidance for study designs

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The binding of a drug to plasma protein reduces free drug in the blood circulation that would otherwise be available for penetration into tissues to reach the therapeutic target or the kidney for elimination. Therefore, the binding event affects drug elimination from the body, efficacy, duration of action and toxicity. Co-administration of other drugs, food and pathological conditions of patients can significantly change percentage binding of the drug and result in serious consequences. Here, we present the largest and newest information on plasma protein binding for 222 drugs, of which 50% show 90–100% binding, a range that could be considered as a favorable element for future lead selection. We also provide critical and comprehensive evaluations on the methods and techniques established to determine plasma protein binding, pinpoint advantages and pitfalls of individual approaches, and offer detailed guidance for experimental designs, including ultrafiltration, equilibrium dialysis, ultracentrifugation, charcoal adsorption, high-performance affinity chromatography, high-performance frontal analysis, solid-phase microextraction and *in vivo* microdialysis.

Introduction

Binding of drugs to plasma proteins is one of many factors that influences drug ADME [1]. Binding of a drug to plasma protein reduces free drug available to penetrate from the blood circulation into tissues to reach the therapeutic target or the kidney for elimination. It is generally accepted that the effect of a drug is related to the exposure of a patient to the unbound concentration of the drug at its action site rather than its total concentration [2]. Drugs can bind to a variety of blood constituents, including albumin, α_1-acid glycoprotein, lipoproteins, red blood cells, leukocytes, platelets and α-, β- and γ-globulins. The interaction of a drug with plasma proteins is electrostatic, hydrophobic, satiable and reversible.

Two plasma proteins are most responsible for binding of drugs: human serum albumin (HSA) and α_1-acid glycoprotein, although some lipophilic basic and neutral drugs (e.g. probucol and etretinate) tend to bind to lipoproteins [very-high-density lipoprotein (VHDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL)].

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HSA has multiple hydrophobic binding sites (e.g. a total of eight for fatty acids) [3], and binds a diverse set of drugs. HSA primarily binds to organic anions (e.g. carboxylic acids, phenols) but it also binds to basic and neutral drugs. Binding to albumin is widely assumed to be merely dominated by the lipophilicity of drugs [4], although it has been argued that lipophilicity is rather poorly correlated to serum albumin binding for a diverse set of molecules, in contrast to congeneric series, where lipophilicity is found to be the dominant factor. It is suggested that specific molecular recognition elements besides physicochemical parameters are essential [5]. HSA is a 66 kDa globular protein of 585 amino acids in a single polypeptide chain. It is the major protein component of plasma and constitutes ~4.5% of the weight of human blood (3.8–4.8 g/100 ml). HSA is the most abundant protein in plasma (i.e. 60% of the total plasma protein) with a concentration ranging from 500 to 750 μM (35–50 mg/ml). HSA is capable of binding endogenous ligands (e.g. fatty acids and metal ions) as well as drugs. There are multiple drug-binding sites on HSA, although two appear to predominate and there is a slight preference for binding acidic drugs. Considering the high concentration of albumin in blood plasma, the free drug available for therapeutic action can be

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effectively reduced for drugs with high binding to plasma proteins, although the affinity of drugs for plasma proteins is often less than for the receptor or enzyme targets. The primary physiological functions of HSA are to maintain blood pH and osmotic pressure and transport molecules throughout the body.

α_1-Acid glycoprotein primarily binds basic drugs (e.g. amines) and it also binds hydrophobic compounds (e.g. steroids). The concentration of α_1-acid glycoprotein in the blood is 15 μM (0.5–1.0 mg/ml). α_1-Acid glycoprotein consists of 181 amino acids in a single polypeptide chain and has a molecular weight (MW) of 44 kDa. It has high carbohydrate content (i.e. 45% of total mass) and an acidic isoelectric point ~3. α_1-Acid glycoprotein has one binding site per molecule that binds drugs primarily by nonspecific hydrophobic interactions. α_1-Acid glycoprotein concentration in blood is thought to be much more sensitive to some disease conditions than HSA. Its primary function is to carry steroids throughout the body.

Drug–plasma protein complexes in the plasma serve as drug reservoirs for the free drug concentration, because the drug is removed from the body by various elimination processes, and prolong the duration of drug action. The drug–plasma protein complex cannot permeate through cell membranes by passive transcellular or paracellular permeation. Only free drug passes through membranes to reach tissues, and only free drug molecules are available for liver metabolism and renal excretion. There are two complementary factors of plasma protein binding (PPB): (i) degree of binding at equilibrium, which is expressed as percent bound or percent unbound in plasma, or equilibrium dissociation constant K_d; and (ii) rate of association and dissociation, which is expressed as association and dissociation rate constants K_a and K_d, respectively. These factors affect the ADME of a drug [6]. For example, if the drug is highly bound (i.e. low percent unbound) and tightly bound (i.e. slow dissociation) to plasma proteins, the effect of the binding on the ADME of the drug can be 'restrictive' to drug retention in plasma and drug distribution into target tissue, can decrease metabolism and clearance but increase half-life, drug brain penetration and the requirement for higher loading doses but lower maintenance doses. By contrast, if the drug has high percentage binding and a fast dissociation rate, low percentage binding and a slow dissociation rate, or low percentage binding but a fast dissociation rate then the effect of the binding on ADME can be 'nonrestrictive' or 'permissive'. High percentage binding can be restrictive or permissive to liver extraction. For instance, propranolol has >90% binding but is permissive to >90% liver extraction. However, warfarin, with 99% binding, is restrictive to <0.3% liver extraction [7].

To determine the binding capacity and affinity of plasma protein to a drug, it is suggested that at least three concentrations of the investigational drug (e.g. 0.1, 0.5 and 2.5 μg/ml, the often-observed *in vivo* blood concentrations of most drugs) be applied and tested [8–10] to clarify concentration-related changes in percentage binding. In general, the percentage binding should not be affected by the changes in the drug concentrations within a limited range [11]. Plasma protein contains at least two independent binding sites with different intrinsic affinities: (i) high affinity, low capacity; and (ii) low affinity, high capacity. It has shown nanomolar binding affinity for high capacity and micromolar affinity for low capacity of the binding sites [11]. The increase in free fraction with increased total drug concentration in blood can imply saturation of the predominant binding site. A decline in plasma protein concentration can result in a new equilibrium and hence a decrease in the bound fraction.

In vivo duration of drug action not only depends on macroscopic pharmacokinetic (PK) properties, such as plasma half-life and the time needed to equilibrate between the plasma and the effect compartments, but is also influenced by long-lasting target binding and rebinding when there is no longer sufficient free drug around to maintain high levels of receptor occupancy [12]. There is not always a direct link between slow dissociation and long-lasting *in vivo* target protection, because the rate of free drug elimination from the effect compartment is also a key influencing factor. Local phenomena that hinder the diffusion of free drug molecules away from their target can cause them to bind consecutively to the same target and/or targets nearby (denoted as 'rebinding'), even when their concentration in the bulk phase has already dropped to insignificant levels [13].

It can be estimated that there are ~1300 frequently described active pharmaceutical ingredients (APIs) and their corresponding formulations [14–16] in the global pharmaceutical market today. Table 1 reports percentage of protein binding for some APIs. This dataset was extracted from the literature [5,8,17–20], and is the largest and the newest data pool of drug–plasma protein binding currently available. No general rules or a trend for protein binding can be derived from physicochemical properties of the drugs. Retrospective analysis of the distribution of the percentage plasma protein binding (Table 1) revealed that 50% of these drugs possess plasma protein binding higher than 90% (Fig. 1), an important feedback obtained from the current analysis of the 222 drugs. The data indicated that good plasma binding had been considered as a favorable element by pharmaceutical developers when they determined a candidate. This consideration should be taken forward into the future lead selection process to avoid pitfalls.

There are numerous methods available for measuring plasma protein binding including ultrafiltration, ultracentrifugation, equilibrium dialysis, high-performance frontal analysis, solid-phase microextraction (SPME), charcoal adsorption and *in vivo* microdialysis, which will be discussed below. The different methods and techniques in this field should be used correctly so that experimental results can be compared and interpreted. We provide our critical and comprehensive overview on these methods, pinpoint advantages and pitfalls of individual approaches and offer the guidance for experimental designs.

Ultrafiltration

This method uses either individual sample vials (Centrifree®) or a 96-well ultrafiltration device (MultiScreen® Ultracel-PPB) to separate free drug from its binding fraction of plasma protein. An aliquot of plasma sample is loaded into the upper chamber of an ultrafiltration apparatus that has a membrane with a certain MW cut-off (MWCO). The device is centrifuged and the solution is ultrafiltered through the membrane by the force of the centrifugation. Unbound drug moves with the liquid through the membrane into the receiver chamber, whereas drug bound to plasma protein remains in the loading chamber. Ultrafiltration is conducted under the assumptions that: (i) the drug does not bind to the membrane; (ii) there is no leakage of plasma protein through the

TABLE 1

Percentage (f_b %) of plasma protein binding of 222 drugs and compounds and their molecular weight

Drugs	MW	f_b (%)	Refs
3-Carboxy-4-methyl-5-propyl-2-furan-propionic acid	240.28	99	[5]
Acenocoumarin	353.35	99	[5]
Acetaminophen	151.17	0	[18]
Alprazolam	308.76	71	[32]
Alprenolol	249.39	85	[5]
Amiloride	229.63	40	[18]
Amiodarone	645.31	99.98	[18]
Amobarbital	226.31	61	[32]
Amphotericin B	924.08	>90	[18]
Aspirin	180.17	55	[5]
Atenolol	266.34	<5	[18]
Atracurium besilate	929.15	82	[18]
Azapropazone	300.40	99	[5]
Benoxaprofen	301.74	99	[5]
Benzylpenicillin	334.42	65	[5]
Betamethasone	392.46	64 ± 6	[18]
Bilirubin	584.73	99.9	[5]
Binedaline	293.45	96	[5]
Bromazepam	182.09	60	[32]
Budesonide	430.53	88	[18]
Bumetanide	364.41	99 ± 0.3	[18]
Bupivacaine	288.48	95 ± 1	[18]
Buprenorphine	467.61	96	[5]
Bupropion	239.74	84 ± 2	[18]
Camptothecin	348.38	98.3	[5]
Captopril	217.29	30 ± 6	[18]
Carbamazepine	236.29	76	[5]
Carbendazim	191.18	60–74	[6]
Carbenicillin	378.40	50	[18]
Carbenoxolone	570.84	99	[5]
Carboplatin	371.25	0	[18]
Carprofen	273.73	99	[5]
Cefaclor	367.83	24.7	[5]
Cefadroxil	363.42	20	[5]
Cefamandole	462.54	74	[18]
Cefazolin	454.54	87.5	[5]
Cefixime	453.45	67 ± 1	[18]
Cefonicid	542.57	98	[18]
Cefoperazone	645.73	91	[5]
Ceforanide	519.56	80–82	[18]
Cefotaxime	455.18	38	[5]
Cefotetan	576.62	85 ± 4	[18]
Cefoxitin	427.48	76.5	[5]
Cefsulodin	532.58	45	[5]
Ceftazidime	546.62	15.5	[5]
Ceftriaxone	554.62	93.8	[5]
Cefuroxime	424.42	31.5	[5]
Cephalexin	347.42	14.5	[5]
Cephaloridine	415.51	20	[5]
Cephalothin	396.46	68	[5]
Cephapirin	423.49	62	[5]
Cephradine	349.44	12	[5]
Cetiedil	349.58	74	[5]
Chlordiazepoxide	299.75	96.5 ± 1.8	[18]
Chlorothiazide	295.73	82.3	[5]
Chlorpromazine	318.89	97.8	[5]
Chlorpropamide	276.76	96	[5]
Cicletanine	261.72	93.5	[5]
Cimoxatone	338.39	94.5	[5]
Clofibrate	242.70	96.5 ± 0.3	[18]
Clofibric acid	214.66	98	[5]
Clometacin	357.81	99	[5]
Clonazepam	315.72	86 ± 0.5	[18]
Clozapine	326.84	95	[32]
Dapsone	248.30	73 ± 1	[18]
Desipramine	266.42	86	[5]
Desmethyldiazepam	270.71	97.5	[18]
Diazepam	284.76	99	[5]
Diclofenac	296.16	99.5	[5]
Diflunisal	218.21	99	[5]
Digitoxigenin	374.57	92.7	[5]
Digitoxin	765.05	93.5	[5]
Digoxin	780.94	25 ± 5	[18]
Disopyramide	325.18	89	[5]
Doxazosin	451.47	98.9 ± 0.5	[18]
Doxycycline	444.48	90.5	[5]
Duloxetine	297.41	95	[5]
Ethambutol	204.31	<5	[18]
Ethinyl estradiol	296.40	95–98	[18]
Ethosuximide	141.17	0	[18]
Etodolac	287.39	99.1	[5]
Etoposide	588.56	96 ± 0.4	[18]
Fenbufen	254.30	98.5	[5]
Fenoprofen	242.29	99	[5]
Fentiazac	329.81	99.5	[5]
Flucytosine	129.09	4	[18]
Fluindione	240.24	99.5	[5]
Flunitrazepam	313.29	79	[32]
Fluorouracil (5-FU)	130.08	11	[5]
Fluoxetine	309.33	94	[18]
Flurazepam	387.88	96.6	[18]
Flurbiprofen	244.28	99	[5]
Fulvestrant	606.77	99	[5]
Furosemide	330.76	98.4	[5]
Fusidicacid	516.79	94.8	[5]

TABLE 1 (*Continued*)

Drugs	MW	f_b (%)	Refs
Gentamicin	477.59	<10	[18]
Glipizide	445.54	98.4	[18]
Halofenate	415.82	99.5	[5]
Haloperidol	375.90	92	[18]
Heroin	369.41	35 ± 2	[18]
Hexobarbital	236.27	42–52	[18]
Ibuprofen	206.31	99	[5]
Imipramine	280.45	92.6	[5]
Indomethacin	357.81	90	[18]
Indoprofen	281.33	98	[5]
Isotretinoin	300.44	99.9	[18]
Itanoxone	300.75	98.5	[5]
Ketoconazole	531.43	99 ± 0.1	[18]
Ketoprofen	254.30	99.2 ± 0.1	[18]
Labetalol	328.41	50	[18]
Levonorgestrel	312.45	37 ± 7	[18]
Lidocaine	234.38	67	[5]
Lomefloxacin	351.35	10	[18]
Loracarbef	349.77	25	[18]
Loratadine	382.88	97	[18]
Lorazepam	321.16	91 ± 2	[18]
Lorcainide	370.92	85 ± 5	[18]
Lovastatin	404.54	95	[18]
Mefloquine	378.31	98.2	[18]
Melphalan	305.20	90 ± 5	[18]
Mepivacaine	246.39	84	[5]
Methicillin	380.45	39 ± 2	[18]
Methohexital	262.31	73	[32]
Methotrexate (RU486)	454.18	46 ± 11	[18]
Metoclopramide	299.80	40 ± 4	[18]
Metocurine	652.82	35 ± 6	[18]
Metronidazole	171.15	11 ± 1	[18]
Midazolam	441.84	95	[32]
Mifepristone	429.59	98	[15]
Meperidine	247.33	58 ± 9	[18]
Morphine	285.34	35 ± 2	[18]
Moxisylyte	279.42	26	[5]
Nabumetone	228.29	>99	[18]
Nadolol	309.40	20 ± 4	[18]
Nafcillin	414.51	89.4	[5]
Nalidixic acid	232.26	90–94	[5]
Naproxen	230.28	99.4	[5]
Nicardipine	479.52	98–99.5	[18]
Nicergoline	484.43	95	[5]
Nimesulide	308.33	99	[5]
Norepinephrine	169.20	50	[5]
Nortriptyline	263.41	93.5	[5]
Novobiocin	612.69	90	[5]
Octreotide	1019.24	65	[18]

TABLE 1 (*Continued*)

Drugs	MW	f_b (%)	Refs
Omeprazole	345.40	95	[18]
Oxazepam	286.73	98.4	[5]
Oxyphenbutazone	324.41	99	[5]
Paclitaxel (Taxol)	853.91	95–98	[18]
Phenobarbital	232.26	51 ± 3	[18]
Phenylbutazone	308.41	97.8	[5]
Phenylethylmalonamide	206.24	8 ± 1	[18]
Phenytoin	252.29	91	[5]
Pindolol	248.32	51	[32]
Piperacillin	517.55	30	[18]
Pipotiazine	475.72	45	[5]
Piretanide	362.43	94	[5]
Pirprofen	251.73	99.8	[5]
Practolol	266.38	30	[5]
Pravastatin	424.53	43–48	[18]
Prazosin	383.40	95 ± 1	[18]
Prednisolone	360.44	90–95	[18]
Pregnenolone	316.53	80	[5]
Procaine	236.35	6	[5]
Propanolol	295.34	87	[5]
Propofol	178.27	95–99	[32]
Propranolol	259.38	87 ± 6	[18]
Quinapril	438.52	97	[18]
Quinidine	324.42	87 ± 3	[18]
Quinine	324.46	93	[5]
Ramipril	416.51	56	[18]
Ribavirin	244.21	0	[18]
Rifabutin	847.01	85 ± 2	[18]
Rifampin	822.94	89 ± 1	[18]
Rimantadine	179.30	40	[18]
Risperidone	410.48	89	[18]
Salicylic acid	138.13	95	[5]
Selegiline	187.28	94	[18]
Sertraline	306.23	99	[18]
Simvastatin	418.57	94	[18]
Sotalol	272.40	17.5	[5]
Streptomycin	581.574	48 ± 14	[18]
Sufentanil	386.55	93 ± 1	[18]
Sulfamethoxazole	253.30	66	[5]
Sulfaphenazole	314.39	95	[5]
Sulfinpyrazone	404.48	98.3 ± 0.5	[18]
Sulfisoxazole	267.33	91.4	[5]
Sulfobromophthalein	796.04	99.9	[5]
Sulindac	356.43	93.5	[5]
Suprofen	260.32	20	[5]
Tacrine	198.26	55	[18]
Tamoxifen	563.65	>98	[18]
Temazepam	300.74	96.8	[5]
Temezepam (R/S)	349.61	97	[32]

TABLE 1 (*Continued*)

Drugs	MW	f_b (%)	Refs
Teniposide	656.65	>99	[18]
Terbutaline	225.28	20	[18]
Testosterone	288.47	60	[5]
Tetracycline	444.48	18	[5]
Tetrahydrocannabinol	314.45	95	[18]
Theophylline	180.17	56 ± 4	[18]
Thiopental	242.37	82.5	[5]
Ticarcillin	384.43	65	[18]
Ticlopidine	263.80	96.5	[5]
Timolol	316.42	35	[32]
Tinoridine	316.45	92	[5]
Tobramycin	467.51	<10	[18]
Tocainide	192.26	10 ± 15	[18]
Tolazamide	311.44	97	[5]
Tolbutamide	270.38	96	[5]
Triamcinolone acetonide	434.18	40	[18]
Triamterene	253.26	61 ± 2	[18]
Triazolam	343.21	90	[32]
Trimethoprim	290.36	41.5	[5]
Tryptophan	204.25	80	[5]
Tubocurarine	624.765	50 ± 8	[18]
Urapidil	387.54	80	[5]
Valproic acid	144.24	93	[5]
Vancomycin	1449.30	30 ± 10	[18]
Venlafaxine	277.40	27 ± 2	[18]
Verapamil	454.67	90 ± 2	[18]
Warfarin	308.35	99	[5]
Zalcitabine	211.22	<4	[18]
Zomepirac	291.75	98.5	[5]

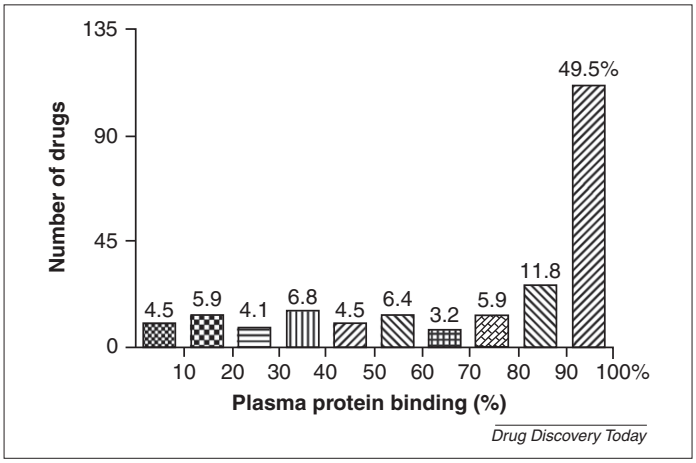

FIGURE 1

Distribution of plasma protein binding (%) for 222 drugs from different therapeutic indications including renal, cardiovascular, central nervous system, inflammation and chemotherapy. The number above each bar indicates the percentage of the 222 drugs that fall in the corresponding binding range. Note, nearly 50% of the 222 drugs show plasma protein binding 90%.

membrane; (iii) the equilibrium constant does not change as the protein is gradually concentrated during the separation process; and (iv) the membrane is equally permeable to the drug and water. In applying the individual vials, many researchers pre-set MWCO at 10,000 Da, drug concentration in plasma, serum or operation buffers at ~10 μM, and loading volume at 400 μl. After centrifugation at 2000 × *g* for 30 min, 60 μl of filtrate (typically less than one-fifth of the filtrate) could be collected [8,21]. The concentration of test drug in the receiver is quantitated, and the fraction unbound is calculated as this ultrafiltrate concentration is divided by the total initial concentration. Although ultrafiltration is a simple and rapid technique that is especially applicable to those unstable drugs, the major disadvantage of this technique is the nonspecific binding of drugs to filter membrane that is composed of cellulose acetate and a plastic device. It is thought that the nonspecific binding can sometimes affect 20–30% of the tested drugs. It is useful to perform mass balance studies to calculate the recovery to evaluate the degree of nonspecific binding to the apparatus.

The nonspecific binding problem can usually be overcome by pretreatment of the filter membrane with 5% Tween 80 (for hydrophobic and acidic drugs) or 5% benzalkonium chloride (for basic drugs) [22]. Tween 80 is a nonionic surfactant, and benzalkonium chloride is a cationic surfactant. Another way to decrease the nonspecific binding of drugs to the filter membrane and device is to 'siliconize' them by simply dipping them in pre-saturated simethicone (a classical siliconizing agent) suspension at 37°C (room temperature might work), and then rinsing them with deionized water before use. It is important in all cases to run a nonspecific binding control to justify the results. Using silanized glassware instead of plastic or synthetic apparatus could be another option.

Eqns (1)–(3) can be used to determine the NSB and % protein binding:

$$\mathrm{NSB} = \frac{C_{BD} - C_{BF}}{C_{BD}} \quad (1)$$

$$\mathrm{Fu} = \frac{C_{SF}}{(1 - \mathrm{NSB})/C_{SD}} \quad (2)$$

$$\%\mathrm{Protein\ binding} = (1 - \mathrm{Fu}) \times 100 \quad (3)$$

where NSB denotes nonspecific binding; C_{BD} the concentration of drug in buffer (PBS; or plasma water); C_{BF} the concentration of drug in filtrate after ultrafiltration of drug in buffer (or plasma water); C_{SD} the concentration of drug in serum (or plasma); C_{SF} the concentration of drug in filtrate after ultrafiltration of drug in serum (or plasma); and Fu is the free fraction of drug.

The plasma water can be prepared as follows [23]: plasma is added to preparatory centrifugal ultrafilters (Centriprep-10), and then the filtrate will be ultrafiltered again using the Centricon-10 to obtain protein-free plasma water. The tested drug can be added quantitatively to the plasma water, which is then equally divided into two aliquots, one for C_{BD} determination and another for C_{BF}.

Assuming binding to plasma proteins is an equilibrium (as opposed to a covalent and/or coordinate binding), and drugs encounter blood, not plasma alone, a drug is simultaneously partitioned into erythrocytes while binding and equilibrating with plasma protein. Schuhmacher *et al.* [24] have modified and improved a method [25] to exploit this situation. The technique is a little more labor-intensive than the standard techniques.

Equilibrium dialysis

Equilibrium dialysis is the most frequently used method and is generally accepted as the 'gold standard'. Fundamentally, one determines the partitioning of drug across a semi-permeable membrane between buffer and serum or purified serum components. Because the passive transit of a substance across a symmetric membrane is proportional to the concentration of that substance, equilibrium will be reached when the concentration of free drug is identical on each side of the membrane. Thus, the drug concentration on the buffer side is a reflection of free drug on the serum side of the membrane. This method serves as a reference technique against other methods such as ultrafiltration where nonspecific binding of drugs to the filtration membrane and other surfaces can cause experimental artifacts.

In conducting the experiment, plasma (0.5–1.0 ml) is added to equilibrium dialysis cells (made with natural cellulose with MWCO of 10,000 Da) with a 1 ml maximum capacity and/or cavity. The plasma compartment is dialyzed at 37°C against Sörensen phosphate buffer (67 mM, pH 7.4) or phosphate buffer saline using an equilibrium dialyzer (Spectra/Por® equilibrium dialyzer) or Dianorm dialyzer. There is no consensus made regarding to which compartment the test drug should be added. The test drugs have been added to the plasma side [26] or buffer side only [27], or equally to both sides [11,28]. Eqn (4) is applicable to the case where the test drug is only added to plasma side.

During equilibrium dialysis an osmotic water shift (also called volume shift) occurs such that the protein in the plasma compartment becomes diluted and the volume on the buffer side of the semi-permeable membrane decreases [27]. If a correction is not made for this volume shift (15–20%) then the concentrations of free and bound drug and the free fraction in nonlinear binding situations refer to lower protein and drug concentrations in the original plasma or serum. The volume shift can be checked by comparing initial plasma concentration with post-dialysis plasma concentration. It has been reported that within a short time period (e.g. 4.5 hours) the volume shift is not significant [28]. To overcome the volume shift problem, however, it has been recommended to add dextran (5% w/v) of high MW (40,000) to the buffer compartment [11]. The possibility of binding of the test drug to dextran should then be clarified by running a parallel experiment with and without the addition of dextran to the buffer side. In cases of linear binding without volume shift interfering Eqn (4) could be used to calculate the % binding.

$$\mathrm{Fu} = \frac{1}{(C_p/C_f) - R} \tag{4}$$

where Fu is the free fraction, C_p is the drug concentration in plasma before dialysis, C_f is the free drug concentration in the buffer compartment at dialysis equilibrium and R = (volume of buffer)/(volume of plasma), both before dialysis.

In running the equilibrium dialysis, several pitfalls that one must pay attention to are: (i) the drug stability at 37°C, the equilibrium dialysis usually requires a long time to reach the equilibrium. Thereby, drug stability under the assay conditions must be clarified; (ii) some drugs can precipitate with the plasma protein during the equilibrium dialysis. Recently, a high-throughput equilibrium dialysis method has been developed by Amika (Columbia, MD) using 10 kDa ultrathin semi-permeable membrane 96-well dispo-equilibrium biodialyzers. This method seems promising [11].

A rapid equilibrium dialysis device has recently been introduced [29] that needs a shorter preparation and dialysis time, and can be automated as a high-throughput assay for the determination of protein binding. Owing to the high membrane surface-to-volume ratio, which allows for rapid dialysis, equilibrium can be reached in four hours with high levels of reproducibility and accuracy. Incubation time can be as short as 100 min with proper agitation.

Ultracentrifugation

This technique avoids the nonspecific membrane binding [30–33]. Briefly, 50 μl of test drug at four concentrations (e.g. 0, 0.1, 0.5 and 2.5 μg/ml) was added to human plasma (10 ml). After vortex, each plasma sample (duplicated) is transferred into Beckman Optiseal polyallomer ultracentrifuge tubes (10 ml) and then subjected to centrifugation at 100,000 × *g* at 37°C for 24 hours. The drug stability in plasma under the same conditions should be checked before ultracentrifugation. The initial drug concentration in plasma and plasma water before spinning should be determined as the 'total plasma' and 'total free' drug concentrations, respectively. After centrifugation, the plasma is separated into three distinct layers [33]: solid protein pellet (lower); aqueous (middle); and lipoprotein (chylomicron) layer (upper). Each layer is isolated and transferred to individual tubes for analysis. The protein pellet stuck to the bottom should be completely dissolved using appropriate solution. Drug in each layer should be extracted, if needed, using either solid-phase extraction or liquid–liquid extraction before analysis. It is assumed that the amount of drug found in the aqueous layer represents the free or unbound fraction of drug, whereas the summation of the lipid and protein layers represents the bound fraction of drug. However, it would be more logical to use Eqn (5) to calculate the % binding of the drug to plasma protein [33].

$$\%\,\mathrm{bound} = \left(1 - \frac{\begin{array}{c}\text{concentration in post-centrifugation}\\ \text{middle layer of plasma}\end{array}}{\begin{array}{c}\text{concentration in post-centrifugation}\\ \text{middle layer of plasma water sample}\end{array}}\right) \times 100 \tag{5}$$

The drug concentration in plasma water before ultracentrifugation should be the same as the original total drug concentration in plasma before ultracentrifugation.

Charcoal adsorption

For those drugs that adsorb nonspecifically to the functional materials of dialysis cells and membranes of equilibrium dialysis and ultrafiltration equipment, an ultracentrifugation or charcoal adsorption method might be an option. The latter is useful for compounds exhibiting extensive nonspecific adsorption, or hav-

ing apparent lipid partitioning into LDL and VLDL [34]. The charcoal adsorption method for determining the extent of protein binding is based on the continuous removal (by adsorption by the charcoal) of unbound drug over a time period and determination of % bound drug at each time point. Association and dissociation of drug with proteins is a dynamic phenomena and charcoal acts as a sink for the removal of free drug. This method is more widely used to separate free and bound ligand in radioimmunoassays as compared with determination of the extent of protein binding [35]. The extent of protein binding (%) is estimated from the decline in percentage drug remaining in the supernatant after adding the charcoal. The method is versatile and can be used for determination of protein binding of similar drugs in different binding matrices such as plasma, serum and pure protein solutions.

Plasma samples are prepared before addition of charcoal to the samples at the concentration of 4 g/100 ml. The suspension is stirred at room temperature for eight hours and centrifuged overnight at 35,000 × *g* in a centrifuge (e.g. Beckman J2-HS) at 4°C. Plasma is filtered using nonsterile Acrodisc filters attached to a 20 cc syringe in the following sequence: 5 μm, 1.2 μm and 0.45 μm, to obtain purer drug-bound plasma protein. Khurana *et al.* modified the method by using dextran-coated charcoal [35]. The dextran-coated charcoal suspension is prepared by adding 600 mg of charcoal to 100 ml of Dulbecco's phosphate buffered saline containing 0.06% dextran. The mixture is stirred with a magnetic stirrer at room temperature until the charcoal is suspended. With continuous stirring 3 ml of charcoal suspension is added to serum. Serial samples (375 μl) are withdrawn from this continuously stirred mixture at different intervals into 0.6 ml polypropylene microcentrifuge tubes and centrifuged immediately at 15,000 × *g* at 37°C using a temperature-controlled microcentrifuge. Aliquots (100 ml in triplicate) of supernatant from each tube were immediately transferred into 3 ml glass centrifuge tubes and stored at 30°C until analyzed for drug content by an applicable analytical method such as HPLC.

The direct use of this method for protein-binding studies can give underestimates because: (i) dilution of protein solution occurs upon charcoal addition; (ii) K_a for protein binding is much lower than the K_a for antigen–antibody interactions; and (iii) drug–protein interaction is a dynamic phenomena and bound drug dissociates from proteins whereas the free drug is removed continuously by charcoal. The kinetic approach applied by Yuan *et al.* [34] and Khurana *et al.* [35], however, takes into account the dynamic feature of protein binding. In fact it takes advantage of this feature where only the % bound is determined at each time point and used to estimate the maximum binding possible at time 0.

High-performance affinity chromatography (HSA column)

When new techniques emerge old ones will be refined to incorporate the new ones, making analytical procedures more efficient with improved time and cost effectiveness. The high-performance affinity chromatography is such an example, using an HSA chromatography column in combination with UV and mass spectrometric detection to determine plasma protein binding [36]. The column contains immobilized albumin that is commercially available and can be used to differentiate binding to various sites. The HSA chiral stationary phase can be purchased from Hypersil (Runcorn, UK) using a 50 × 4.6 mm I.D. [37]. A moderate correlation (R^2 = 0.661) between the plasma protein binding, determined by traditional techniques of equilibrium dialysis or ultrafiltration, and chromatographic factor has been observed [19]. Essentially identical data had been obtained for compounds analyzed in singlet or cassette for minimally or highly bound (>90% bound) compounds. Analysis of cassettes containing compounds with plasma protein binding >90% did not cause column overload, even at drug concentrations up to 100 μg/ml [19]. Mass spectrometry offers the advantages of greater sensitivity and selectivity for the method, which increases throughput by combining several compounds in one cassette injection [38]. The improved sensitivity should enable analysis of a smaller amount of each compound, which would minimize errors from competitive displacement or column overloading [19]. However, the overall use of the HSA column model as an HTS device seems limited [19]. Another factor that should affect the general use of the HSA column is that acidic drugs have been shown to displace various test compounds bound to albumin but basic drugs have not [39].

High-performance affinity chromatography is based on examining the retention and competition of drugs as they pass through an immobilized HSA column, in which the zonal elution and frontal affinity chromatography or a combination of both are often used to examine association constants of various drugs. This method has been of particular interest in recent years owing to its notable advantages of high precision, automation, speed and good correlation compared with other traditional methods [40,41]. The principles of the method are described here.

First, the HSA support needs to be prepared by using the functional material, such as Nucleosil Si-300 silica. Generally, the Nucleosil Si-300 silica is converted into a diol-bonded form. This diol-bonded silica (2.0 g) is then converted to an aldehyde form through oxidation with sodium periodate (2.0 g) in 90% glacial acetic acid (20 ml). The HSA is then immobilized for four days in a cold room using a Schiff base method by mixing 0.5 g of aldehyde silica with 100 mg HSA in the presence of 50 mg sodium cyanoborohydride in 10 ml of potassium phosphate buffer (KPB) (67 mM, pH 7.4). The silica-containing HSA is then washed with PBS buffer, and treated with three portions of 10 mg sodium borohydride, respectively, to convert the excess aldehyde groups on the support into alcohol. The support is then washed several times with PBS buffer and stored in the cold room until use. Nonspecific interaction between a drug and the modified silica support is measured using a control support. The latter is prepared by the same Schiff base reaction except that no HSA is added during the immobilization step.

During chromatographic operation, the immobilized HSA silica and control support are downward slurry packed at 3000 psi (207 bar) into separate stainless steel columns (30 mm × 4.6 mm I.D.) using KPB as the packing solution. For high affinity HSA-binding compounds, a shorter column (10 mm × 2.1 or 4.6 mm I.D.) should be used. Each column should be placed onto a column oven for temperature control at 37°C. All drugs are dissolved in ethanol or KPB at a concentration of 10 mM as stock solutions and further diluted with KPB for working solutions. Drugs dissolved in ethanol should be stored in a cold room (4°C) for no more than

one week whereas drugs dissolved in KPB should be prepared daily. Chromatographic mobile phase uses KPB and should be degassed for 20 min before use.

The so-called frontal analysis is performed using KPB which can accommodate various drugs at different concentrations. This solution flows at a slow rate of 0.5 ml/min, which is appropriate for establishing a local equilibrium in the HSA column. The retained compounds will be eluted and the column will be regenerated between studies by washing with KPB through the column. The amount of drugs required to saturate a column are determined from the mean position of the resulting breakthrough curve. The results obtained for the control column are subtracted from those obtained for an HSA column of identical size to correct for the column void time and to correct for secondary interactions between drugs and the support. A correction for the system void time is made by performing similar experiments using sodium nitrate as a non-retained solute.

$$\frac{1}{m_{\mathrm{Lapp}}} = \frac{1}{(K_a m_L[A])} + \frac{1}{m_L} \tag{6}$$

where m_L is related to the true number of binding sites on the column, m_{Lapp} is the apparent moles of solute required to reach the mean point of the breakthrough curve. K_a is the association equilibrium constant for the binding of a drug to HSA, and [A] is the concentration of drug applied to the column.

Zonal elution is typically performed at 1.0 ml/min (4.6 mm I.D. column) or 0.2 ml/min (2.1 mm I.D. column). The concentrations of injected compounds range from 1.0 to 30 μM. These levels are found to be sufficiently low enough to avoid significant changes in the retention factor caused by overloading effects. The mean retention time is obtained by calculating the statistical moment for each peak (Eqn (7)). The column void time can be found by injecting sodium nitrate as a non-retained solute.

$$k' = \frac{t_r - t_m}{t_m} \tag{7}$$

where k' is the retention factor of injected solute while t_r, t_m are retention time, void time, respectively.

High-performance frontal analysis (HPFA)

HPFA is a novel analytical method that enables simultaneous determination of total and unbound drug concentrations under drug–plasma protein binding conditions by combining frontal analysis with separation systems such as HPLC and CE (high-performance capillary electrophoresis). HPFA enables a simple analysis following direct sample injection and does not suffer from undesirable drug adsorption on membrane or leakage of bound drug through the membrane, compared with conventional ultrafiltration and dialysis methods. In addition, by coupling HPFA with a chiral HPLC column, the high-performance capillary electrophoresis/frontal analysis (HPCE/FA), which enables to determine unbound concentrations enantioselectively with ultramicro injection volume, is hence useful especially for the binding study of drugs that are scarce and difficult to obtain.

Below is the procedure of HPFA. An increased volume of drug–HSA mixed solution is directly injected on to the HPFA column. The protein peak is eluted first from the column and the unbound drug is eluted later as a trapezoidal peak having a plateau region. The plateau height and the peak area correspond to the unbound drug concentration and total drug concentration, respectively, and the unbound drug concentration is determined from the plateau height or by online or offline analysis of the plateau region. The appearance of a plateau region is a must for HPFA (Eqn (8)).

$$\%\text{bound drug} = \left[1 - \left(\frac{\text{unbound drug concentration}}{\text{total drug concentration}}\right)\right] \times 100 \tag{8}$$

Solid-phase microextraction (SPME)

SPME is a well-known approach for sampling and sample preparation [42]. It completely eliminates the use of organic solvents and offers several advantages such as small sample size, short analysis time, possibility to automate and ability to study complex samples directly. Recently, SPME has been applied to protein-binding analysis including the plasma protein binding or drug binding to whole blood [43,44].

In SPME, the fiber is exposed to an aqueous or gaseous sample until binding equilibrium is established between the analyte in the sample and on the polymer-coated fused fiber that is used as a stationary phase. The analyte is then desorbed from the fiber at a high temperature in a gas chromatograph injector to a chromatography column and subsequently analyzed by gas chromatographic (GC) detector. Thermally unstable compounds should be desorbed by a suitable desorption fluid, and then analyzed following direct injection into the HPLC systems.

It has been long recognized that plasma pH has a significant impact on method reproducibility and the extent of drug binding to plasma proteins. Accordingly, several methods for controlling the pH have been investigated, such as incubation with 10% CO_2 or dilution at 1:10 with isotonic PBS which is suitable for highly bound drugs and should be applied only when the concentration of the drug is significantly lower than that of the binding proteins.

In vivo microdialysis

Microdialysis sampling is an *in vivo* technique for sampling free drugs in biological fluids, tissues and organs of the body [45,46]. The technique is based on the passive diffusion of compounds down a concentration gradient across the semi-permeable membrane of a microdialysis fiber. Commonly used membranes include a 100 kDa MWCO polyethersulfone membrane. Although several techniques have been used to determine free unbound drugs from biological fluids such as ultrafiltration and equilibrium dialysis, only microdialysis enables simultaneous sampling of free drugs *in vivo* which brings high temporal resolution compared with conventional methods for PK and pharmacodynamic (PD) studies [47].

Microdialysis sampling is a diffusion-based sampling method that has been used for nearly three decades as a means to access the extracellular fluid space of many different tissues. During microdialysis sampling, a small semi-permeable hollow fiber is attached to inlet and outlet tubing through which a perfusion fluid is passed at low μl/min flow rates. During microdialysis sampling, there are three regions that contribute to analyte diffusive mass transport resistance: dialysate, membrane and tissue or sample. Figure 2 represents a typical structure and working mechanism of the microdialysis probe: the probe tip covered with a dialysis

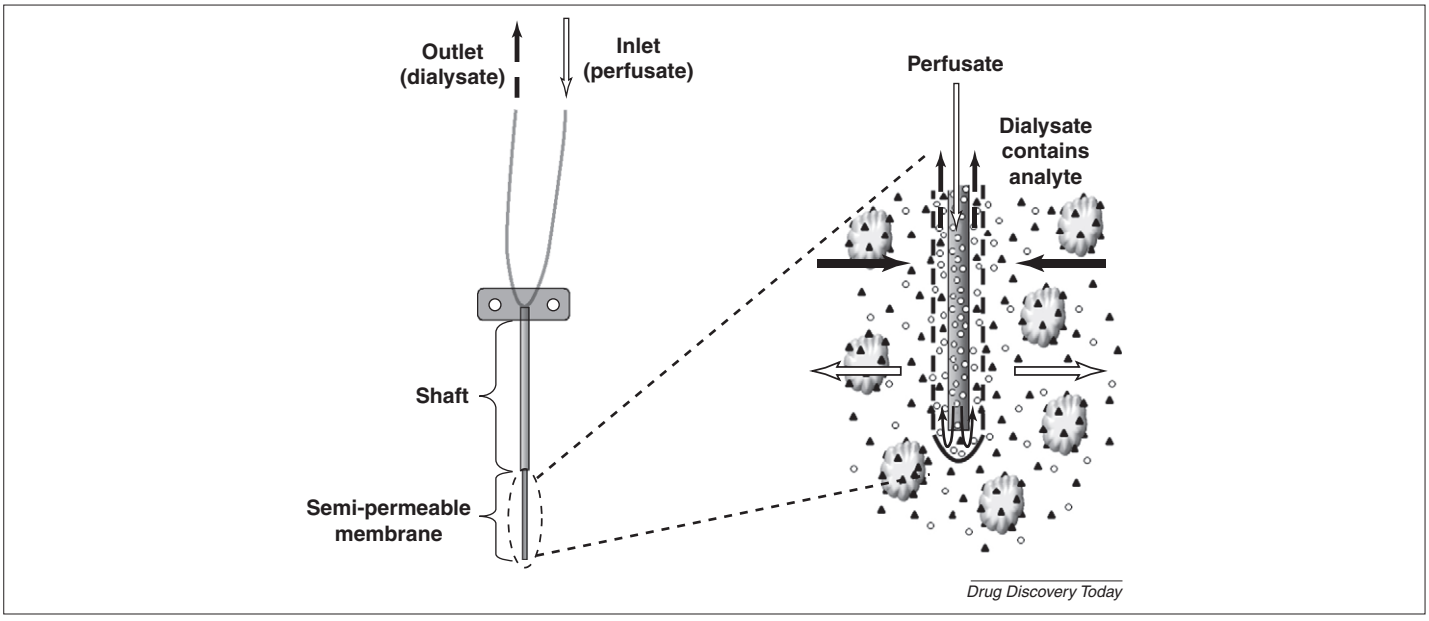

FIGURE 2

A schematic representation of the structure and the working mechanism of a microdialysis probe. The left panel outlines the working mechanism of the probe: the physiological perfusate flows under pressure of a pneumatic pump into the microdialysis tip, which is covered by the dialysis membrane, and then leaves the tip with various analytes containing the tested free drug. The right panel delineates directions of passive diffusion of analytes and their corresponding calibrates down the concentration gradient.

membrane is implanted into blood vessel, tissues or organs, and then perfused with a suitable perfusate at a constant flow rate. Free drugs diffuse along a concentration gradient toward the probe lumen or away from the probe [47]. Microdialysate samples can be collected for subsequent analysis or assayed online by HPLC or other suitable techniques and the total drug concentration in plasma including the free and protein-bound drug can be obtained by a classical blood sampling method. As a result, the % protein-bound drug can be estimated (Eqn (9)).

$$\%\text{Protein binding} = \left(1 - \frac{\text{dialysate concentration}}{\text{total plasma concentration}}\right) \times 100 \quad (9)$$

Recently, a modification was made to the methodology that introduces chemical binding affinity agents in the perfusion fluid that serve to 'lure' the targeted analytes to the probe and prevent their back diffusion across the dialysis membrane. Heparin, a blood anticoagulant agent, has the highest negative charge density of any known biological molecule and has been used for this purpose [48]. It provides the advantage of being reusable as an enhancement agent and serves to improve recovery of microdialysis.

Concluding remarks and future perspectives

The importance of plasma protein binding in drug discovery has recently been fully appreciated. Binding of a drug to plasma protein affects the elimination half-life of the drug, active concentrations of the unbound free drug in blood, the time needed for equilibration between the plasma and the effect compartments (target binding and rebinding), and subsequent elimination from the effect compartments. As a result, the efficacy of the drug, *in vivo* duration of action and toxicity related to free fraction of drug will be influenced accordingly. Co-administration of other drugs and food (especially lipids) and pathological conditions of patients could significantly modify the binding percentage of the investigational drug to plasma protein. This is especially crucial in cases where the drug has a relatively narrow therapeutic index, and a small change in free drug concentration could result in serious consequences.

Despite the wealth of data available for plasma protein binding, only few prediction models are reported for this ADME property in the literature. Plasma protein binding of drugs is difficult to classify and no general implications for the design of lead-like compounds could be derived by the examination of the properties of existing drugs. For most of the drugs, one has to consider the whole physicochemical and PK profile of the drug candidate to decide individually the acceptable amount of protein binding. In practice, there can be more confounding factors [49] and attention must be given to possible sources of artifacts including drug binding to the apparatus, drug insolubility, drug instability, slow equilibrium (time dependence), directional differences, lot-to-lot plasma variability, recovery of drug from plasma and analytical specificity. Nevertheless, it is usually possible to carry out carefully controlled measurements with liquid chromatography–mass spectrometry quantitation and generate reproducible data. Table 2 provides a general comparison among various methods used for determining drug–plasma protein binding.

Despite of the lack of general guidelines for plasma protein binding, the recognition of potential submicromolar to nanomolar binders is important in early drug discovery to prioritize leads and avoid later complications in selecting the initial doses in animal and human trials. The demand for plasma protein binding data on increased numbers of compounds has stimulated attempts

TABLE 2

Comparison of various methods used for plasma protein binding study

Method	Advantages	Disadvantages
Equilibrium dialysis	Temperature controlled; commonly used; a standardized method	Time to reach equilibrium; drug stability concerns
Ultrafiltration	Technically simple, rapid and inexpensive; suitable for unstable drugs; most commonly used	Uncontrolled temperature; adsorption of drugs to filter membrane; dissociation of the bound drugs
Ultracentrifugation	No nonspecific adsorption to membrane	Time consuming in sample preparation (overnight); sedimentation, back diffusion; expensive equipment
Immobilized protein columns	Technically simple and inexpensive	Binding limited to albumin; limited physiological relevance
Charcoal adsorption	No nonspecific adsorption; suitable for drugs bound to lipoproteins; determine bound fraction	More procedures for sample preparation
***In vivo* microdialysis**	*In vivo* and simultaneous sampling, especially appropriate for tissue distribution studies	Difficult to determine probe recovery; tissue damage caused by probe implantation; low temporal and spatial resolution
High-performance affinity chromatography	Rapid, ultramicro injection volume; no undesirable adsorption to the membrane	Relatively large quantity of drug compound; limited to specific albumin
Solid-phase microextraction (SPME)	Simple, rapid automation; solventless; good linearity and high sensitivity	Long desorption times; carryover effects; non-exhaustive sample preparation

to streamline existing methodology as well as to develop new methods. An approach involving the pooling of dialysates for multiple compounds to reduce the number of liquid chromatography–mass spectrometry runs has been described [50]. As stated before, methods involving HPLC on columns with immobilized albumin have also been reported [51]. Recently, the use of surface plasmon resonance has enabled the interaction of drugs with albumin to be characterized thermodynamically (K_d) and kinetically (K_{on} and K_{off}) [51]. All these efforts will continue in the future.

As interest in the role of protein binding in drug action has increased in the medicinal chemistry field, plasma protein binding has become an area of interest to computational medicinal chemists. Multiple approaches have been investigated in an attempt to elucidate the molecular determinants of binding to albumin and to develop predictive models for binding [52–54]. These studies point out the high correlation between the degree of plasma protein binding and lipophilicity within a chemical series, and also show that lipophilicity is not the sole determinant because there is a wide range of binding across a series for compounds with the same lipophilicity.

Photoaffinity labeling, site-directed mutagenesis and X-ray crystallography [55,56] are helpful tools to reveal the binding sites for a drug on plasma protein. Increased understanding of the significance of plasma protein binding to drug action should lead to improved drug candidates. The potential exists to identify and differentiate drug candidates based on their plasma protein binding values.

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