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Impact of pH on Plasma Protein Binding in Equilibrium Dialysis

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Abstract: Many pharmacokinetic analyses require unbound plasma concentrations, including prediction of clearance, volume of distribution, drug−drug interactions, brain uptake analysis, etc. It is most often more convenient to measure the total drug concentration in plasma rather than the unbound drug concentration. To arrive at unbound plasma concentrations, separate in vitro determinations of the plasma protein binding of a drug are usually carried out in serum or in plasma, and the plasma pharmacokinetic results are then mathematically adjusted by this fraction unbound ($f_{u,p}$). Plasma protein binding or the drug fraction unbound in plasma ($f_{u,p}$) is known to be affected by protein, drug, free fatty acid concentrations, lipoprotein partitioning, temperature, pH, and the presence or absence of other drugs/displacing agents within plasma samples. Errors in $f_{u,p}$ determination caused by lack of adequate pH control in newer assay formats for plasma protein binding (e.g., 96-well equilibrium thin walled polypropylene dialysis plates) will have significant drug-specific impact on these pharmacokinetic calculations. Using a diverse set of 55 drugs and a 96-well equilibrium dialysis plate format, the effect of variable pH during equilibrium dialysis experiments on measured values of $f_{u,p}$ was examined. Equilibrium dialysis of human plasma against Dulbecco's phosphate buffered saline at 37 °C under an air or 10% CO_2 atmosphere for 22 h resulted in a final pH of approximately 8.7 and 7.4, respectively. The ratio of $f_{u,p}$ at pH 7.4 (10% CO_2) vs pH 8.7 (air) was $\geq$2.0 for 40% of the 55 compounds tested. Only one of the 55 compounds tested had a ratio $<$0.9. Select compounds were further examined in rat and dog plasma. In addition, physicochemical properties were calculated for all compounds using ACD/Labs software or Merck in-house software and compared to plasma protein binding results. Changes in plasma protein binding due to pH increases which occurred during the equilibrium dialysis experiment were not species specific but were drug-specific, though nonpolar, cationic compounds had a higher likely hood of displaying pH-dependent binding. These studies underscore the importance of effectively controlling pH in plasma protein binding studies.

Keywords: Plasma protein binding; fraction unbound; equilibrium dialysis; pharmacokinetics; physicochemical properties; pH

Introduction

The free drug hypothesis states that the unbound drug equilibrates between tissues and binds to drug targets. In pharmacokinetics, it is generally assumed that unbound drug concentrations should be used to predict parameters like drug clearances, volumes of distribution, and half-lives[1–4] and drug interaction potential in terms of inhibition and induction of enzyme activities.[5–7] For nearly all drugs, it is more convenient to measure the total drug concentration in plasma rather than the unbound drug concentration. Separate in vitro determinations of the plasma protein binding of a drug are usually carried out in serum or in plasma at total drug concentrations covering the range observed in in vivo samples. The plasma pharmacokinetic results are then mathematically adjusted by the fraction unbound in plasma ($f_{u,p}$).

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Potential limitations and factors to be considered when designing and executing a drug to plasma protein binding experiment are the affinities of the drug for the different plasma proteins, multiple binding sites on proteins, binding to the membranes and walls of the devices employed (nonspecific binding), drug concentrations, volume shifts and equilibration time during dialysis, and pH control over the time course of the experiment. In recent years, the trend in industry to increase sample throughput has led to the translation of equilibrium dialysis (ED) ligand binding experiments to a multiwell plate format.

Recent publications have shown the dramatic changes in plasma pH upon incubation alone[8] and during ED experiments even when dialysis buffers of reasonably high ionic strength are employed on the buffer side of dialysis cells.[9] Upon incubation, as well as with sample storage, the loss of carbon dioxide (CO_2) from biological samples occur with time. The loss of CO_2 has a dramatic impact on the pH of plasma because a major buffering component of plasma is carbonic acid or bicarbonate.

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

With the loss of CO_2, the pH of plasma will rise. Changes in pH during ED experiments were historically a concern. Traditionally, sealed, thick-walled Teflon cells (e.g., Dianorm equilibrium dialysis cells) were employed to minimize pH changes occurring due to carbon dioxide loss from plasma and biological samples.

There are two main drug-binding proteins in plasma, albumin and α_1-acid glycoprotein (AGP), present at concentrations of approximately 700 and 20 μM, respectively.[10,11] Unlike AGP, human serum albumin (HSA) has been shown to have several different pH-dependent structural conformations.[12] Of particular interest is the neutral or normal (N) conformation (pH 5–7) and the basic (B) conformation (>pH 8). Within these conformations reside at least six different binding sites. Binding sites labeled as I and II are thought to bind most drug molecules.[11] Interestingly, binding site I (warfarin binding site; Sudlow's site I) has been shown to be altered upon the conformational transition of human albumin from the N to B conformation, resulting in increased drug binding to site I.[13] This suggests that a drug with binding affinity to site I of albumin will have a decreased $f_{u,p}$ with increasing pH. In addition, albumin binding sites have also been reported to be different across species.[14] More specifically, dog albumin does not have a binding site similar to human albumin site I, suggesting that not only may pH-dependent binding be site I specific, but also species specific.[15]

Here, we explore the measurement of the plasma protein binding for a set of 55 structurally diverse drugs. The measurements were made using commercially available 96-well polypropylene ED plates. Dialysis experiments were carried out in a 37 °C incubator under either an air or 10% carbon dioxide atmosphere to prevent carbon dioxide loss (and concomitant elevation in plasma pH) from plasma samples. The potential species difference in binding of several drugs was also examined.

Experimental Section

Chemicals and Reagents. Chemicals were obtained from an in-house source (MRL's Compound Management Group: aprepitant, bosentan, and moricizine) or from commercial sources [Moravek Biochemicals (Brea, CA) and Sigma-

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Aldrich (St. Louis, MO)]. The buffer used for all experiments was Dulbecco's phosphate-buffered saline (DPBS) 1X without calcium and magnesium from Mediatech, Inc. (Herndon, VA). According to the manufacturer, this buffer contains 0.20 g/L of KCl, 0.20 g/L of KH_2PO_4, 8.00 g/L of NaCl, and 1.15 g/L of anhydrous Na_2HPO_4, which translates to a total of 9.6 mM PO_4 (8.1 mM HPO_4 + 1.5 mM H_2PO_4). Plasma used in experiments was obtained either from a commercial source (lot of frozen male human plasma with K_2EDTA, pool of 10 donors, Bioreclamation, Hicksville, NY) or in-house sources (fresh and frozen rat and dog plasma with K_2EDTA).

Plasma Incubations. A volume of approximately 10 mL of fresh rat plasma, obtained from centrifugation of blood from CO_2-anesthetized female Sprague–Dawley rats collected in tubes containing K_2EDTA, was incubated in an open glass scintillation vial and a shaking water bath set to a temperature of 37 °C. The pH of the plasma was measured at selected time points using a Corning 445 pH meter equipped with a 3-in-1 combo RJ electrode.

Equilibrium Dialysis Experiments. Equilibrium dialysis (ED) was performed using a 96-well equilibrium dialyzer with MW cutoff of 5K (Harvard Apparatus, Holliston, MA) and placed in dual-plate rotator set to maximum speed (Harvard Apparatus, Holliston, MA) located in a 37 °C incubator with either normal atmospheric conditions (~0.04% CO_2) or a 10% CO_2 atmospheric environment. Frozen male human plasma was thawed and either used as is, or the pH was adjusted to 7.4 using a pH meter and 1 N HCl (~300 μL of HCl to ~60 mL of plasma). Frozen male rat and dog plasma was thawed and adjusted to a pH of 7.2. Plasma was then spiked with 2 μL of 2.5 mM stock solutions in DMSO (except nelfinavir and saquinavir, 1.25 mM in DMSO) to give 10 μM (5 μM for nelfinavir and saquinavir) in both pH-adjusted and nonadjusted plasma. Final DMSO concentration in spiked plasma samples was 0.4%. ED was performed according to the protocol recommended by the manufacturer. Briefly, 200 μL of plasma samples and buffer were added to the respective sides of the 96-well dialysis plate, wells were capped, and the plate was then placed in the rotator and incubator and allowed to dialyze for 22 h. Following 22 h of dialysis, 25 μL of buffer and plasma were removed from each side of dialysis plate and mixed with 25 μL of the opposite matrix in a 96-deep well plate. Samples were then stored at −20 °C for future analyte quantitation. Remaining buffer in wells from dialysis plate was removed and pooled. The same was done for the plasma side; pH values were then determined on this pooled buffer and plasma using a Corning 445 pH meter equipped with a 3-in-1 combo RJ electrode. In a separate experiment, the change in pH of rat and human plasma during a 20 h equilibrium dialysis experiment was investigated by removing plasma and buffer at designated times over the course of the experiment and measuring the pH.

Ultracentrifugation Experiment. Frozen human plasma was thawed, and the pH was measured, with half of the plasma having the pH adjusted using 1 N HCl. Aliquots (1 mL) of pH-adjusted and nonadjusted plasma were placed in separate 11 × 34 mm polycarbonate centrifuge tubes (Beckman Instruments, Palo Alto, CA). A total of 10 tubes were placed in a MLA-130 rotor and then spun for 4 h at 37 °C and 100000 rpm in a Optima Max centrifuge (Beckman Instruments, Palo Alto, CA). Centrifuge tubes, rotor, and ultracentrifuge were preheated to 37 °C prior to addition of plasma to tubes. Following centrifugation, tubes were removed from the rotor, the first 0.6 mL of ultrasupernatant was removed from each tube and pooled according to starting pH, and finally, the pH of the ultrasupernatant was measured using the pH meter described above.

Analyte Quantitation. Post-ED samples were thawed and then mixed with 10 μL of solvent (50% acetonitrile) and 200 μL of acetonitrile containing 1 μg/mL internal standard (cortisone), with vortexing and sonication after each step. Samples were then spun in a centrifuge for 15 min at 3000 rpm and 10 °C. Fifty microliters of the supernatant were transferred to a 96-well microtiter plate and mixed with 100 μL of 3% acetonitrile. Samples were then subjected to LC−MS/MS analyses. Standard curves and quality controls (QCs) for analytes of interest were prepared in the same manner except 10 μL of stock standards or QCs (in 50% acetonitrile) was added instead of solvent.

Calculations. Concentrations of analytes from each side of the dialysis plate were determined using a standard curve constructed in the same matrix. The fraction unbound in plasma ($f_{u,p}$) was calculated by dividing the drug concentration in the buffer side of the ED plate by the drug concentration in the plasma side. Drug recovery values were also calculated by adding the drug concentrations in buffer and plasma post dialysis, dividing this sum by the drug concentration in plasma prior to dialysis, and then multiplying by 100 to give a percent recovery. Fraction unbound values under normal atmospheric (air) and 10% CO_2 conditions were compared by calculating the ratio of $f_{u,p}$ in 10% CO_2 to $f_{u,p}$ in air. Statistical analysis was performed on the $f_{u,p}$ data using the t test function in Microsoft Excel (two-tailed and paired) and data with p values <0.01 were deemed significant.

Physicochemical parameters, mainly "rule-of-five" type molecular parameters,[16] were estimated for each compound using ACD/Labs (Advanced Chemistry Development, Inc.) and Merck in-house software and compared visually using Spotfire. Parameters include pK_a; overall charge at pH 7.4 and 9.0; number of hydrogen bond acceptors (HBA) and donors (HBD); the Klopman−Wang log P (K log P); log D at pH 7.4 and pH 9.0; bond flexibility, an estimation of the number of flexible and partially flexible bonds; normalized bond flexibility, the fraction of flexible bonds in the molecule; normalized polarity, the fraction of polar atoms in the molecule; the number of nonhydrogen atoms; total surface area and percent polar surface area (PSA) calculated at pH

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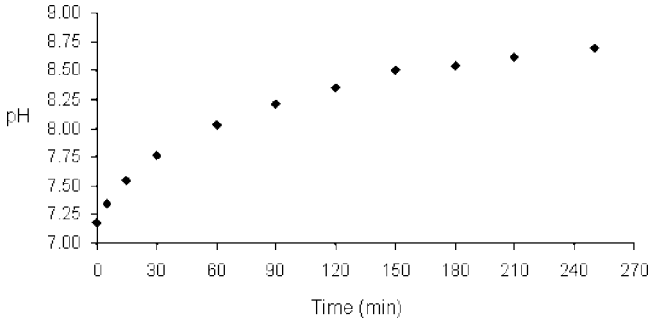

Figure 1. Change in pH of rat plasma upon incubation at 37 °C. Plasma obtained from CO_2-anesthetized female rats.

7.4 and 9.0 using the ACD Laboratories predicted protonation state to create conformations for the Clark PSA calculation method, a three-dimensional method.[17,18] The difference between log *D* values at pH 7.4 and pH 9.0 was calculated by subtracting log $D_{9.0}$ from log $D_{7.4}$.

Results

pH of Incubated Plasma. Incubation of fresh rat plasma, obtained from CO_2 anesthetized female animals, at 37 °C in normal atmospheric conditions resulted in an increase in pH with time (Figure 1). The pH at $t = 0$ min was 7.2 and increased to 8.0 after 60 min of incubation, to 8.3 after 120 min, and to 8.7 after 250 min of incubation. These results are consistent was those previously reported.[8]

pH in Equilibrium Dialysis. Frozen plasma was thawed and then either dialyzed as is (nonadjusted) or had the pH adjusted to ~7.4 prior to dialysis. At selected time points, buffer and plasma were removed from a column of wells from the equilibrium dialysis plates and pooled according to matrix, and the pH was measured for each pool of wells minutes after removal (buffer, plasma, atmosphere, time point). The pH of both buffer and plasma was measured during the course of equilibrium dialysis experiments conducted at 37 °C and either at normal atmospheric conditions (air) or a 10% CO_2 atmosphere. In air, the pH of both the plasma side (shown in Table 1) and buffer side (not reported) increase with time. Adjusting the initial plasma pH to 7.4 did little to keep the final plasma or buffer side pH near physiological values, both the plasma-side and buffer-side pH was between 8.90 and 8.97. The rate of change in pH was similar in both dialyzed plasma (Table 1) and that for rat plasma (Figure 1), displaying a rapid increase in pH before approaching a plateau.

Following 22 h of ED in a 10% CO_2 environment, both the plasma compartment and buffer compartment had pH values close to the physiological pH of 7.4 (Table 1). Results indicate that the presence of CO_2 controls the pH over time.

pH in Ultracentrifugation. Ultracentrifugation is performed under vacuum although the internal sample chamber of the rotor is sealed via an O-ring gasket. In ultracentrifugation experiments there is no practical way to control the pH of experiment by using a CO_2 atmosphere, as is possible in ED experiments. Following ultracentrifugation (4 h at 37 °C and 100,000 rpm) of human plasma with an initial pH of 7.2 (adjusted with HCl) and a pH of 7.7 (nonadjusted), the resulting "ultra" supernatant had pH values of 8.1 and 8.4, respectively (Table 1).

Measured Fraction Unbound in Plasma, $f_{u,p}$. The fractions unbound of 55 compounds in human plasma were determined following equilibrium dialysis in the presence and absence of 10% CO_2, with the results presented in Table 2. Comparison of human $f_{u,p}$ results indicates a significant difference between the air (pH ~8.7) and 10% CO_2 (pH ~7.4) environments for 23 of the 55 compounds examined ($p < 0.01$, two-tailed paired *t* test). The ratio of $f_{u,p}$ determined in the 10% CO_2 and air was >1.3 for 37 compounds and ranged from 2.0 to 10.5 for 23 of the 55 compounds (40% of the compounds tested). Seven of the 55 compounds had either low recovery (<50%) following equilibrium dialysis and or >20% difference in recovery (10% CO_2 recovery minus air recovery) following equilibrium dialysis, and of these seven compounds, four had $f_{u,p}$ ratios ranging from 2.3 to 7.5 and

Table 1. pH during Plasma Protein Binding Experiments at 37°C

experimental conditions			pH at time (h)[c]					
initial plasma	method[a]	atmosphere[b]	0	0.5	2	4	20	22
frozen human	ED	air	7.4[d]					8.5, 9.0
		CO_2	7.4[d]					7.4 ± 0.2
frozen human	ED	air	8.1					8.9
		CO_2	8.1					7.6
frozen human	ED	air	8.5	8.7	8.7	8.7	9.0	
		CO_2	8.5	8.3	8.3	8.0	7.7	
fresh rat	ED	air	7.4	8.2	8.3	8.3	8.8	
		CO_2	7.4	8.1	8.0	8.0	7.7	
frozen human	UC	air	7.2[d]			8.1		
		air	7.7			8.4		

[a] ED = equilibrium dialysis of plasma using Harvard Apparatus 96-well equilibrium dialyzer against Dulbecco's phosphate-buffered saline. UC = ultracentrifugation of plasma at 100000 rpm and 37 °C for 4 h. [b] ED was conducted in an incubator containing air (~0.04% CO_2) or 10% CO_2. In UC experiments, air is evacuated from the centrifuge although it is believed the rotor is sealed and contains air. [c] pH measured by first pooling samples according to starting pH and matrix and then measuring pH using a pH meter equipped with a 3-in-1 combination electrode. pH at time 0 h is starting plasma; all other times correspond to pH of plasma dialyzate (ED) or top three of five fractions following ultracentrifugation (UC). Mean ± SD, $n = 3$; all others $n = 1$. [d] Plasma pH adjusted using 1 N HCl.

Table 2. Fraction Unbound ($f_{u,p}$) of Compounds at 10 μM in Human Plasma Following Equilibrium Dialysis under Normal Atmospheric Conditions or 10% CO_2

		human $f_{u,p}$[c]		
compd	pK_a[a]	air (pH ~8.7)	10% CO_2 (pH ~7.4)	$f_{u,p}$ ratio (CO_2/air)
amprenavir		0.105 ± 0.008	0.15 ± 0.01	1.4
antipyrine		0.61 ± 0.06	0.89 ± 0.02	1.5*
aprepitant	4.0, 8.1	0.0026 ± 0.0007	0.0036 ± 0.0003	1.4
artemisinin[b]		0.21 ± 0.02	0.27 ± 0.01	1.3*
azithromycin	8.2, 8.6	0.58 ± 0.10	0.80 ± 0.10	1.4
biperiden[b]	9.8	0.026 ± 0.010	0.20 ± 0.02	7.5***
bosentan	6.0	0.018 ± 0.002	0.021 ± 0.003	1.1
bupropion[b]	7.2	0.16 ± 0.03	0.57 ± 0.03	3.6**
carbamazepine		0.215 ± 0.017	0.55 ± 0.02	2.6**
cimetidine	7.1	0.95 ± 0.05	0.88 ± 0.02	0.9
clarithromycin	8.2	0.44 ± 0.07	0.42 ± 0.05	0.9
clindamycin	8.7	0.087 ± 0.003	0.29 ± 0.03	3.3**
clotrimazole	6.3	0.0026 ± 0.0002	0.0035 ± 0.0002	1.3
delavirdine	4.0, 7.7, 9.2	0.022 ± 0.002	0.020 ± 0.001	0.9
dexamethasone		0.17 ± 0.02	0.34 ± 0.01	2.0**
diazepam		0.023 ± 0.007	0.020 ± 0.019	0.9
diclofenac	4.2	0.0036 ± 0.0004	0.0038 ± 0.0003	1.1
diltiazem[b]	8.9	0.23 ± 0.05	0.54 ± 0.02	2.3
efavirenz	10.2	0.0042 ± 0.0006	0.0063 ± 0.0002	1.5**
felbamate		0.384 ± 0.016	0.84 ± 0.02	2.2***
felodipine		0.0032 ± 0.0003	0.0048 ± 0.0002	1.5*
fenofibrate		0.008 ± 0.002	0.009 ± 0.006	1.1
fluconazole	11.0	0.84 ± 0.07	0.99 ± 0.09	1.3
fluoxetine	10.1	0.021 ± 0.001	0.069 ± 0.004	3.3**
fluvoxamine	9.4	0.138 ± 0.014	0.281 ± 0.011	2.0**
griseofulvin[b]		0.135 ± 0.005	0.22 ± 0.02	1.6*
ibrolipim		0.043 ± 0.001	0.077, 0.082	1.9
ketoconazole	5.5, 6.9	0.018 ± 0.003	0.018 ± 0.001	1.0
L-365260		0.0051 ± 0.0003	0.0121 ± 0.0002	2.4**
metyrapone	4.6	0.63 ± 0.03	0.80 ± 0.05	1.3*
mibefradil	6.0, 9.3	0.0048 ± 0.0012	0.019 ± 0.001	4.0**
mifepristone	5.5	0.0056 ± 0.0016	0.0062 ± 0.0002	1.1
moricizine	6.9	0.063 ± 0.003	0.116 ± 0.005	1.8**
mycophenolic acid	4.7, 9.9	<0.01 (LOQ)	0.018 ± 0.007	>1.8
nafcillin[b]		0.059 ± 0.015	0.171 ± 0.016	2.9**
nefazodone	5.9, 6.7	0.0056 ± 0.0005	0.0088 ± 0.0005	1.6*
nelfinavir	7.5, 9.6	0.0018 ± 0.0004	0.0024 ± 0.0001	1.4
olanzapine	6.1, 7.8	0.024 ± 0.001	0.25 ± 0.02	10.5**
oxybutynin	8.2	0.0029 ± 0.0010	0.028 ± 0.002	9.7**
paclitaxel[b]		0.10 ± 0.03	0.12 ± 0.03	1.2
phenytoin	8.3 ± 0.5	0.181 ± 0.002	0.202 ± 0.006	1.1*
quinidine	4.8, 9.3	0.15 ± 0.03	0.39 ± 0.04	2.7**
rifampicin	5.0, 7.3, 8.8, 9.9	0.07 ± 0.02	0.19 ± 0.01	2.6**
ritonavir		0.0058 ± 0.0009	0.0181 ± 0.0011	3.1***
rosiglitazone	6.3, 6.5	0.0021 ± 0.0002	0.0045 ± 0.0007	2.2*
saquinavir	7.6, 11.0	0.0031 ± 0.0010	0.0136 ± 0.0009	4.4**
simvastatin[b]		0.0143 ± 0.0013	0.0092 ± 0.0008	0.6**
sulfasalazine	9.5	0.0033 ± 0.0014	0.0043 ± 0.0013	1.3*
sulfinpyrazone		0.0129 ± 0.0011	0.0138 ± 0.0008	1.1
tamoxifen	8.7	0.00026 ± 0.00002	0.0006 ± 0.0001	2.3*
ticlopidine	7.1	0.00108 ± 0.00002	0.0029 ± 0.0003	2.7**
verapamil	9.0	0.08 ± 0.03	0.27 ± 0.01	3.5**
vinblastine	5.6, 7.6	0.18 ± 0.03	0.26 ± 0.03	1.5
R-warfarin	4.5	0.0063 ± 0.0001	0.015 ± 0.003	2.4*
S-warfarin	4.5	0.0070 ± 0.0003	0.012 ± 0.002	1.7*

[a] pK_a values estimated using ACD/Labs software, estimated error ranged from ± 0.1–1.0 [b] recovery was ≤50% or >20% difference in recovery between air and CO_2 environments. [c] $f_{u,p}$ values reported as mean ± s.d. (n = 3). * $p < 0.05$ using two-tailed paired t test. ** $p < 0.01$ using two-tailed paired t test. *** $p < 0.001$ using two-tailed paired t test.

one compound (simvastatin) had a $f_{u,p}$ ratio of 0.6. Low recovery values may be the result of insolubility at 10 μM, compound instability over 22 h of dialysis, and/or nonspecific binding to the dialysis apparatus.

Table 3. Fraction Unbound ($f_{u,p}$) of Compounds at 10 μM in Rat and Dog Plasma Following Equilibrium Dialysis under Normal Atmospheric Conditions or 10% CO_2

	rat $f_{u,p}$[b]			dog $f_{u,p}$[b]		
compd	air (pH ~8.6)	10% CO_2 (pH ~7.2)	$f_{u,p}$ ratio (CO_2/air)	air (pH ~8.6)	10% CO_2 (pH ~7.2)	$f_{u,p}$ ratio (CO_2/air)
biperiden[a]	0.012 ± 0.009	0.31 ± 0.02	25.5**	0.02 ± 0.02	0.194 ± 0.005	9.7**
clindamycin	0.20 ± 0.01	0.44 ± 0.02	2.2***	0.055 ± 0.005	0.20 ± 0.01	3.7**
diazepam	0.109 ± 0.005	0.140 ± 0.007	1.3**	0.043 ± 0.008	0.042 ± 0.003	1.0
efavirenz	0.011 ± 0.003	0.008 ± 0.001	0.7	0.006 ± 0.001	0.006 ± 0.001	1.0
fluoxetine	0.027 ± 0.002	0.076 ± 0.004	2.8**	0.009 ± 0.002	0.035 ± 0.002	3.9**
fluvoxamine	0.11 ± 0.01	0.22 ± 0.01	2.0**	0.15 ± 0.02	0.172 ± 0.001	1.1
L-365260	0.019 ± 0.001	0.022 ± 0.001	1.2	0.014 ± 0.003	0.017 ± 0.001	1.2
mibefradil[a]	0.013 ± 0.003	0.037 ± 0.003	2.7**	0.011 ± 0.004	0.023 ± 0.003	2.0
olanzapine[a]	0.13 ± 0.01	0.28 ± 0.04	2.2	0.15 ± 0.04		
oxybutynin	0.008 ± 0.002	0.045 ± 0.005	5.4*	0.005 ± 0.001	0.038 ± 0.007	8.1*
ritonavir	0.018 ± 0.002	0.027 ± 0.004	1.5	0.007 ± 0.001	0.009 ± 0.001	1.3
verapamil	0.036 ± 0.002	0.24 ± 0.01	6.7***	0.054 ± 0.007	0.23 ± 0.02	4.2**
R-warfarin	0.011 ± 0.001	0.012 ± 0.001	1.1	0.066, 0.061	0.033 ± 0.002	0.5
S-warfarin	0.011 ± 0.001	0.0056 ± 0.0004	0.5*	0.067, 0.060	0.022 ± 0.001	0.3

[a] Recovery was ≤50% or >20% difference in recovery between air and CO_2 environments. [b] $f_{u,p}$ values reported as mean ± s.d. ($n = 3$). * $p < 0.05$ using two-tailed paired t test. ** $p < 0.01$ using two-tailed paired t test. *** $p < 0.001$.

The fraction unbound in rat and dog plasma was examined for 14 compounds following the same procedure as that for human plasma (Table 3). The compounds were chosen based on the ratio of $f_{u,p}$ in human or known binding location on albumin. Similar ratios of $f_{u,p}$ were observed in all species for nearly all compounds. Warfarin (dog plasma) and simvastatin (human plasma) are the only compounds that exhibited significant decreases in binding with increasing pH.

Discussion

According to the free drug hypothesis, the fraction of drug not bound to plasma proteins is the fraction available to distribute outside the plasma compartment. Though the unbound drug concentration is of interest, the total concentration of drug in plasma is often determined due to ease of measurement. The unbound or free drug concentration is then calculated by multiplying the total drug concentration by the fraction unbound in plasma. This fraction unbound or the fraction of free to total drug concentration in plasma ($f_{u,p}$) is often assumed to be constant for a drug, and is a value used in many pharmacokinetic (PK) calculations to more realistically represent the fraction of total drug available for bioactivity or clearance.

There are many variables that influence a drug's affinity for plasma proteins. Such variables include drug and protein concentration, fatty acid concentration, lipoprotein partitioning, temperature, and pH. Plasma pH is often assumed to be controlled in ED experiments by preadjustment of plasma pH for freeze–thawed plasma samples and dialysis against a buffer of suitable ionic strength. A recent report attempted to examine the in vivo relationship between blood pH and $f_{u,p}$ and found most drugs show a pH-dependent binding to plasma proteins with in the range of arterial blood pH of 6.7–8.0.[19] Benet and Hoener also discuss changes in plasma protein binding caused by a drug-drug interaction or disease state and the minimal impact in the clinical setting, but highlight that differences in fraction unbound need to be considered when scaling PK parameters from animal and in vitro data.[20]

Wan and Rehngren recently reported that pH is often not controlled and thus not constant over the course of a plasma protein binding experiment when using equilibrium dialysis to determine $f_{u,p}$.[9] Here, we have verified that plasma pH cannot be assumed to remain constant during a simple incubation or during an equilibrium dialysis or ultracentrifugation experiment. As shown in Figure 1 and Table 1, pH rises with time and this is thought to be due to the loss of CO_2. We suspect the newer, thin walled polypropylene ED plates may have sufficient permeability to carbon dioxide such that adequate pH control is not maintained. With the addition of CO_2 to the atmosphere of the incubation chamber housing the 96-well equilibrium dialysis plate, the pH of the plasma and buffer compartments can be maintained close to a physiological value (7.4). In the absence of such control (lack of CO_2), the pH of the system rises, and for >65% of the drugs tested using equilibrium dialysis, binding to plasma proteins is greater in the absence of CO_2 (Table 2). This suggests that pH does affect the fraction unbound, and at higher pH the fraction unbound is nearly always less than at lower pH. Similar results and observations were obtained for drugs initially dissolved in plasma with a pH of 8.1 (data not shown), which suggests that pH-dependent changes in plasma protein binding are reversible.

In addition to the Hinderling and Hartmann work on pH-dependent binding within the range of arterial blood pH,[19] only a few other observations of pH-dependent plasma protein binding have been previously reported.[19,21,22] With

(17) Clark, D. E. Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. 2. Prediction of blood-brain barrier penetration. *J. Pharm. Sci.* **1999**, *88* (8), 815–21.

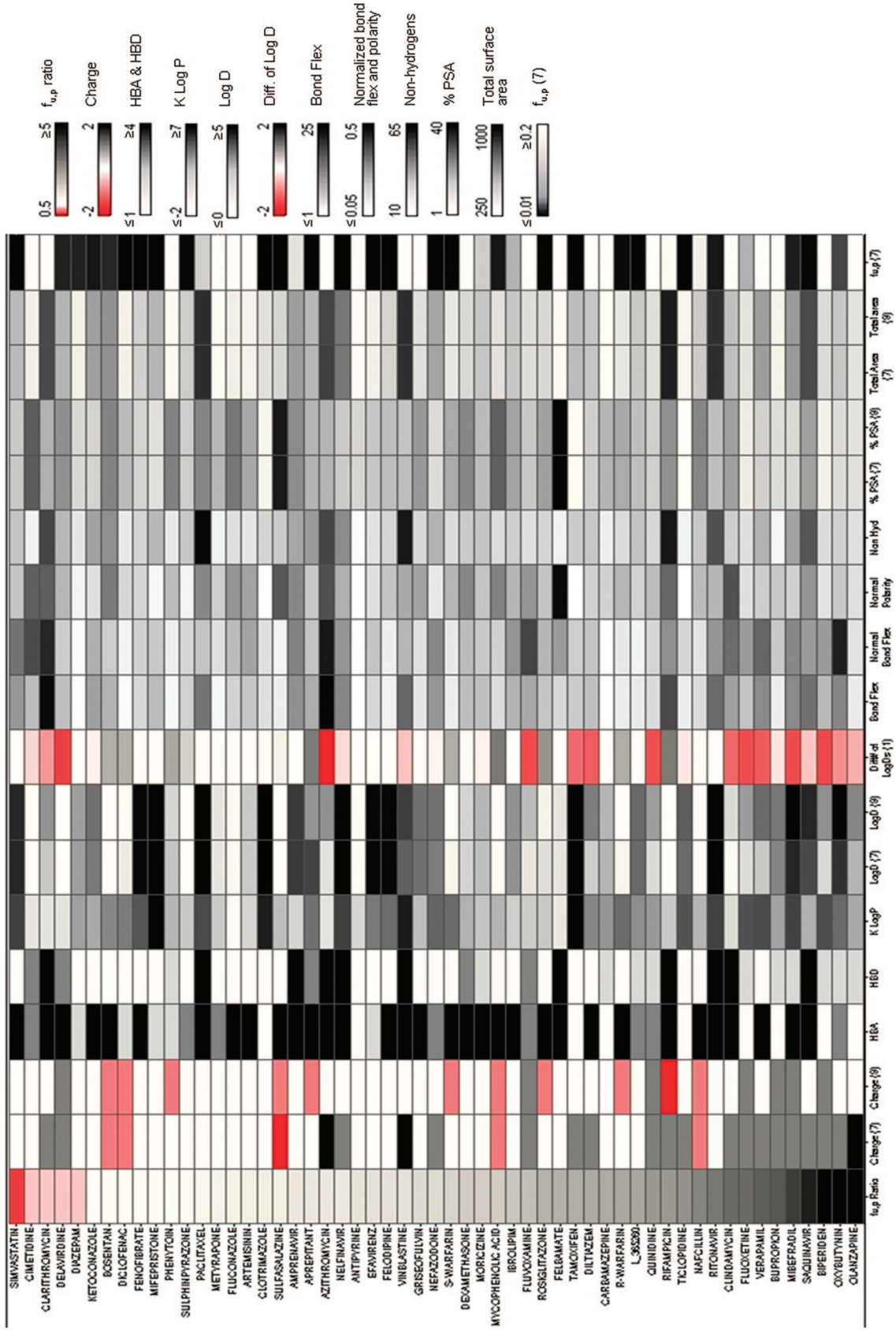

Figure 2. Visual comparison of Tables 4 and 5 using Spotfire. Comparison of the effect of pH on fraction unbound ($f_{u,p}$) of drugs in plasma and their physicochemical properties. The property values are represented by color shading for each physicochemical property as indicated by the color key.

efforts to increase the throughput of plasma protein binding measurements, perhaps less attention has been paid to fundamental issues such as pH control. Few recent literature reports have listed the initial or more importantly, the final pH of the experimental system used to determine a fraction unbound. The results of our current study, as well as those reported by Wan and Rehngren showing the inability of different buffering systems to control the pH during an equilibrium dialysis experiment,[9] add uncertainty to many of the reported fraction unbound measurements.

Initially, our hypothesis was that the influence of pH on the fraction unbound of a drug would likely be the result of a change in the physicochemical properties of the ligand, centered around a ligand's pK_a. However, the estimated pK_a of the drug compounds in this study and their respective fraction unbound in plasma under normal and 10% CO_2 atmosphere (Table 2) appears uncorrelated. Upon closer examination of several estimated physicochemical properties in Figure 2 and Tables 4 and 5 there appear to be some general trends in the $f_{u,p}$ ratio with respect to the charge of the molecule at pH 7.4, the difference between log *D* at pH 7.4 and 9.0, and to a lesser extent, the percent polar surface area (% PSA) of the molecule. Positively charged compounds, shaded gray for +1 and black for +2 in the "Charge (7)" column of Figure 2, tend to be the drugs that displayed pH-dependent plasma protein binding. This can be seen when comparing the "Charge (7)" column with the "$f_{u,p}$ Ratio" column of Figure 2; the cationic compounds tend to have the greater $f_{u,p}$ ratio (gray/black). In addition, compounds with a larger predicted log *D* at pH 9.0 as compared to at pH 7.4, displayed as shades of red in the "Diffs of LogD" column of Figure 2, also tend to have a greater $f_{u,p}$ ratio. Finally, a slight trend may exist for compounds with a low % polar surface area (light gray/white cells in %PSA column of Figure 2) to have a greater $f_{u,p}$ ratio and display pH-dependent plasma protein binding. Taken all together, the results presented in Tables 4 and 5 and Figure 2 suggest the fraction unbound in plasma for nonpolar cationic compounds will have a higher likelihood of being influenced by changes in pH.

An alternate hypothesis is that changes in $f_{u,p}$ may be the result of changes in the characteristics of plasma proteins rather than changes in the ionization and physicochemical properties of the compound. In fact, HSA has been shown to have several pH-dependent conformations,[12] whereas AGP has not within the physiological pH range. A recent review by Ascenzi et al. discusses pH and other allosteric modulation of ligand binding to HSA.[11] Of particular interest is the neutral or normal (N) and basic (B) conformations of HSA. The N-conformation of albumin is thought to exist in plasma and resides in this conformation between the pH 4.3–8.0. The B-conformation exists above pH 8. It is thought that with the transition between these two conformations (N−B transition) the binding sites or pockets within albumin may also be altered. HSA has been shown to have several binding sites, with two major sites (Sudlow's site I and II) for binding xenobiotics. Sudlow's binding site I (warfarin binding site) has been shown to be most affected by a change in albumin conformation,[13] and so one would expect those drugs that bind to site I to have the most pronounced changes in $f_{u,p}$ when altering the pH. However, little is known about the exact binding nature of compounds in plasma or even to albumin. Warfarin is thought to bind to site I and diazepam is thought to bind to site II, but few other compounds have been shown to bind exclusively to either one of these sites.[11] Because of this, it is difficult to relate our results of $f_{u,p}$ changes with binding site affinities.

There appear to be species differences with regard to albumin structure.[14,15,23] Kosa et al. reports similar N−B transition mechanisms between species, but differences in structural characteristics and ligand binding properties. They go on to suggest that rat albumin contains a drug binding site similar to human albumin binding site I, and dog albumin has a binding site similar to human binding site II. If this holds true, along with the suggestion that albumin's binding site I is most affected by pH, we would then expect compounds that bind to site I to have a similar change in pH-dependent $f_{u,p}$ for human and rat albumin but not for dog. Following comparison of Tables 2 and 3, it appears that for those compounds that had significant changes in human $f_{u,p}$, they also had significant changes in $f_{u,p}$ for both rat and dog plasma protein binding. These findings were somewhat unexpected based on results from Kosa et al. where they show species differences with respect to pH effects on the albumin binding of site-specific drugs.[15] Warfarin and diazepam plasma binding seem to follow the same trends across species as found by Kosa et al. with serum albumin.

The data presented here and by Hinderling and Hartmann clearly show the impact of pH on the $f_{u,p}$ for drugs.[19] Much of the data variability reported in the literature may be the result from differences in measurement methodologies. Equilibrium dialysis, ultracentrifugation, or ultrafiltration may take anywhere from 1 h to 1 day to complete the experiment

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Table 4. Fraction Unbound and Estimated Physicochemical Properties of Drugs

molecule	$f_{u,p}$ ratio[a]	$f_{u,p}$ (7)[b]	charge (7)[b,d]	charge (9)[c,d]	HBA[b,e]	HBD[c,f]	log P[g]	log D (7)[b,h]	log D (9)[c,h]	diff of log D's[i]
amprenavir	1.4	0.15	0	0	6	4	1.43	4.2	4.2	0
antipyrine	1.5	0.89	0	0	1	0	1.23	0.3	0.3	0
aprepitant	1.4	0.0036	0	−1	4	3	4.35	4.1	3.1	1.01
artemisinin	1.3	0.27	0	0	5	0	1.29	2.3	2.3	0
azithromycin	1.4	0.8	2	0	12	7	0.27	1.3	3.1	−1.84
biperiden	7.5	0.2	1	1	1	2	5.14	1.7	3.1	−1.46
bosentan	1.1	0.021	−1	−1	11	1	3.84	−0.2	−0.8	0.63
bupropion	3.6	0.57	1	1	1	2	3.23	3.3	3.5	−0.19
carbamazepine	2.4	0.55	0	0	1	2	3.55	2.7	2.7	0
cimetidine	0.9	0.88	0	0	3	3	0.09	−0.1	0.3	−0.30
clarithromycin	0.9	0.42	1	0	13	5	0.27	2.3	3.1	−0.77
clindamycin	3.3	0.29	1	0	5	5	0.87	0.5	1.6	−1.16
clotrimazole	1.3	0.0035	0	0	1	0	6.11	5.4	5.4	−0.02
delavirdine	0.9	0.02	1	1	4	3	0.27	−3.3	−1.7	−1.54
dexamethasone	1.8	0.34	0	0	5	3	2.93	1.9	1.9	0
diazepam	0.9	0.02	0	0	2	0	3.3	3.0	3.0	0
diclofenac	1.1	0.0038	−1	−1	2	1	4.12	1.0	0.3	0.61
diltiazem	2.3	0.54	1	0	4	1	3.87	2.1	3.4	−1.26
efavirenz	1.5	0.0063	0	0	2	1	4.18	4.8	4.8	0.03
felbamate	2.2	0.84	0	0	4	4	−0.02	1.2	1.2	0
felodipine	1.5	0.0048	0	0	4	1	4.45	4.8	4.8	0
fenofibrate	1.1	0.009	0	0	4	0	4.95	4.8	4.8	0
fluconazole	1.3	0.99	0	0	5	1	−1.94	0.5	0.5	0
fluoxetine	3.3	0.069	1	1	1	2	5.05	1.6	3.0	−1.44
fluvoxamine	2.0	0.281	1	1	3	3	1.96	1.2	2.6	−1.43
griseofulvin	1.6	0.22	0	0	6	0	2.6	3.5	3.5	0
ibrolipim	1.9	0.077	0	0	5	1	1.8	3.1	3.1	0
ketoconazole	1.0	0.018	0	0	5	0	3.1	3.4	3.5	−0.11
L_365260	2.4	0.0121	0	0	3	2	3.74	3.6	3.6	0
metyrapone	1.2	0.8	0	0	3	0	2.04	1.2	1.2	0
mibefradil	4.0	0.019	1	1	4	2	5.4	4.4	5.8	−1.42
mifepristone	1.1	0.0062	0	0	2	1	6.8	4.9	4.9	0
moricizine	1.8	0.116	0	0	4	2	2.46	2.6	2.7	−0.11
mycophenolic acid	1.8	0.018	−1	−1	6	1	2.84	0.3	−0.8	1.03
nafcillin	2.9	0.171	−1	−1	5	1	2.81	−0.2	−0.2	0.03
nefazodone	1.6	0.0088	1	0	3	1	4.65	3.4	3.5	−0.10
nelfinavir	1.4	0.0024	1	0	4	5	5.43	6.6	6.9	−0.26
olanzapine	10.5	0.25	2	0	1	2	3.1	2.7	3.3	−0.58
oxybutynin	9.7	0.028	1	0	3	2	4.51	4.3	5.1	−0.83
paclitaxel	1.2	0.12	0	0	14	4	5.2	7.4	7.4	0.01
phenytoin	1.1	0.202	0	−1	2	2	2.81	2.5	1.8	0.69
quinidine	2.6	0.39	1	1	3	2	3.44	1.6	3.0	−1.40
rifampicin	2.6	0.19	1	−2	13	7	2.15	−1.4	−2.5	1.02
ritonavir	3.1	0.0181	0	0	7	4	5.4	5.3	5.3	0
rosiglitazone	2.2	0.0045	0	−1	4	1	2.16	1.5	0.6	0.83
R-warfarin	2.4	0.015	0	−1	4	1	4.47	0.6	−0.04	0.65
saquinavir	4.4	0.0136	1	0	6	7	2.95	4.0	4.4	−0.40
simvastatin	0.6	0.0092	0	0	5	1	5.42	4.4	4.4	0
sulfasalazine	1.3	0.0043	−2	−1	9	1	2.63	0.05	−0.07	0.12
sulfinpyrazone	1.1	0.0138	0	0	3	0	2.19	−0.4	−1.8	0.38
S-warfarin	1.7	0.012	0	−1	4	1	4.47	0.6	−0.04	0.65
tamoxifen	2.3	0.0006	1	0	1	1	7.2	6.6	7.7	−1.14
ticlopidine	2.7	0.0029	1	0	0	1	5	3.6	3.8	−0.17
verapamil	3.5	0.27	1	0	5	1	5.18	2.3	3.6	−1.28
vinblastine	1.5	0.26	2	0	9	5	6.17	3.7	4.2	−0.43

[a] $f_{u,p}$ ratio is the ratio of $f_{u,p}$ at pH 7.4 over $f_{u,p}$ at pH 9. [b] pH of 7.4, signified by (7). [c] pH of 9.0, signified by (9). [d] Predicted charge on molecule at corresponding pH calculated using ACD/Labs. [e] Number of hydrogen bond acceptors (HBA) at pH 7.4. [f] Number of hydrogen bond donors (HBD) at pH 7.4. [g] Klopman−Wang log P prediction. [h] Predicted log D using ACD/Labs software. [i] Difference of predicted log D at pH 9.0 from log D at pH 7.4.

depending on the time of centrifugation and or incubation. Of these three methods, only equilibrium dialysis employs the use of a dialysis buffer with pH buffering capacity. As we and others have shown, the carbonate−bicarbonate buffering system in plasma is subject to changes due to the volatilization of carbon dioxide during incubations. The pH of plasma samples can change significantly during the course of even a short incubation, no matter the measurement methodology employed, as demonstrated with the incubation of rat plasma and with ultracentrifugation experiments. To

Table 5. Additional Estimated Physicochemical Properties of Drugs

molecule	bond flex[a]	normal bond flex[b]	normal polarity[c]	non hyd[d]	total area (7)[e,f]	% PSA (7)[e,g]	total area (9)[f,h]	% PSA (9)[g,h]
amprenavir	11.0	0.3	0.29	35	593.9	22.0	594.0	22.0
antipyrine	0.9	0.06	0.07	14	253.0	11.2	253.0	11.2
aprepitant	8.1	0.2	0.19	37	529.9	16.9	527.9	16.7
artemisinin	5.8	0.25	0.25	20	322.1	18.5	322.1	18.4
azithromycin	24.1	0.45	0.37	52	827.1	16.9	815.3	16.8
biperiden	6.8	0.26	0.13	23	410.8	4.9	410.8	4.9
bosentan	7.5	0.18	0.31	39	624.0	23.0	624.6	23.0
bupropion	3.0	0.19	0.19	16	316.7	9.4	316.9	9.5
carbamazepine	1.4	0.07	0.17	18	281.8	16.8	281.8	16.8
cimetidine	6.5	0.38	0.35	17	327.8	27.9	328.0	27.8
clarithromycin	23.1	0.43	0.35	52	803.8	17.6	802.7	17.3
clindamycin	9.3	0.33	0.37	27	508.5	19.5	507.1	19.4
clotrimazole	4.0	0.14	0.04	25	386.2	4.4	385.9	4.3
delavirdine	6.3	0.18	0.22	32	537.5	21.5	537.0	21.5
dexamethasone	6.4	0.21	0.29	28	418.5	22.4	418.5	22.4
diazepam	1.8	0.08	0.1	20	328.4	10.6	328.4	10.6
diclofenac	2.0	0.1	0.16	19	319.8	15.9	319.7	15.9
diltiazem	6.3	0.2	0.17	29	494.2	12.5	493.7	12.5
efavirenz	4.6	0.2	0.14	21	327.3	14.3	327.2	14.3
felbamate	4.5	0.26	0.47	17	293.8	39.4	293.8	39.3
felodipine	2.9	0.11	0.2	25	440.0	15.7	440.2	15.8
fenofibrate	4.5	0.17	0.16	25	445.2	11.6	445.1	11.6
fluconazole	5.0	0.21	0.27	22	328.2	24.6	328.3	24.6
fluoxetine	6.0	0.26	0.14	22	376.9	6.1	377.0	6.1
fluvoxamine	8.5	0.39	0.27	22	395.9	14.0	395.6	14.0
griseofulvin	3.6	0.14	0.25	24	392.2	20.3	391.6	20.3
ibrolipim	7.5	0.27	0.22	27	486.8	19.0	487.0	19.0
ketoconazole	9.3	0.23	0.14	36	592.7	11.8	592.7	11.7
L_365260	3.3	0.1	0.17	30	478.0	15.6	478.0	15.6
metyrapone	2.5	0.14	0.18	17	291.1	16.6	290.5	16.6
mibefradil	11.5	0.29	0.17	36	602.1	10.6	600.5	10.4
mifepristone	6.7	0.19	0.09	32	532.4	8.7	532.5	8.7
moricizine	6.3	0.19	0.2	30	493.9	15.9	493.7	16.0
mycophenolic acid	4.1	0.17	0.3	23	384.2	27.4	385.0	27.4
nafcillin	4.3	0.13	0.21	29	467.9	22.6	468.2	22.6
nefazodone	10.3	0.29	0.12	33	575.9	9.6	575.3	9.8
nelfinavir	11.8	0.27	0.23	40	692.2	13.3	691.4	13.4
olanzapine	3.2	0.13	0.14	22	380.5	8.7	380.2	8.2
oxybutynin	11.8	0.44	0.19	26	485.1	9.6	483.1	9.6
paclitaxel	13.5	0.2	0.29	62	868.0	22.7	865.8	22.7
phenytoin	2.4	0.11	0.21	19	297.4	23.4	297.2	23.3
quinidine	4.3	0.16	0.21	24	397.7	12.6	397.9	12.5
rifampicin	17.4	0.28	0.34	59	900.3	21.4	904.4	21.5
ritonavir	14.5	0.27	0.22	50	865.4	15.5	862.6	15.4
rosiglitazone	5.9	0.22	0.2	25	421.1	19.6	419.0	19.3
R-warfarin	3.1	0.12	0.22	23	362.4	18.2	363.4	18.4
saquinavir	13.8	0.26	0.27	49	801.0	19.2	801.2	19.4
simvastatin	10.2	0.32	0.2	30	522.0	13.7	521.7	13.7
sulfasalazine	3.0	0.1	0.36	28	427.6	35.5	431.7	35.6
sulfinpyrazone	5.1	0.16	0.1	29	465.0	12.9	465.2	12.9
S-warfarin	3.1	0.12	0.22	23	362.4	18.2	363.4	18.4
tamoxifen	5.5	0.18	0.07	28	516.2	3.1	513.9	3.1
ticlopidine	3.0	0.16	0.06	17	311.1	1.8	310.0	1.5
verapamil	11.5	0.34	0.18	33	594.7	12.2	592.6	12.1
vinblastine	14.9	0.22	0.24	59	862.1	15.7	865.3	15.1

[a] Bond flexibility is an estimate of the amount of flexible and partially flexible bonds. [b] Normalized bond flexibility is the fraction of bonds in the molecule that are flexible. [c] Normalized polarity is the fraction of atoms that are polar. [d] Number of nonhydrogen atoms. [e] pH of 7.4, signified by (7). [f] The estimated total surface area of the molecule. [g] The predicted percent of total surface area that is polar using the Clark approximation. [h] pH of 9.0, signified by (9).

stabilize pH, the addition of CO_2 to the atmosphere of an incubation chamber, standard practice in cell biology experiments, utilizes the native bicarbonate buffering system.

Inaccuracies in plasma protein binding adversely impact the interpretation of in vivo pharmacokinetic/ADME studies. Determining $f_{u,p}$ under nonphysiological conditions

(high pH) may result in misleading results. For example, the species differences in $f_{u,p}$ for biperiden at pH 7.4 is not seen at high pH, and the artificial species differences for verapamil were observed at high pH but not at pH 7.4 (Tables 2 and 3). For most pharmacokinetic, pharmacodynamic, and drug−drug interaction predictions, plasma concentrations are corrected by multiplying by $f_{u,p}$. Since many pharmacokinetic processes occur at subsaturating drug concentrations, a 2-fold error in $f_{u,p}$ will often translate into a 2-fold error in the predicted property. Therefore, we suggest that either measures be taken to ensure constant pH during the course of a plasma protein binding experiment, or to report the final pH of the dialyzed, ultrafiltrate, or ultrasupernatant sample along with the measured $f_{u,p}$ values.

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