Control and Measurement of Plasma pH in Equilibrium Dialysis: Influence on Drug Plasma Protein Binding

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ABSTRACT:
Past publications have highlighted the influence of postdialysis plasma pH on the measured fraction unbound in plasma (fup). There is disparity in the industry as to which of two main methods is more suitable for controlling postdialysis plasma pH: the use of either a stronger buffer or a CO2 atmosphere for the incubation. In the current study, it has been found that 10% CO2 could be too high for the buffering capacities of both 100 mM sodium phosphate (pH 7.40 decreased to pH 6.90 after a 6-h incubation) and plasma (decreased below pH 7.40 after a 6-h incubation). To provide appropriate control over the postdialysis plasma pH, for a range of species, it is proposed that a standard phosphate buffer strength (100 mM) and pH (7.40) in combination with a 5% CO2 atmosphere be used for equilibrium dialysis. Furthermore, statistically significant differences in fup values obtained with a pH difference of less than 0.32 pH unit have been demonstrated. An acceptance range for postdialysis plasma pH in routine in vitro fup screening assays of pH 7.40 ± 0.10 is recommended.

Introduction
Determining the extent of drug binding to plasma proteins is an essential step in the drug discovery process for assessing drug disposition (Lombardo et al., 2002; Musteata et al., 2006; Trainer, 2007; van Steeg et al., 2009). Quantification of a drug’s plasma protein binding [and hence fraction unbound in plasma (fup)] is essential for extrapolation of preclinical efficacy data, prediction of in vivo clearance from in vitro data, and estimation of free drug concentration in tissues (Kalvass and Maurer, 2002; Summerfield et al., 2008; He et al., 2009). Interspecies differences in plasma protein binding (PPB) need to be considered for prediction of volume of distribution and clearance in humans (Benet and Hoener, 2002).

Equilibrium dialysis is a well recognized and preferred method for determining the binding of drugs to plasma proteins (Chuang et al., 2009; Howard et al., 2010). Older equilibrium dialysis equipment, for example, systems from Dianorm (Munich, Germany), can be labor-intensive and time-consuming to use and difficult to automate. Several 96-well format equilibrium dialysis devices have been developed and validated, thus facilitating assay automation (Kriv et al., 2001; Banker et al., 2003; Wong et al., 2009; http://www.HTDialysis.com; http://www.harvardappartus.com). However, these methods still require soaking of membranes and washing and assembly of equipment, thereby increasing the probability of leakage and contamination. With the 96-well plate for RED (Thermo Fisher Scientific, Loughborough, UK), there are no washing or assembly steps. Several groups are using this plate (Waters et al., 2008; see http://www.piercenet.com for poster presentations).

It has been observed that buffer choice in equilibrium dialysis assays affects fup (Guentert and Oie, 1982). It was subsequently reported that there is a difference in pH between fresh plasma and stored plasma (Fura et al., 2003). This was attributed to rapid CO2 loss during plasma collection, storage, and incubation, causing an increase in pH. The equilibria of CO2 in water (eqs. 1a and 1b) are widely known (Roughton and Booth, 1938) and are nonionic and slow, with an equilibration time of several minutes. The reaction time is catalyzed to milliseconds by carbonic anhydrase, which is not present in plasma (Kitching and Edge, 2002).

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \] (1a)

\[
\text{CO}_2 + \text{OH}^- \leftrightarrow \text{HCO}_3^- \] (1b)

It has been shown that an increase in plasma pH can have a significant influence on the extent of drug binding to plasma proteins (Paxton and Calder, 1983; Kremer et al., 1988; Hinderling and Hartmann, 2005; Wan and Rehngren, 2006; Kochansky et al., 2008). In attempts to address this issue, Wan and Rehngren (2006) determined that stronger buffers were required to minimize pH shifts, whereas others advocated the use of a 10% CO2 atmosphere (Fura et al., 2003; Musteata et al., 2006; Kochansky et al., 2008) or initial purging of plasma with CO2 followed by ultracentrifugation for determination of fup (Nilsson and Schmidt, 2001). There is further discussion in the literature, supporting the use of either stronger buffer or incubation in a CO2 atmosphere (Banker and Clark, 2008). Past studies have also examined the effect of a relatively wide postdialysis plasma pH range (of between pH 6 and 9) on fup (Hinderling and Hartmann, 2005; Wan...
and Rehngren, 2006). In a normal physiological state the in vivo arterial blood pH range is much narrower, within pH 7.35 and 7.45 (Eckert et al., 1997; Porter and Kaplan, 2006; Troy, 2006; Waugh and Grant, 2007). This is due to the homeostatic control of blood hydrogen ion concentration by complex acid-base balance mechanisms. The range of postdialysis plasma pH values between pH 6 and 9 is only observed in abnormal circumstances (e.g., hypoxia and hyperventilation) or in disease states such as metabolic acidosis or alkalosis (Davenport, 1974).

In the current study, 22 marketed drugs were chosen for a series of investigations using the disposable 96well RED plate and ultrapressure liquid chromatography (UPLC)-tandem mass spectrometry for the determination of fup. These compounds had a broad range of physicochemical properties and protein binding. Although the use of either a stronger buffer or incubation in a CO2 atmosphere resulted in improved pH control (Banker and Clark, 2008), we demonstrate that it is necessary to use both of these factors in combination. Moreover, there are other factors such as predialysis buffer pH that influence accurate control of postdialysis plasma pH. These factors have been taken into consideration and applied to a range of species. It is proposed that the postdialysis plasma pH is the most important measurement to obtain rather than a during-dialysis plasma pH (or pH shift from start to finish) because it has been postulated that pH-dependent changes in plasma protein binding are reversible (Kochansky et al., 2008). Furthermore, data are presented to highlight the fact that the method of postdialysis pH measurement is critically important. The impact of a postdialysis plasma pH variation of less than 0.50 pH unit on the obtained value of fup is discussed.

Materials and Methods

Materials. Pooled human plasma (EDTA as coagulant) was obtained fresh from in-house donors (n = 3) or from BioChemed Services (Winchester, VA) and stored at −20°C. Plasma from all other species (cynomolgus monkey, Wistar rat, Sprague-Dawley rat, ICR mouse, C57 mouse, and beagle dog) was supplied by Harlan Seralab (Haywards Heath, UK) and stored at −20°C. Test compounds (bupivacaine, carbamazepine, cinacalcet, clozapine, diclofenac, diltiazem, fluoxetine, haloperidol, imipramine, midazolam, milnacipran, mirtazapine, nicardipine, paroxetine, propranolol, quinidine, risperidone, tranylcypromine, trimipramine, verapamil, and warfarin) were obtained from Sigma-Aldrich (Dorset, UK) with measured pKa values being taken from the literature. The RED plates, 96-well plates for analysis, gas-permeable lid seals, Eppendorf tubes, and lids were purchased from Thermo Fisher Scientific. Deionized water was obtained from a purification system (Milli-Q Academic with a Quantum Ex Ultrapure Organex cartridge; Millpore Corporation, Watford, UK). High-performance liquid chromatography-grade acetonitrile, formic acid, ammonium formate (aqueous), methanol, isopropanol, and DMSO were purchased from Thermo Fisher Scientific. The pH of the buffers, which were supplied in sacs, was determined in-house (predialysis pH) because the range stipulated by manufacturers was too wide (e.g., pH 7.4 ± 0.2).

Buffers. The following four buffers were used in this study: buffer 1, 10 mM phosphate-buffered saline, 140 mM NaCl, and 2.7 mM KCl (predialysis pH 7.43; Sigma-Aldrich); buffer 2, 100 mM sodium phosphate and 150 mM NaCl (predialysis pH 7.00; Thermo Fisher Scientific); buffer 3, 100 mM sodium phosphate and 150 mM NaCl [made in-house to pH 7.40 (unless otherwise stated) as detailed below]; and buffer 4, 8.3 mM phosphate-buffered saline, 140 mM NaCl, and 2.7 mM KCl [made in-house to pH 7.40 as detailed below (used by Kochansky et al., 2008)].

Buffer 3 was prepared by the following method. A basic solution was made by dissolving 14.2 g/l Na2HPO4 and 8.77 g/l NaCl in deionized water. An acidic solution was made by dissolving 15.6 g/l NaH2PO4·2H2O and 8.77 g/l NaCl in deionized water. The basic solution was then titrated with the acidic solution to pH 7.40 (or as required).

Buffer 4 was prepared as per buffer 3 with adjusted constituents and concentrations: basic solution, 0.2 g/l Na2HPO4; 0.2 g/l KCl; and 8 g/l NaCl; and acidic solution, 1.15 g/l KH2PO4, 0.2 g/l KCl, and 8 g/l of NaCl.

Dialysis Method. An initial in-house validation that showed no fup difference between singly incubated and coincubated (cassette) compounds (data not shown) was completed. Results were consistent with the findings of Wan and Rehngren (2006). Measurement of fup was performed using a disposable 96-well RED plate with cassettes of five compounds (pooling of compounds predialysis), each run in triplicate. The RED plate and all buffer and plasma solutions were heated to 37°C before dialysis. All compounds were freshly prepared from powder at a stock concentration of 10 mM in DMSO. Cassettes were prepared by mixing 10 µl of each 10 mM test compound stock (five test compounds per cassette) and 50 µl of DMSO to yield a cassetted compound concentration of 1 mM. Subsequently, 10 µl of the cassette was spiked into 990 µl of plasma and thoroughly mixed, resulting in a final compound concentration of 10 µM for dialysis. The spiked plasma (300 µl) was added to the donor side of the RED plate. Buffer (500 µl) was added to the receiving well. The RED plate was sealed with a gas-permeable lid, and dialysis was performed at a shaking speed of 800 rpm (Eppendorf Thermostom Comfort; Thermo Fisher Scientific) within a 5% CO2 incubator for 6 h at 37°C. Previous in-house validation showed that equilibrium was achieved within a 6-h incubation time. After dialysis, 20 µl of sample from the plasma chamber of the RED plate was added to 80 µl of fresh buffer in a clean 96-well analytical plate, and 80 µl of sample was removed from the buffer chamber and added to 20 µl of fresh blank plasma. These samples were immediately quenched with 300 µl of acetonitrile containing internal standard (positive ion mode: desipramine at 0.5 µg/ml; negative ion mode: tolbutamide at 0.25 µg/ml). The plate was shaken for 10 min using a Siemens MicroMix 5 (EURO/DPC Ltd., Gwynedd, UK) set at amplitude 9, form 20. The plate of quenched samples was stored for 15 min at ~80°C to facilitate protein precipitation and subsequently centrifuged at 3000g at 4°C for 10 min. After centrifugation, a portion of the resulting supernatant (100 µl) was transferred to a clean 96-well analytical plate, and 100 µl of deionized water was added.

Several experimental studies were performed in three stages. The first stage (1 and 2 below) was to investigate the impact of sealing or not sealing the RED plate within a CO2 incubator. In addition, the influence of CO2 incubation on buffer and plasma solutions with and without dialysis was studied. The next stage (3 and 4 below) was to implement observations from stage 1 and to investigate further the selection of standard buffer/atmospheric conditions conducive to reaching a postdialysis plasma of pH 7.40 for a range of species. The third and final stage (5 below) was to compare the fup values obtained using proposed standard buffer/atmospheric conditions (from results generated in stages 1 and 2) to another three widely used buffer/atmospheric conditions (the difference in fup values is attributed to postdialysis plasma pH differences).

1. Influence of incubation in a CO2 atmosphere on volume loss from the system and pH of buffer and plasma. Because of the unavailability of a commercially supplied buffer of strength similar to that of buffer 2 but starting at pH 7.40, it was made in-house (buffer 3, see above). An experiment was conducted without dialysis in which 500 µl of buffers 1, 2, 3, or 4 or 300 µl of either human or Wistar rat plasma only were placed in Eppendorf tubes. These tubes were then incubated under normal atmospheric conditions (nonshaking water bath) or in a 5 or 10% CO2 atmosphere (thermoshaker), all at 37°C. The pH of the separate buffers or plasmas was measured in situ after 6 h. Further pH measurements were made for buffer 3, human plasma, and Wistar rat plasma every 5 min for 30 min after removal from the CO2 incubator.

To measure volume loss over the 6-h dialysis period used in this study, the RED plate was weighed (a) empty, (b) after the addition of assay components, and (c) after the 6-h incubation period. The percent volume lost was calculated according to eq. 2:

\[
\text{% Volume loss} = 100 - ((c - a)/(b - a))
\]

This calculation was used to compare the volume loss under different conditions: with no lid in the 5% CO2 incubator, with a gas-permeable lid in the 5% CO2 incubator, and with an adhesive foil lid under normal atmospheric conditions (within a closed lid thermostaker).

2. Postdialysis plasma pH: Influence of CO2 and method for pH determination. Human plasma from different batches was dialysed against either buffer 3 or 4 and incubated at either 5% or 10% CO2. pH readings were made at 37°C using two methods for comparison: pH measurement...
method 1: pH was measured in situ, in triplicate, immediately after a 6-h dialysis and averaged; and pH measurement method 2: plasma from triplicate wells was aspirated by pipette and pooled into a clean 96-well analytical plate, resulting in the pH measurement being taken after approximately 20 min after a 6-h dialysis.

3. Applicability of standard conditions to plasma from seven species.

Plasma from seven species was separately dialysed using four buffer/ atmospheric combinations. All pH readings were made in triplicate (see pH method 1 above) at 37°C after a 6-h dialysis. The assay was repeated on 3 separate days.

4. Influence of buffer predialysis pH on postdialysis plasma pH.

Buffer 3 was prepared with various predialysis pH values and dialysed against human plasma in a 5% CO2 environment for 6 h with a mixture of five drugs (warfarin, carbamazepine, propranolol, nicardipine, and diazepam) to mimic normal assay conditions. All pH readings were made in duplicate (see pH method 1 above) at 37°C after a 6-h dialysis.

5. Influence of postdialysis plasma pH on fup. Analyses were performed as per dialysis method (see above), with alteration of buffer/atmospheric conditions. Paired t-tests were performed using Minitab software (Minitab Ltd., Coventry, UK) for each buffer/atmospheric condition and each compound. All pH readings were made in triplicate (see pH method 1 above) at 37°C after a 6-h dialysis.

Instrumentation and Analytical Method.

The Eppendorf Thermomixer Comfort was set to 42°C. Initial tests were performed in situ to confirm that this temperature was required to achieve and maintain the liquids in the RED plate at 37°C. pH measurements were recorded using a Beckman pH meter equipped with a Hamilton Minitorde pH electrode, which was calibrated before every experiment using commercially available standard buffers of known pH (phthalate, pH 4; phosphate, pH 7; and borate, pH 10; Thermo Fisher Scientific). A nonhumidified incubator with a variable CO2 concentration setting (phthalate, pH 4; phosphate, pH 7; and borate, pH 10; Thermo Fisher Scientific).

A nonhumidified incubator with a variable CO2 concentration setting was used at 37°C (LEEC, Nottingham, UK).

All analyses were performed on a Waters Acquity ultra-pressure liquid chromatograph coupled to a Quattro Premier XE mass spectrometer with electrospray ionization (Waters, Manchester, UK). The acquisition and UPLC run time was 0.9 min. The aqueous mobile phase (A) was 10 mM ammonium formate adjusted to pH 3.5 with formic acid, and the organic mobile phase (B) was acetonitrile. The flow rate was 0.8 ml/min through an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7-μm particle size; Waters) maintained at 45°C. The proportion of mobile phase B was initially 5% and increased linearly (curve 6) to 100% over 0.4 min where it was held for 0.3 min and then was decreased (curve 1) back to 5% and finally held at this for the remaining 0.2 min to reequilibrate the column. The injection volume was 3 μl (positive ionization mode) with the autosampler operating in partial loop with needle overfill mode. As an alternative, 10 to 20 μl (negative ionization mode or for compounds with poor sensitivity) was used in full loop mode. Between injections the autosampler needle was washed with 0.8 ml of a strong wash mixture comprising equal proportions of water, acetonitrile, methanol, and isopropanol with 0.1% formic acid. The source temperature of the mass spectrometer was maintained at 150°C with a desolvation temperature of 450°C. The most abundant product ion of each pseudomolecular ion, or [M + H]+ (1 + 20, v/l) to matrix with the final sample composition. Regression models were fitted using linear regression with 1/x weighting. If this model produced a biased percent error residuals plot, then quadratic regression with 1/x weighting was used. Percent PPB and fup were calculated according to eqs. 3 and 4, respectively.

\[
\% \text{PPB} = 100 - \left(\frac{D_b \times C_i}{D_i \times C_p}\right)
\]

(3)

where \(C_i\) and \(C_p\) represent the calculated concentration of the compound from the buffer and plasma chambers, respectively, and \(D_i\) and \(D_p\) represent the final dilution of the buffer and plasma samples upon normalization (which accounts for sample matrix matching).

\[
\text{fup} = \frac{(D_b \times C_i) + (D_i \times C_p)}{10}
\]

(4)

Recovery was measured to ensure robustness of repeat measurements. This was calculated as in eq. 5.

\[
\% \text{Recovery} = \frac{(D_b \times C_i) + (D_i \times C_p)}{10}
\]

(5)

Results

Influence of Incubation in a CO2 Atmosphere on Volume Loss from the System and pH of Buffer and Plasma. The effect of incubation in a CO2 atmosphere on the pH of separate buffers and plasmas (i.e., with no dialysis) is shown in Table 1. pH measurement was made in situ immediately after 6 h of incubation at 37°C. Plasma with no cap showed an increase in pH under normal atmospheric conditions and tended toward pH 7.40 under a 5% CO2 atmosphere. Buffers with no cap exhibited a decrease in pH under a CO2 atmosphere with the stronger buffers (2 and 3) giving the smallest decrease. A 10% CO2 atmosphere produced a larger decrease in pH than 5% CO2 for all buffers. Postincubation plasma increased in pH upon removal from the CO2 incubator after a 6-h incubation, as did buffer 3 (Fig. 1).

A 6-h dialysis with the RED plate in 5% CO2 at 37°C with no lid and with a gas-permeable lid resulted in volume losses of 16.6 and 0.1%, respectively. Under normal atmospheric conditions in a thermostaker, the volume loss was minimal (0.1%).

Postdialysis Plasma pH: Influence of CO2 and Method for pH Determination. After observation of the effect on pH of incubation in a CO2 atmosphere on separate buffers and plasmas (1 above), dialysis

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Influence of a 6-h incubation in different atmospheric conditions on buffer and plasma pH in both capped and uncapped tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preincubation pH</td>
<td>Normal Atmosphere</td>
</tr>
<tr>
<td>Buffer 1 (10 mM)</td>
<td>7.43</td>
</tr>
<tr>
<td>Buffer 2 (100 mM)</td>
<td>7.00</td>
</tr>
<tr>
<td>Buffer 3 (100 mM)</td>
<td>7.39</td>
</tr>
<tr>
<td>Buffer 4 (8.3 mM)</td>
<td>7.39</td>
</tr>
<tr>
<td>Human plasma</td>
<td>7.96</td>
</tr>
<tr>
<td>Human plasma*</td>
<td>7.04</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>7.44</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>7.92</td>
</tr>
<tr>
<td>Wistar rat plasma</td>
<td>7.14</td>
</tr>
</tbody>
</table>

* Donor of n = 1.

Δ denotes the pH change between preincubation and postincubation.
experiments were undertaken to compare the effect of two different CO2 incubation concentrations on postdialysis plasma pH. Buffers 3 and 4 (different strengths but both starting at pH 7.40) were incubated at either 5 or 10% CO2. This process was repeated on different days to assess reproducibility. For buffer 3, this incubation was also performed using five (warfarin, carbamazepine, propranolol, nicardipine, and diazepam), one (warfarin), or no compounds to assess any compound or cassetting influence on the postdialysis plasma pH. Incubation in a 10% CO2 atmosphere resulted in postdialysis plasma pH values less than pH 7.40 for both buffers (Table 2). Buffer 3 under a 5% CO2 atmosphere produced postdialysis plasma pH values close to pH 7.40. The postdialysis plasma pH measurement method 2 (aspirating and pooling) resulted in pH values 0.2 to 0.3 unit higher than method 1 (in situ). On the basis of these observations from stage 1 (1 and 2 above), 5% CO2 and buffer 3 (100 mM at pH 7.40) are suggested as optimal and hereafter referred to as the standard conditions.

Applicability of Standard Conditions to Plasma from Seven Species. A comparison of postdialysis plasma pH values for seven different species was made. The plasmas were dialysed against buffers 2 and 3 (both having same strength but differing in predialysis pH) either in a 5% CO2 atmosphere or under normal atmospheric conditions. Nine separate measurements were made (n = 3 on 3 separate days) and the data are shown in Fig. 2. The standard conditions (c) consistently reached close to pH 7.40 for all seven species. Use of the same buffer (buffer 3) with a lower predialysis pH and/or under normal atmospheric conditions did not achieve a postdialysis pH of 7.40.

Influence of Buffer Predialysis pH on Postdialysis Plasma pH. To assess the robustness of the standard conditions (1–3 above), buffer 3 was subsequently prepared at eight different predialysis pH values, and the respective postdialysis human plasma pH values were obtained (Fig. 3). The predialysis plasma pH was 7.61. Buffer with a predialysis pH of 7.40 produced postdialysis plasma of the same pH (7.40), whereas all other predialysis buffer pH values produced disparate postdialysis plasma pH values, which tended toward pH 7.40.

Influence of Postdialysis Plasma pH on fup. To obtain a comparison of fup values at differing postdialysis plasma pH values, a set of 22 drugs were dialysed in human plasma against four buffer/atmospheric combinations (Table 3). A control was included in every cassette with mean fup values and S.D. taken across the assay to monitor repeatability: warfarin (fup CV was 21%) and carbamazepine (fup CV was 14%). Compound recovery was >70% with the exception of midazolam (63%). The buffer/atmospheric combinations and resulting postdialysis plasma pH values were as follows: buffer 1 with normal atmospheric conditions (pH 7.72); buffer 2 with normal atmospheric conditions (pH 7.22); buffer 3 with 5% CO2 (pH 7.40) (standard conditions); and buffer 4 with 5% CO2 (pH 7.63).

The impact of postdialysis plasma pH on fup measurement can be assessed by calculating the fup fold difference of two buffer/atmospheric combinations (one fup divided by the other; where no difference translates to fold difference = 1). This result has been displayed for decreasing postdialysis plasma pH differences (Figs. 4, a–c). The statistical significance of the fup fold difference for each comparison was determined using paired t tests.

Discussion

Influence of Incubation in a CO2 Atmosphere on Volume Loss from the System and pH of Buffer and Plasma. There are some technical difficulties associated with performing equilibrium dialysis in a CO2 atmosphere. It is necessary to have the incubations uncapped (to allow access of CO2); however, the use of a gas-permeable lid with a foil seal lid. Whereas increasing levels of CO2 can result in increased formation of carbamino groups on plasma proteins (Eckert et al., 1997), the effect on PPB is unknown. Consistent with previously published findings (Fura et al., 2003; Kochansky et al., 2008), incubation in a CO2 atmosphere prevented the increase in plasma pH observed under normal atmospheric conditions.

### Table 2

<table>
<thead>
<tr>
<th>CO2 in Incubation</th>
<th>Buffer</th>
<th>No. of Compounds in Cassette</th>
<th>Buffer Predialysis pH</th>
<th>Plasma Predialysis pH</th>
<th>Average Postdialysis Plasma pH*</th>
<th>Pooled Postdialysis Plasma pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% CO2</td>
<td>3</td>
<td>5</td>
<td>7.39</td>
<td>7.65</td>
<td>7.41 ± 0.01</td>
<td>7.52</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>7.39</td>
<td>8.01</td>
<td>7.37 ± 0.01</td>
<td>N.D.</td>
</tr>
<tr>
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<td>3</td>
<td>5</td>
<td>7.39</td>
<td>7.76</td>
<td>7.40 ± 0.02</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>7.40</td>
<td>7.63</td>
<td>7.41 ± 0.01</td>
<td>7.52</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>7.39</td>
<td>8.02</td>
<td>7.42 ± 0.02</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>7.39</td>
<td>7.65</td>
<td>7.63 ± 0.01</td>
<td>7.86</td>
</tr>
<tr>
<td>10% CO2</td>
<td>3</td>
<td>5</td>
<td>7.41</td>
<td>7.74</td>
<td>7.28 ± 0.02</td>
<td>7.43</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>7.41</td>
<td>7.96</td>
<td>7.33 ± 0.01</td>
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<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>7.38</td>
<td>7.74</td>
<td>7.22 ± 0.02</td>
<td>7.53</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>7.38</td>
<td>7.96</td>
<td>7.34 ± 0.03</td>
<td>7.54</td>
</tr>
</tbody>
</table>

* N.D., not determined.

a Predialysis plasma pH was dependent on the day/batch.

b n = 3, measured in situ immediately after a 6-h dialysis (pH measurement method 1).

c Plasma from triplicate wells was aspirated and pooled (pH measurement method 2).
The observation that CO₂ concentration influences the postdialysis plasma pH may highlight the requirement for interlaboratory investigation of an optimal CO₂ level of approximately 5% because there are variations in seals, models, and set-up of incubators and thereshakers, which may affect CO₂ permeation into liquids. It was found that the pooling or addition of compounds did not affect the postdialysis plasma pH (Table 2).

Influence of Predialysis Buffer pH on Postdialysis Plasma pH. It can be deduced from Figs. 2 and 3 that, in addition to using a stronger buffer, the predialysis buffer pH for incubation in a 5% CO₂ atmosphere must be at pH 7.40 to consistently reach the target postdialysis plasma pH of 7.35 to 7.45. This value has been found to be appropriate for the range of species and predialysis plasma pH values routinely observed. For human plasma, when the predialysis buffer pH was not pH 7.40, there was a tendency for the postdialysis plasma pH to deviate toward pH 7.40.

Fig. 2. Influence of buffer choice and atmospheric conditions on postdialysis pH of plasma from seven species. Buffer/atmospheric conditions selected were buffer 2 with 5% CO₂ (a), buffer 2 with normal atmospheric conditions (b), buffer 3 with 5% CO₂ (c), and buffer 3 with normal atmospheric conditions (d). Across the species, the pH measurement percent CV ranges for each set of incubation conditions were 0.1 to 0.8% (a), 0.3 to 0.7% (b), 0.4 to 0.7% (c), and 0.5 to 1.0% (d).

Fig. 3. Influence of predialysis pH choice of buffer 3 on postdialysis plasma pH. It was found that, using buffer 4, a 10% CO₂ environment, and pH measurement method 2 (i.e., pH measurement made approximately 20 min after removal from the incubator) the postdialysis plasma pH was pH 7.4 ± 0.2. In the current study, using pH measurement method 2, postdialysis pH values of 7.53 and 7.54 were found (assays performed on separate days). However, when the pH was measured immediately in situ (method 1), pH values of 7.22 and 7.34 were found. Thus, it is possible that the pH value of 7.4 ± 0.2 reported by Kochansky et al. (2008) could have arisen from a lower postdialysis plasma pH and subsequent loss of CO₂ before pH determination.

The observation that CO₂ concentration influences the postdialysis plasma pH may highlight the requirement for interlaboratory investigation of an optimal CO₂ level of approximately 5% because there are variations in seals, models, and set-up of incubators and thereshakers, which may affect CO₂ permeation into liquids. It was found that the pooling or addition of compounds did not affect the postdialysis plasma pH (Table 2).
(Fig. 3). As discussed previously, this result could be due to the ability of plasma proteins to act as a weak buffer. It was also discovered that commercially available buffer mixes could not be prepared within the necessary tolerance (pH 7.4 ± 0.2) to ensure the required reproducibility of postdialysis plasma pH.

**Influence of Postdialysis Plasma pH on fup**. This study confirms the findings of Wan and Rehgregen (2006) that compound class and pKa play an important role in the influence of pH on fup (Fig. 4, a–c). In general, the degree of protein binding increases with hydrophobicity (Colmenarejo et al., 2001); therefore, for basic drugs, an increase in pH results in an increase in the ratio of un-ionized (hydrophobic) species and therefore potentially a decrease in fup. This principle and the application of the Henderson-Hasselbalch equation (which quantifies the relative ratio of un-ionized to ionized species at pH 7.40 of a drug with a known pKa) can be used to predict an increase or decrease in fup for a drug at a given postdialysis plasma pH compared with pH 7.40. In addition, it could be predicted that, for the pH range studied, a greater difference in fup would be observed for compounds within a pKa range of 6.4 to 8.4 (e.g., nicardipine has a pKa of 7.4; at pH 7.40, 50% will be un-ionized; at pH 7.90, 69% will be un-ionized). This observation is substantiated by the data shown in Fig. 4, a–c, for which basic drugs in this pKa range show the most prominent shift in fup fold difference. For acids, the opposite

<table>
<thead>
<tr>
<th>Marker in Fig. 4</th>
<th>Compound</th>
<th>pK_a</th>
<th>Class</th>
<th>Buffer 2, Normal Atmospheric Conditions; Postdialysis Plasma pH 7.72</th>
<th>Buffer 3, 5% CO_2; Postdialysis Plasma pH 7.40</th>
<th>Buffer 4, 5% CO_2; Postdialysis Plasma pH 7.63</th>
<th>Buffer 1, Normal Atmospheric Conditions; Postdialysis Plasma pH 7.72</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Diazepam</td>
<td>3.4</td>
<td>Neutral</td>
<td>0.019 ± 0.002</td>
<td>0.017 ± 0.003</td>
<td>0.019 ± 0.003</td>
<td>0.023 ± 0.001</td>
</tr>
<tr>
<td>B</td>
<td>Diclofenac</td>
<td>4.0</td>
<td>Acid</td>
<td>0.003 ± 0.001</td>
<td>0.007 ± 0.000</td>
<td>0.005 ± 0.002</td>
<td>0.004 ± 0.002</td>
</tr>
<tr>
<td>C</td>
<td>Cinoxacin</td>
<td>4.7</td>
<td>Acid</td>
<td>0.251 ± 0.104</td>
<td>0.214 ± 0.016</td>
<td>0.230 ± 0.039</td>
<td>0.266 ± 0.025</td>
</tr>
<tr>
<td>D</td>
<td>Warfarin</td>
<td>4.9</td>
<td>Acid</td>
<td>0.009 ± 0.002</td>
<td>0.014 ± 0.003</td>
<td>0.013 ± 0.003</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>E</td>
<td>Nicardipine</td>
<td>7.2</td>
<td>Base</td>
<td>0.008 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td>0.008 ± 0.002</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>F</td>
<td>Mirtazapine</td>
<td>7.3</td>
<td>Base</td>
<td>0.320 ± 0.025</td>
<td>0.286 ± 0.034</td>
<td>0.266 ± 0.058</td>
<td>0.160 ± 0.045</td>
</tr>
<tr>
<td>G</td>
<td>Buspirone</td>
<td>7.3</td>
<td>Base</td>
<td>0.333 ± 0.022</td>
<td>0.277 ± 0.022</td>
<td>0.261 ± 0.039</td>
<td>0.214 ± 0.041</td>
</tr>
<tr>
<td>H</td>
<td>Clozapine</td>
<td>7.5</td>
<td>Base</td>
<td>0.101 ± 0.012</td>
<td>0.081 ± 0.011</td>
<td>0.058 ± 0.011</td>
<td>0.042 ± 0.009</td>
</tr>
<tr>
<td>I</td>
<td>Diltiazem</td>
<td>8.0</td>
<td>Base</td>
<td>0.417 ± 0.038</td>
<td>0.349 ± 0.015</td>
<td>0.350 ± 0.030</td>
<td>0.298 ± 0.038</td>
</tr>
<tr>
<td>J</td>
<td>Quinidine</td>
<td>8.1</td>
<td>Base</td>
<td>0.274 ± 0.020</td>
<td>0.218 ± 0.001</td>
<td>0.299 ± 0.060</td>
<td>0.251 ± 0.026</td>
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<tr>
<td>K</td>
<td>Tranylcypromine</td>
<td>8.2</td>
<td>Base</td>
<td>0.630 ± 0.027</td>
<td>0.612 ± 0.107</td>
<td>0.530 ± 0.051</td>
<td>0.490 ± 0.096</td>
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<tr>
<td>L</td>
<td>Haloperidol</td>
<td>8.3</td>
<td>Base</td>
<td>0.223 ± 0.032</td>
<td>0.161 ± 0.018</td>
<td>0.164 ± 0.027</td>
<td>0.124 ± 0.014</td>
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<tr>
<td>M</td>
<td>Risperidone</td>
<td>8.3</td>
<td>Base</td>
<td>0.276 ± 0.029</td>
<td>0.186 ± 0.030</td>
<td>0.222 ± 0.051</td>
<td>0.163 ± 0.044</td>
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<tr>
<td>N</td>
<td>Trimipramine</td>
<td>8.4</td>
<td>Base</td>
<td>0.106 ± 0.016</td>
<td>0.054 ± 0.013</td>
<td>0.052 ± 0.004</td>
<td>0.037 ± 0.016</td>
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<tr>
<td>O</td>
<td>Verapamil</td>
<td>8.7</td>
<td>Base</td>
<td>0.233 ± 0.035</td>
<td>0.160 ± 0.016</td>
<td>0.154 ± 0.022</td>
<td>0.137 ± 0.021</td>
</tr>
<tr>
<td>P</td>
<td>Midazolam</td>
<td>9.0</td>
<td>Base</td>
<td>0.034 ± 0.005</td>
<td>0.036 ± 0.004</td>
<td>0.041 ± 0.009</td>
<td>0.019 ± 0.006</td>
</tr>
<tr>
<td>Q</td>
<td>Imipramine</td>
<td>9.4</td>
<td>Base</td>
<td>0.220 ± 0.016</td>
<td>0.181 ± 0.030</td>
<td>0.172 ± 0.043</td>
<td>0.105 ± 0.034</td>
</tr>
<tr>
<td>R</td>
<td>Paroxetine</td>
<td>9.5</td>
<td>Base</td>
<td>0.136 ± 0.011</td>
<td>0.099 ± 0.026</td>
<td>0.089 ± 0.020</td>
<td>0.074 ± 0.006</td>
</tr>
<tr>
<td>S</td>
<td>Propranolol</td>
<td>9.6</td>
<td>Base</td>
<td>0.315 ± 0.021</td>
<td>0.231 ± 0.046</td>
<td>0.213 ± 0.015</td>
<td>0.216 ± 0.031</td>
</tr>
<tr>
<td>T</td>
<td>Milnacipran</td>
<td>9.7</td>
<td>Base</td>
<td>0.809 ± 0.048</td>
<td>0.837 ± 0.071</td>
<td>0.777 ± 0.110</td>
<td>0.828 ± 0.024</td>
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<tr>
<td>U</td>
<td>Fluoxetine</td>
<td>10.1</td>
<td>Base</td>
<td>0.079 ± 0.015</td>
<td>0.056 ± 0.006</td>
<td>0.063 ± 0.011</td>
<td>0.040 ± 0.013</td>
</tr>
<tr>
<td>V</td>
<td>Carbamazepine</td>
<td>Neutral</td>
<td>0.303 ± 0.021</td>
<td>0.291 ± 0.029</td>
<td>0.288 ± 0.053</td>
<td>0.299 ± 0.023</td>
<td></td>
</tr>
</tbody>
</table>

*Fig. 4. Comparison of fup values obtained with various postdialysis pH values. Data are expressed as fup fold difference for each of three combinations of dialysis conditions and are plotted against the pKa of the test compounds. a, Δ pH 0.50 with fup fold difference for buffer 2, normal atmospheric conditions (postdialysis plasma pH 7.72) versus buffer 1, normal atmospheric conditions (postdialysis plasma pH 7.72). †, 16 of 22 are statistically significantly different. b, Δ pH 0.32 with fup fold difference for buffer 3, 5% CO_2 (postdialysis plasma pH 7.40) versus buffer 1, normal atmospheric conditions (postdialysis plasma pH 7.72). ‡, 12 of 22 are statistically significantly different. c, Δ pH 0.23 with fup fold difference for buffer 3, 5% CO_2 (postdialysis plasma pH 7.40) versus buffer 4, 5% CO_2 (postdialysis plasma pH 7.63). †, 1 of 22 is statistically significantly different. fup measurements were made in triplicate for all buffers and on two separate days for buffer 2, normal atmospheric condition (R² = 0.989). Horizontal lines at 0.5 and 2.0 represent a 2-fold difference. All compounds are basic except for compound A, which is effectively neutral, and B, C, and D are acidic. Carbamazepine (neutral, data not plotted) did not show a significant change in fup (comparison a; p = 0.787).*
would be expected; however, other groups have shown that some acids exhibit a decrease in fup with an increase in pH (Panjehshahin et al., 1992; Hindering and Hartmann, 2005). We also observed this phenomenon for warfarin, which could potentially be due to a change in ionization of albumin, a charge-charge interaction with the lysine residue on albumin, or a change in conformation of albumin (Kasai-Morita et al., 1987). It is important to highlight the fact that this last explanation could also influence the binding of neutral drugs.

The current study has shown a statistically significant difference in fup for 16 of 22 compounds at postdialysis plasma pH 7.22 compared with pH 7.72 (pH difference of 0.50) (Fig. 4a). This result indicates that a postdialysis plasma pH acceptance range of pH 7.40 ± 0.25 is too wide. However, with a pH difference range of 0.23 (Fig. 4c) only 1 of the 22 compounds produced a statistically significant difference in fup, indicating that a range of pH 7.40 ± 0.10 may be acceptable. The influence of a smaller pH range on fup values would be difficult to investigate given inherent pH electrode, experimental, and instrumental errors. Some of the compounds exhibiting statistically significant differences in fup are highly bound to plasma proteins, and these differences could therefore substantially influence interpretation of pharmacological and pharmacokinetic data.

To conclude, to improve the reproducibility of in vitro fup measurements, we propose that dialysis be performed using a minimum strength plasma proteins, and these differences could therefore substantially influence pH range on fup values would be difficult to investigate given inherent pH measurement of central nervous system exposure. Xenobiotica 39:687–693. Hindering PH and Hartmann D (2005) The pH dependency of the binding of drugs to plasma proteins in man. Ther Drug Monit 27:71–85.


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Authorship Contributions

Participated in research design: Curran, Claxton, Hutchison, and Littlewood. Conducted experiments: Curran, Claxton, and Hutchison. Performed data analysis: Curran, Claxton, Hutchison, and Harradine. Wrote or contributed to the writing of the manuscript: Curran, Claxton, Hutchison, Harradine, Martin, and Littlewood.

References


