The Importance of pH Control for Accurate Assessment of in vitro Protein Binding

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Abstract
The measurement of plasma protein binding is generally considered a routine in vitro study to provide information on drug binding that can be incorporated into further processing of both in vitro and in vivo data. However, recent publications have highlighted the complexities of the methodology and indicate that temperature and pH need to be carefully controlled to provide meaningful data.1 One major factor is ensuring that the pH remains at a physiological level (pH 7.4 ± 0.2) throughout the incubation (typically 6-8 hours) to prevent any conformational changes to plasma proteins that may alter the extent of protein binding. In this study, the pH was monitored after incubating mouse, rat, rabbit, dog and human plasma in the presence and absence of 5% CO₂ at 37°C for up to 6 hours in a RED device (ThermoFisher Scientific, Waltham, MA). The effect of pH on the protein binding of three well-characterized compounds—warfarin, testosterone and caffeine—was also assessed. In the absence of CO₂, the pH of rat, dog, rabbit and human plasma increased to greater than pH 7.9 after a 6-hour incubation, whilst the pH in human plasma reached a maximum of pH 8.4. Conversely, the pH of mouse plasma remained constant over the 6-hour incubation (maximum of pH 7.8). In the presence of CO₂, the pH was controlled for all species within the range of pH 7 ± 0.2, with the exception of mouse plasma where a decrease to pH 7.12 was observed after 6 hours. The free fraction was determined in all species at 6 hours in the presence and absence of CO₂. For caffeine, the binding was consistent between the two conditions in all species. However, for warfarin and testosterone, a difference in the free fraction was observed in rat, rabbit and human plasma where an increase in the free fraction was observed in the presence of CO₂ (>1.3-fold). The greatest difference was observed in rat plasma, where a 2-fold increase in the free fraction was observed for both compounds at 6 hours in the presence of CO₂. In summary, this indicated that there was a species difference in pH control such that the pH of mouse plasma was maintained over the 6-hour incubation without additional pH control. For the other species investigated, however, incubations in the presence of CO₂ were required to maintain the pH at physiological levels. The current study confirmed the importance of controlling pH during the plasma protein binding incubations to obtain an accurate assessment of the free fraction of compounds in vitro.

Objectives
- Assess the change of pH in plasma from mouse, rat, rabbit, dog and human over a typical 6-hour dialysis using the Thermo RED device.
- Determine if plasma pH can be controlled within the range of pH 7.4 ± 0.2 using a 5% CO₂ environment.
- Determine the effect of pH on the fraction unbound of three control compounds: warfarin, caffeine and testosterone.

Methods
Plasma was obtained from mouse, rat, rabbit, dog and human and the pH was monitored prior to the incubation. Plasma and 0.01M phosphate buffered saline was pre-warmed at 37°C for up to 6 hours. Plasmafrom each species was spiked with either radiolabelled testosterone, caffeine or warfarin and 2 individual RED plates were loaded using an identical plate plan. Both plates were sealed with a BreathEasy® membrane and incubated at approximately 37°C. One plate was incubated in the absence of CO₂ and the other plate was incubated in the presence of 5% CO₂. The pH in each well of the dialysis plate was monitored at 0, 2, 4 and 6 hours. After the incubation was complete, the % fraction unbound of each compound was calculated.

Results
Change in Plasma pH in the Presence of CO₂ (Figure 1)
- Rat, rabbit, dog and human pH was controlled within the range of pH 7.4 ± 0.2 over the 6-hour dialysis period.
- Between 4 and 6 hours the pH was comparable, indicating that equilibrium between CO₂ and water had been reached for rat, dog and human plasma.
- Plasma pH was maintained within an acceptable range for up to 4 hours, after which there was a decline in the pH.
- This may be important to consider if a longer dialysis time is required.
- In mouse plasma a decrease in the pH was observed over the dialysis time such that after 4 hours the pH was outside the acceptable range (pH 7.12 at 6 hours).

The Effect of Plasma pH on Caffeine, Testosterone and Warfarin Unbound Fractions (Figures 3 and 4)
- Plasma pH had no impact on the fraction unbound of caffeine (fraction unbound ~84%).
- The fraction unbound of warfarin and testosterone changed by up to 2-fold when plasma from rat, rabbit and human was incubated in the presence of CO₂.
- In all cases an increased fraction unbound was observed in the presence of CO₂.
- For mouse plasma the fraction unbound was generally comparable between the two incubation conditions.

Conclusions
- Plasma pH over a 6-hour dialysis time varied between species, with the exception of mouse plasma where the pH was maintained over the 6-hour incubation without additional pH control.
- For rat, rabbit, dog and human, 5% CO₂ was required to maintain the pH at physiological levels.
- Fraction unbound for testosterone and warfarin was dependent on the pH in rat, rabbit and human plasma.
- Indicates that these compounds may be dependent on binding sites which are affected by an increase in the pH (>7.8).
- Our recommendation is to carry out plasma protein binding studies in a 5% CO₂ environment to maintain a physiological pH.

Figure 1. Change in plasma pH after incubation in the presence of 5% CO₂ for up to 6 hours.

Figure 2. Change in plasma pH after incubation in the absence of 5% CO₂ for up to 6 hours.

Figure 3. Calculated fraction unbound for Testosterone after 6-hour dialysis in the presence and absence of 5% CO₂ (* indicates >1.3-fold difference between 2 values).

Figure 4. Calculated fraction unbound for Warfarin after 6-hour dialysis in the presence and absence of 5% CO₂ (* indicates >1.3-fold difference between 2 values).

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5-7 September 2012
Loughborough, UK

Presented at the
DMDG Open Meeting

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