A Method to Quantify Protein Binding Kinetics and Supersaturated Solutions Precipitation Kinetics

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INTRODUCTION

Drug disposition is governed by the dissolved, free (available) drug concentration. For rapid processes like precipitation or protein binding, conventional measurement methods can be too slow to characterize the kinetics or may overestimate the available drug concentration (for instance, by allowing nuclei or other small structures to pass through filters). Pulsatile microdialysis (PMD) is a novel technique that rapidly measures the available concentration in situ, making it ideal for systems where free drug levels change quickly and total concentration measurements are misleading.

This study applies PMD to two key challenges in pharmaceutics, demonstrating its utility in detecting formulation-induced shifts in free drug availability.

- Quantifying supersaturation and precipitation kinetics
- Determining the binding and release kinetics of drug-carrier systems and fitting a kinetic model to the release data

LEARNING OBJECTIVES

- Understand the principles and advantages of PMD for measuring free drug in complex systems.
- Learn how PMD reveals dynamic kinetic processes in supersaturation and protein binding.
- Explore how PMD supports modeling, AI integration, and formulation screening.

METHODS

Pulsatile Microdialysis (PMD)

- PMD utilizes probes comprising semi-permeable tubular membranes through which a dialysate liquid is passed in a push-rest-collect cycle. Free drug molecules pass through membrane pores from the donor and accumulate vs. time in the dialysate, with PMD sample collection as often as every 7 seconds.
- All collected PMD samples were immediately analyzed for the drug concentration by HPLC.
- The free drug concentration in the donor $C_{D,f}$ is calculated from the PMD sample concentration C_S as $C_S = F_R\, C_{D,f}$ where the fractional recover F_R is determined for each probe using a "dynamic" probe calibration procedure.
- Dynamic calibrations were done to establish F_R when the donor medium concentration changed rapidly.
  - An appropriate "donor" medium of known initial volume and drug concentration (typically zero) at a chosen temperature, pH, etc., was selected.
  - PMD probe(s) were immersed in the donor and allowed to stand for 30 minutes. PMD was started, with samples collected every 7 to 30 seconds.
  - After 30-60 seconds, a controlled infusion of a concentrated drug solution (water + cosolvents, if needed) was started into the stirred donor. The next 2-3 PMD samples were discarded.
  - The PMD sample concentrations were plotted vs. the calculated donor concentration (C_S vs. $C_{D,f}$). The slope was taken as the F_R.

Study A: Supersaturation & Precipitation Kinetics

- Ibuprofen: A solution (2.5 mg/mL in 50:50 MeOH:H_2O) was infused at 1.0 mL/min into pH 2 phosphate buffer.
- Dipyridamole: A solution (1.5 mg/mL in pH 2 buffer) was infused at 0.75 mL/min into pH 7 phosphate buffer.
- The dissolved free drug concentrations C_f were measured every 30 seconds using PMD to characterize the "spring and parachute" profile.
- Data analysis:
  - The total drug mass M_T and concentration C_T at any time t were calculated as
    - $M_T(t) = C_{in}\, qt$ $\quad C_T(t) = M_T(t) / V(t)$ $\quad V(t) = V(0) + qt$
      C_{in} = infused drug solution conc. $\quad q$ = infusion flow rate $\quad V_0$ = initial solution volume $\quad V(t)$ = solution volume at time t
    - $M_P(t) = [C_{Total} - C_{free}] \times V(t)$ (Mass precipitated at any time)
    - $V_P = M_P\ /\ \rho$ (ρ = estimated density of precipitate)
    - The precipitation vs. time is characterized by t_N (from extrapolation of the linear portion of M_P or V_P vs. time to the time axis).

Study B: Warfarin-BSA Binding Kinetics

- Warfarin sodium was rapidly added to bovine serum albumin (BSA) phosphate buffer solutions (pH 7.4 and 37 °C).
- The final total warfarin concentrations after mixing were ~100 µg/mL (~0.300mM) in all kinetic experiments.
- Total BSA concentrations after mixing were 1.2%, 1.6%, 2.0% w/v (0.185mM, 0.246mM, 0.308 mM) for BSA.
- Free warfarin concentrations were measured every 9 seconds using PMD.
- A kinetic model (2nd order binding, 1st order release) was fit to the free warfarin data to estimate the binding (k_{on}) and release (k_{off}) rate constants.

Binding model

$$\begin{bmatrix}\text{Free Drug}\\ C_r\end{bmatrix} + \begin{bmatrix}\text{Empty Sites}\\ P_r\end{bmatrix} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \begin{bmatrix}\text{Bound Drug}\\ C_B\end{bmatrix}$$

C_r = total drug concentration $\quad P_r = \nu P_{r,M}$ = total binding site conc $\quad \nu$ = binding sites per molecule

At equilibrium,

$$K = \frac{k_{on}}{k_{off}} = \frac{C_{B,e}}{C_{F,e} P_{F,e}} \qquad P_{r,e} = \nu P_{r,M} - C_{B,e} \qquad K = \text{equilbrium constant} \qquad (1)$$

$$r = \frac{C_{Bc}}{P_{Tm}} \qquad \frac{r}{C_{Fe}} = \nu K - Kr \qquad K = -\text{slope and } \nu = -\text{intercept / slope} \qquad (2)$$

Kinetic rate equation

$$\frac{dC_F}{dt} = -k_{on} C_F P_T + k_{off} C_B \qquad (3)$$

$$C_F = C_{F,e} + \frac{aA_0 \exp(-ak_{on}t)}{1 - A_0 \exp(-ak_{off}t)} \qquad A_0 = \exp(aA^*), \quad 0 < A_0 < 1 \qquad (4)$$

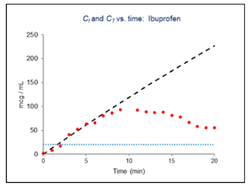
$$a = P_{T,e} + C_{F,e} + \frac{1}{K} = P_T - C_T + 2C_{F,e} + \frac{1}{K} > 0 \qquad (5)$$

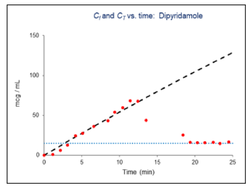
- Data analysis
  - The equilibrium binding constant K and binding stoichiometry ν were determined from equilibrium data and Equation (2).
  - K and total molarities of binding sites (PT) and warfarin (CT) were used to calculate the parameter a for each experiment from Equation (5).
  - Equation (3) was solved using a relaxation method (approach to equilibrium) instead of using initial conditions.
    - Initial conditions (time = 0) are uncertain because it takes several seconds for the warfarin and BSA to become uniformly mixed.
    - The relaxation method mathematically uses the equilibrium data (which are more certain).
    - The parameter A_0 (which depends on the initial conditions) is not used. Instead the additional information is supplied by the equilibrium constant K.
  - Equation (4) was fit to the kinetic data (C_F vs. time) to estimate A_0 and k_{on}. Then, k_{off} was calculated from Equation (1) as $k_{off} = k_{on} / K$.

RESULTS

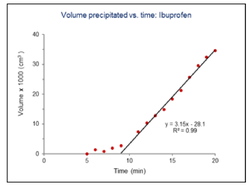
Supersaturation & Precipitation Kinetics

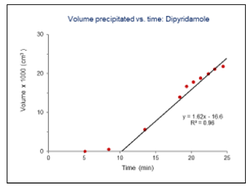
- The dissolved concentration profiles showed "spring and parachute" patterns.
- For both drugs, the dissolved concentration C_f peaked after the onset of nucleation, and within ~1–2 minutes of t_N.
- t_N may be more characteristic of the timing of the precipitation "growth" phase (vs. the onset of nucleation)





- Circles: measure free drug concentration C_f
- Rising dashed line: calculated total drug conc. C_T
- Horizontal dotted line: drug solubility





- Circles: precipitated volume V_P
- Solid line, linear portion of V_P vs. time
- t_N = intercept of solid line and time axis

Ibuprofen

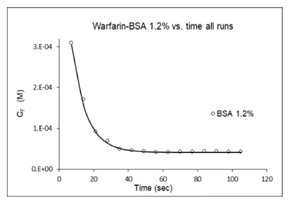
- Solubility ~20 µg/mL
- C_f peaked at ~ 93 µg/mL at ~ 9 min
- Nucleation onset ~ 6 min and t_N ~ 9 min
- Precipitate density was taken as 1.1 g/cm³

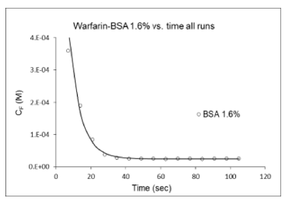
Dipyridamole

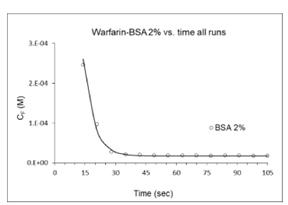
- Solubility ~ 10 µg/mL
- C_f peaked at ~ 70 µg/mL at ~11 min
- Nucleation onset ~ 8 min and t_N ~ 10 min
- Precipitate density was taken as 1.1 g/cm³

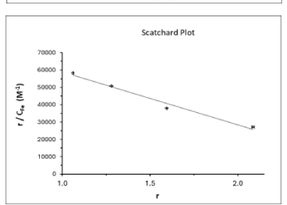
Warfarin–BSA binding kinetics

- Binding was rapid for all BSA concentrations, with > 85% of the warfarin being bound to BSA within 28-35 seconds of mixing.
- Equilibrium data from 0.8%, 1.2%, 1.6% and 2.0% BSA were used in the Scatchard Plot → K = 31700 (1800) M^{-1} and ν = 2.85 (0.03) sites/molecule
- Kinetic analyses were performed on data from 1.2%, 1.6% and 2.0% BSA, which provided at least five data points meeting the following criteria:
  - Free concentrations above $3x10^{-4}$ M were excluded as exceeding the total warfarin concentration (before warfarin and BSA were well mixed)
  - For times longer than 49 seconds, the changes in free warfarin became small, so those times were excluded from fitting analyses
- The estimated average (Std Dev) for the rate constants were k_{on} = 344 (29) $M^{-1}s^{-1}$ and k_{off} = 0.0109 (0.0009) s^{-1}









- Plots of averaged free warfarin C_F for 1.2%, 1.6%, 2.0% BSA. The line represents a fit of the Equation (4) to the averaged data.
- The free warfarin C_F dropped very rapidly in the seconds
- Only C_F data below $3x10^{-4}$ M were used in fits to estimate the kinetic parameter k_{on} (limited to 1.2%, 1.6%, 2.0% BSA)
- Both K and k_{on} were used to estimate k_{off}.)

- Scatchard Plot using 0.8%, 1.2%, 1.6% and 2.0% BSA

DISCUSSION

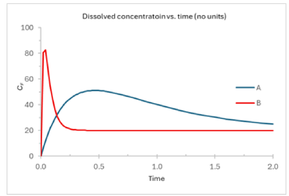
The two studies presented demonstrate that PMD is a robust platform for generating high-quality kinetic data from complex, dynamic systems. The successful fitting of these data to established models confirms that the method is a viable tool for quantitative physicochemical analysis.The key scientific findings support both the experimental method and the analytical models used.

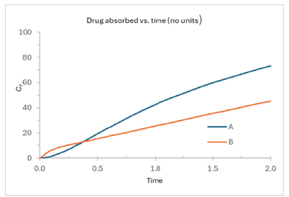
- Differentiating phases of precipitation.
  - The PMD-derived free concentration data for ibuprofen and dipyridamole allows a more nuanced view of precipitation than simple observation.
    - The data allows distinguishing between 1) the initial visual onset of nucleation, and 2) subsequent more rapid growth phase characterized t_N.
    - This allows more accurate modeling and prediction of the supersaturation loss (spring and parachute).
    - This can be done in systems beyond simple solutions, such as simulated gastric (fed and fasted) and simulated intestinal fluid, etc.
- PMD data quality is appropriate to support and characterize the kinetic model
  - The excellent fit of the model to the experimental data for the three BSA concentrations visually confirms that the second-order binding / first-order release model is appropriate for this system.
  - The consistency of the calculated rate constants k_{on} and k_{off} across the BSA concentrations shows that PMD provides consistent data for determining the physicochemical parameters of the drug-protein interaction.

SIGNIFICANCE AND BROADER APPLICATIONS

Application 1: Optimizing Oral Absorption from Supersaturating Formulations

- The kinetic data from PMD allows for the precise characterization of "spring and parachute" profiles, which is critical for selecting the best enabling formulation.
- Simply achieving a higher peak concentration (C_{max}) in vitro does necessarily imply better total bioavailability. The example below compares predicted absorption from two formulations, A and B, for a drug with solubility of 20 (arbitrary units)
  - Formulation A achieves a higher in vitro C_{max} than Formulation B, but less total absorption (bioavailability).
  - Its higher C_{max} leads to more rapid precipitation, which limits the time available for absorption.
  - Formulation B, despite a lower Cmax, maintains a greater dissolved concentration for a longer duration compared to Formulation A. T
  - This leads to a greater area under the curve (AUC) above the solubility value of 20 (arbitrary units), predicting greater overall absorption and bioavailability.
- This type of analysis enables rational formulation design, moving beyond simple metrics to optimize for true *in vivo* potential.





Application 2: Predicting Drug-Drug Displacement from Protein Binding

- Measuring the binding and release rate constants (k_{on} and k_{off}) for different drugs provides information to model and predict complex *in vivo* scenarios.
- For example, if the rate constants are known for two drugs in an appropriate medium ($k_{on,1}$ and $k_{off,1}$ and $k_{on,2}$ and $k_{off,2}$), competitive binding and displacement can be predicted.
- For competitive binding, the binding model would be extended to include two drugs competing for the same total number of binding sites, and two rate equations would be written:

Binding model

$$\begin{bmatrix}\text{Free Drugs}\\ C_{F,1} \text{ and } C_{F,2}\end{bmatrix} + \begin{bmatrix}\text{Empty Sites}\\ P_r\end{bmatrix} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \begin{bmatrix}\text{Bound Drug}\\ C_{B,1} \text{ and } C_{B,2}\end{bmatrix} \qquad \frac{dC_{F1}}{dt} = -k_{on1} C_{F1} P_r + k_{off,1} C_{B,1} \qquad \frac{dC_{F2}}{dt} = -k_{on,2} C_{F1} P_r + k_{off,2} C_{B,2} \qquad P_T = P_r + C_{B,1} + C_{B,2}$$

- For competitive binding, the rate of binding of drug-1 vs. drug-2 would primarily depend on the relative values of $k_{on,1}$ and $k_{on,2}$.
- For displacement (for example, drug-2 displaces drug-1), the rate of displacement would primarily depend on $k_{off,1}$ and $k_{on,2}$.
- The effects of the binding environment on the rate constants is key information for in silico predictions of these types of in vivo scenarios and clinical drug interactions.

Application 3: Support for Predictive & *In Silico* Models

The experimental data generated by PMD provides a viable real-world complement to computational tools.

- PBPK Models: Measured precipitation kinetics can be used to replace less realistic theoretical assumptions (e.g., Classical Nucleation Theory) in PBPK models for more accurate oral absorption predictions.
- Artificial intelligence (AI) and computational models: This "wet chemistry" data can be used to train, validate, and refine AI models, grounding their *in silico* predictions in physical reality and enhancing their predictive power.

CONCLUSION

PMD is a versatile platform that enables the accurate, *in situ* measurement of free drug concentrations in rapidly changing systems. It provides useful kinetic data that supports the rational design of dosage forms, supports predictive modeling, and allows for a deeper mechanistic understanding of drug behavior.

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