Supplemental Appendix 1:
Competitive Serum Binding
Analytical Solution

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1 Introduction

Here we present an analytical framework for the steady-state solution of a simple model of binding kinetics. The model considers three biochemical species: a drug (D), its target protein (T), and serum protein (S). The drug can bind to either the intended target protein or to the serum protein according to the equation:

$$\mathrm{T} + \mathrm{DS} \underset{\mathrm{k_{on,S}}}{\overset{\mathrm{k_{off,S}}}{\rightleftarrows}} \mathrm{S} + \mathrm{D} + \mathrm{T} \underset{\mathrm{k_{off,T}}}{\overset{\mathrm{k_{on,T}}}{\rightleftarrows}} \mathrm{DT} + \mathrm{S} \qquad \{1\}$$

We consider two cases of this model. The first, the 'noncompetitive' framework, includes only the drug and serum protein. This model represents a typical percent protein bound (PPB) equilibrium dialysis experiment. The second, the 'competitive' framework, includes all three species. This model represents our serum-shift assays, and captures the more complicated dynamics played out *in vivo*. For both cases, we solve for the steady-state concentration of unbound drug (D_{ss}) below.

2 Serum Binding (Noncompetitive)

We begin by considering the first case, where only drug and serum protein are present. It can be represented by a reduced version of reaction {1}:

$$\mathrm{DS} \underset{\mathrm{k_{on,S}}}{\overset{\mathrm{k_{off,S}}}{\rightleftarrows}} \mathrm{S} + \mathrm{D} \qquad \{2\}$$

The resulting differential equation for the unbound drug (D) is:

$$\frac{dD}{dt} = k_{off,S} \cdot DS - k_{on,S} \cdot D \cdot S \qquad (1)$$

2.1 Equilibria

We now aim to find the steady-state value of unbound drug (D). According to mass conservation, $D_{tot} = D + DS$ and $S_{tot} = S + DS$. Given that at steady-state $\frac{dD}{dt} = 0$, we have:

$$k_{off,S}(D_{tot} - D) - k_{on,S} \cdot D \cdot (S_{tot} - D_{tot} + D) = 0 \tag{2}$$

Let $K_S = \frac{k_{off,S}}{k_{on,S}}$. Then, equation (2) simplifies to:

$$D^2 + (S_{tot} - D_{tot} + K_S) \cdot D - K_S D_{tot} = 0 \tag{3}$$

Thus,

$$D_{ss} = \frac{-S_{tot} + D_{tot} - K_S \pm \sqrt{(S_{tot} - D_{tot} + K_S)^2 + 4K_S D_{tot}}}{2} \tag{4}$$

2.2 Stability

The above system has two equilibria [Equation (4)]. The stability of these fixed points can be assessed by linearization. Let $f(D) = \frac{dD}{dt}$ (Equation 2). Then,

$$f'(D) = -2k_{on}D - k_{on}S_{tot} + k_{on}D_{tot} - k_{off} \tag{5}$$

Evaluating at D_{ss}, we have

$$f'(D_{ss}) = \mp k_{on}\sqrt{(S_{tot} - D_{tot} + K_S)^2 + 4K_S D_{tot}} \tag{6}$$

Given that $k_{on} > 0$, $f'(D_{ss})$ is positive for the smaller of the two roots and negative for the larger of the two roots. Thus, the unique stable fixed point of the system is the larger root:

$$D_{ss} = \frac{-S_{tot} + D_{tot} - K_S + \sqrt{(S_{tot} - D_{tot} + K_S)^2 + 4K_S D_{tot}}}{2} \tag{7}$$

3 Serum and Target Binding (Competitive)

We now consider the second case, where drug, serum proteins, and target proteins are present. This represents a competitive binding system, since the drug has the potential to bind to either serum proteins or its target protein. It is be represented by reaction 1. The resulting differential equation for unbound drug (D) is:

$$\frac{dD}{dt} = k_{off,S} \cdot DS - k_{on,S} \cdot D \cdot S + k_{off,T} \cdot DT - k_{on,T} \cdot D \cdot T \tag{8}$$

3.1 Equilibria

Again, we aim to find the steady-state value of unbound drug (D). Under the principle of mass conservation, the following are true: $D_{tot} = D + DS + DT$, $S_{tot} = S + DS$, and $T_{tot} = T + DT$. The system has two degrees of freedom and therefore we must consider two differential equations to effectively describe it. In addition to Equation (8), we consider the ODE governing concentration of the drug-target complex (DT), given by the law of mass action:

$$\frac{dDT}{dt} = k_{on,T} \cdot D \cdot T - k_{off,T} \cdot DT \tag{9}$$

Using the above mass conservation equations and the equilibrium condition $\frac{dDT}{dt} = 0$, we can write the steady-state concentration of DT in terms of D:

$$DT_{ss} = \frac{D \cdot T_{tot}}{D + K_T} \tag{10}$$

where $K_T = \frac{k_{off,T}}{k_{on,T}}$.

We now use the mass conservation equations and Equation (10) to evaluate Equation (8), at steady-state, with D as the only unknown variable:

$$\begin{aligned} 0 = k_{off,S}(D_{tot} - D - \frac{D \cdot T_{tot}}{D + K_T}) - k_{on,S} \cdot D \cdot (S_{tot} - D_{tot} + D + \frac{D \cdot T_{tot}}{D + K_T}) + \\ k_{off,T}\frac{D \cdot T_{tot}}{D + K_T} - k_{on,T} \cdot D \cdot (T_{tot} - \frac{D \cdot T_{tot}}{D + K_T}) \end{aligned} \tag{11}$$

Given that $D + K_T \neq 0$, we multiply both sides of Equation (11) by the denominator in Equation (10). Simplifying and recalling that $K_S = \frac{k_o ff,S}{k_o n,S}$, we have:

$$\begin{aligned} 0 = D^3 + (-D_{tot} + S_{tot} + T_{tot} + K_S + K_T) \cdot D^2 + \\ (-D_{tot} \cdot K_S - D_{tot} \cdot K_T + S_{tot} \cdot K_T + T_{tot} \cdot K_S + K_S \cdot K_T) \cdot D - D_{tot} \cdot K_S \cdot K_T \end{aligned} \tag{12}$$

The steady-state concentration concentration can be solved for using the cubic formula:

$$\begin{aligned} D_{ss} = \sqrt[3]{\frac{-b^3}{27} + \frac{b \cdot c}{6} - \frac{d}{2} + \sqrt{(\frac{-b^3}{27} + \frac{b \cdot c}{6} - \frac{d}{2})^2 + (\frac{c}{3} - \frac{b^2}{9})^3}} + \\ \sqrt[3]{\frac{-b^3}{27} + \frac{b \cdot c}{6} - \frac{d}{2} - \sqrt{(\frac{-b^3}{27} + \frac{b \cdot c}{6} - \frac{d}{2})^2 + (\frac{c}{3} - \frac{b^2}{9})^3}} - \frac{b}{3} \end{aligned} \tag{13}$$

where $b = -D_{tot} + S_{tot} + T_{tot} + K_S + K_T$, $c = -D_{tot} \cdot K_S - D_{tot} \cdot K_T + S_{tot} \cdot K_T + T_{tot} \cdot K_S + K_S \cdot K_T$, and $d = -D_{tot} \cdot K_S \cdot K_T$.

3.2 Stability

The above system has three equilibria [Equation (13)]. Again, we can linearize to evaluate stability. Given that this system has two degrees of freedom, we return to the ODEs for $\frac{dD}{dt}$ [Equation (8)] and $\frac{dDT}{dt}$ [Equation (9)]. To linearize, we derive the Jacobian for $\mathbf{F}(\mathbf{x}) = [F_D \; F_{DT}]^T = [\frac{dD}{dt} \; \frac{dDT}{dt}]^T$ below:

$$\nabla \mathbf{F} = \begin{bmatrix} \frac{\partial F_D}{\partial D} & \frac{\partial F_D}{\partial DT} \\ \\ \frac{\partial F_{DT}}{\partial D} & \frac{\partial F_{DT}}{\partial DT} \end{bmatrix} \tag{14}$$

where

$$\frac{\partial F_D}{\partial D} = -2k_{on,S} \cdot D - k_{off,S} - k_{on,S} \cdot (DT + S_{tot} - D_{tot}) - k_{on,T} \cdot (-DT + T_{tot})$$

$$\frac{\partial F_D}{\partial DT} = -k_{off,S} - k_{on,S} \cdot D + k_{off,T} - k_{on,T} D$$

$$\frac{\partial F_{DT}}{\partial D} = k_{on,T} \cdot (-DT + T_{tot})$$

$$\frac{\partial F_{DT}}{\partial DT} = -k_{on,T} \cdot D - k_{off,T}$$

Stability is indicated by the real parts of the eigenvalues evaluated at each fixed point. The characteristic polynomial of the Jacobian is:

$$\begin{aligned} 0 = \lambda^2 - & \\ [-2k_{on,S} \cdot D - k_{off,S} - k_{on,S} \cdot (DT + S_{tot} - D_{tot}) - k_{on,T} \cdot (-DT + T_{tot}) - k_{on,T} \cdot D - k_{off,T}] \cdot \lambda + & \\ [-2k_{on,S} \cdot D - k_{off,S} - k_{on,S} \cdot (DT + S_{tot} - D_{tot}) - k_{on,T} \cdot (-DT + T_{tot})] \cdot [-k_{on,T} \cdot D - k_{off,T}] - & \\ [k_{off,S} - k_{on,S} \cdot D + k_{off,T} - k_{on,T} D] \cdot [k_{on,T} \cdot (-DT + T_{tot})] & \end{aligned} \tag{15}$$

Thus, the eigenvalues can be solved using the quadratic formula:

$$\lambda = -\frac{1}{2} B \pm \frac{1}{2} \sqrt{B^2 + 4 \cdot C} \tag{16}$$

where

$$\begin{aligned} B = & (-2k_{on,S} - k_{on,T}) \cdot D + (-k_{on,S} + k_{on,T}) \cdot DT \\ & - k_{off,S} - k_{off,T} - k_{on,S} \cdot (S_{tot} - D_{tot}) - k_{on,T} \cdot T_{tot} \\ C = & -2k_{on,S} \cdot k_{on,T} D^2 - k_{on,S} \cdot k_{on,T} \cdot D \cdot DT + \\ & k_{on,T} \cdot (-k_{off,S} - k_{on,S} \cdot S_{tot} + k_{on,S} \cdot D_{tot}) \cdot D + (-2k_{on,T} \cdot k_{off,T} + k_{on,S} \cdot k_{off,T}) \cdot DT + \\ & k_{off,T} \cdot k_{off,S} + k_{off,T} \cdot k_{on,S} S_{tot} - k_{off,T} \cdot k_{on,S} \cdot D_{tot} + 2k_{off,T} \cdot k_{on,T} \cdot T_{tot} \end{aligned} \tag{17}$$

The fixed point $[D_{ss} \; DT_{ss}]^T$ that satisfies $Re\lambda < 0$ for $\lambda \in \sigma(\nabla \mathbf{F})$ is stable.

4 Conclusion

These calculations offer exact solutions to the steady-state concentrations of unbound (D_{ss}) and target-bound (DT_{ss}) drug. In the competitive case, the sum of these two quantities gives the concentration of drug not bound to serum proteins. Thus the ratio of $D_{ss} + DT_{ss}$ of the competitive case to D_{ss} of the noncompetitive case indicates the theoretical degree to which PPB equilibrium dialysis experiments underestimate the amount of drug available for target binding. Using the calculations presented above, this ratio can be determined for a range of parameters. Figure 2B of the main text provides a heat map of this ratio as a function of serum- and target-binding affinities. The corresponding MATLAB code can be found on GitHub.