Brief Report

On the temperature dependence of the unbound drug fraction in plasma: Ultrafiltration method may considerably underestimate the true value for highly bound drugs

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ABSTRACT: The value of the unbound drug fraction in plasma is a common characteristic required for drug discovery and development. The communication considers an important possibility that the unbound drug fraction at physiological temperature (37˚C) could be substantially greater (more than 5 times) than the value measured by traditionally set ultrafiltration method with the incubation of drug plasma solution at 37˚C followed by the centrifugation at room temperature. The consideration is based on the general thermodynamic theory of chemical equilibrium, which is applied to the particular problem of determination of protein binding by ultrafiltration method.

***Keywords*:** Protein binding, Unbound drug fraction, Equilibrium dissociation constant, Ultrafiltration, Pharmacokinetics, Thermodynamics

Introduction

Binding of drugs to plasma proteins is an important feature affecting their pharmacodynamic and pharmacokinetic properties. Generally the free drug fraction is available for diffusion and transport across cell membranes to reach the activity site. The ultrafiltration and equilibrium dialysis are the methods traditionally used for determination of protein binding, which are based on the separation of free drug from bound one at equilibrium (*1*). The nonspecific adsorption of drugs (especially lipophylic ones) to the separation membrane and a relatively long and uncertain time of reaching equilibrium for the equilibrium dialysis are the major inconveniences in using these methods. In equilibrium dialysis method the initial total drug concentration in plasma decreases during the dialysis, so that there is no control on what would be the drug concentration at which protein binding is measured. This creates a complexity when binding is nonlinear and requires additional computational adjustments (*2*).

A quite short equilibration time and also the ability to measure protein binding at a given drug concentration are the advantages of the commonly used ultrafiltration method. Ultrafiltration method does not have control over temperature and provides the value of protein binding at room temperature because of a very quick temperature adjustment of equilibrium. For a typical value of the on-rate binding constant (*3*) $k_{on} \gtrsim 3.5\times10^4\ M^{-1}\ s^{-1}$ and albumin concentration $P_o = 670\ \mu M$, the time scale of reaching equilibrium is about $\tau \sim (k_{on}P_o + k_{off})^{-1} \lesssim 4.2\times10^{-2}$ s (the off-rate constant k_{off} was taken as zero in this estimation). A more accurate calculation of τ requires knowing the k_{off}, which strongly depends on the binding energy and can be estimated (*4*). For the binding energy of 10 kcal/mole $k_{off} \sim 4\times10^4\ s^{-1}$, which makes the time scale of reaching equilibrium extremely short $\tau \lesssim 10^{-4}$ s. If incubation is done at 37˚C, and after that plasma is transferred to ultrafiltration device at room temperature for centrifugation, then the temperature of plasma would drop to room temperature with consequent instant adjustment of drug-protein binding equilibrium. Thus after centrifugation we would be measuring the free drug concentration, which corresponds to the equilibrium at room temperature. Only if all procedures were performed at 37˚C until plasma ultrafiltrate containing the free drug fraction is separated, then we would be able to measure the protein binding at controlled temperature. Thus to obtain the value of protein binding at physiological conditions actually requires doing not only the incubation, but also plasma transfer to ultrafiltration device and subsequent centrifugation in a 37˚C room.

Though the influence of the temperature on protein binding is known, the analysis of the dependence of

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possible error in determination of the unbound drug fraction on temperature variation and the strength of protein binding was not reported in literature. The goal of this communication is to show that temperature dependence of protein binding (decrease of fraction bound at 37˚C compared the value measured at room temperature) might be quite significant and should be taken into account, especially for the highly bound drugs. This is considered in detail in the next section.

Determination of the temperature dependence of the unbound drug fraction

Generally there are several binding sites on each type of plasma protein (commonly albumin and α_1 - acid glycoprotein) involved in drug binding. The equilibrium binding to each binding site i of a single type of protein

$$R + P_i \rightleftharpoons Ci$$

is characterized by the affinity K_i (equilibrium dissociation constant), so that according to the mass action law

$$K_i = R*P_i/C_i \quad (1)$$

where R is the equilibrium concentrations of the unbound drug, P_i and C_i are respectively the concentrations of free and occupied i-th binding site. The mass balance equation for a drug and each binding sites can be written as $R_o = R + \Sigma C_i$, $P_o = P_i + C_i$, where R_o is the total drug concentration in plasma, and P_o is the plasma concentration of the protein. The unbound drug fraction is defined as $f_u = R/R_o$. Using Eq. 1 and the mass balance relation, we get

$$f_u = R/(R + \Sigma C_i) = 1/(1 + \Sigma P_i/K_i) \quad (2)$$

The concentration of free i-th binding site $P_i = P_o - C_i > P_o - R_o$ because $C_i < R_o$. The protein binding experiment is commonly performed at drug concentration much less than that of a protein, $R_o << P_o$. The unbound protein concentration P_i can then be assumed equal to P_o, so that Eq. 2 yields the minimum value of the unbound drug fraction, f_u^o, which corresponds to $R_o \rightarrow 0$

$$f_u^o = 1/(1 + k) \quad (3)$$

where $k = P_o\Sigma 1/K_i$. For a drug, which binds to several types of proteins in plasma, similarly as done above (*5*), we would obtain $f_u^o = 1/(1 + k_p)$, where k_p is equal to the sum of the parameters k, which characterize drug affinities of individual proteins.

Let us estimate the influence of temperature on the value of the unbound drug fraction. The traditional ultrafiltration method, when the separation of the unbound drug fraction is commonly performed at room temperature, yields the value of drug protein binding at t = 20˚C. The unbound drug fraction at body temperature (t = 37˚C) is actually pertinent to drug development. To estimate f_u^o (37˚C) from the known value of f_u^o (20˚C) let us assume that all n binding sites of the protein have the same affinity, *i.e.* $K_i = K_d$ in Eq. 1, so that the unbound drug fraction calculated by Eq. 3 is then equal to

$$f_u^o = 1/(1 + nP_o/K_d). \quad (4)$$

The temperature dependence of the equilibrium constant (at constant ambient pressure) is given by the Vant Hoff equation (*6*)

$$d(\ln K_d)/dT = \Delta H^o/(RT^2) \quad (5)$$

where ΔH^o is the enthalpy change of the reaction for each binding site ($\Delta H^o > 0$ for dissociation), R = 1.98 cal/(mole$*^o$K) is the gas constant and T = t + 273.15 is the absolute temperature. Integrating Eq. 5, using a common assumption that ΔH^o is constant in the narrow temperature interval between 20˚C and 37˚C, yields

$$K_d(37^\circ C) = K_d(20^\circ C)*\exp[\Delta H^o(T_2 - T_1)/(RT_1T_2)] \quad (6)$$

where $T_1 = 20 + 273.15 = 293.15$ ^{o}K and $T_2 = 37 + 273.15 = 310.15$ ^{o}K. The unbound drug fraction at 37˚C is calculated as

$$f_u^o(37^\circ C) = 1/[1 + nP_o/K_d(37^\circ C)] \quad (7)$$

Substituting K_d(37˚C) from Eq. 6 in Eq. 7 and taking into account Eq. 4 written for f_u^o(20˚C), we obtain the equation for the calculation of the unbound drug fraction at 37˚C from the known value at 20˚C

$$f_u^o(37^\circ C) = 1/\{1 + (1/f_u(20^\circ C) - 1)*\exp[\Delta H^o (T_1 - T_2)/(RT_1T_2)]\} \quad (8)$$

or the bound fraction $f_b^o = 1 - f_u^o$

$$f_b^o(37^\circ C) = 1/\{1 + (1/f_b(20^\circ C) - 1)*\exp[\Delta H^o (T_2 - T_1)/(RT_1T_2)]\} \quad (9)$$

Eq. 8 readily allows to estimate the temperature change of f_u^o using the values of f_u^o (20˚C) and ΔH^o. The decrease of f_u^o with temperature change from 20˚C to 37˚C strongly depends on the enthalpy change of the reaction. This is shown in Figure 1. Typically the value of ΔH^o ranges from 4 to 18 kcal/mole (*7*), though it could be as large as 30 kcal/mole (*8*). The largest relative increase of f_u^o (37˚C)/f_u^o (20˚C) occurs for highly bound drugs, $f_b^o \rightarrow 1$. For instance for a drug with f_u^o (20˚C) = 0.01 (99% protein binding) and ΔH^o = 12 kcal/mole (which corresponds to the two strong hydrogen bonds) the unbound drug fraction at 37˚C would be f_u^o (37˚C)

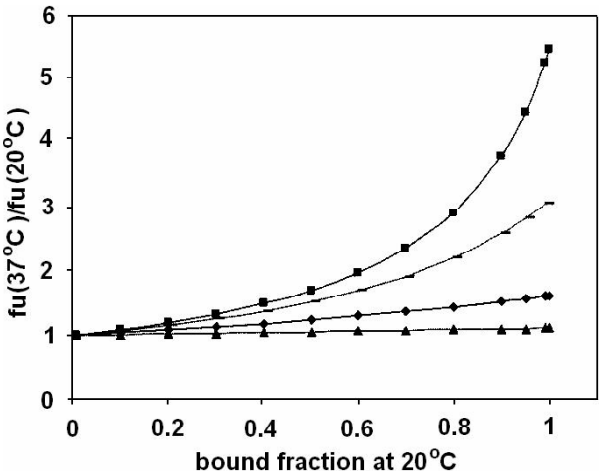

Figure 1. Temperature dependence of the unbound drug fraction. The relative increase of the unbound drug fraction, $f_u^o(37^\circ C)/f_u^o(20^\circ C)$, with temperature change from 20˚C to 37˚C as a function of the enthalpy change of drug - protein complex dissociation reaction (Eq. 8): ▲, ΔH^o = 1 kcal/mole; ◆, ΔH^o = 5 kcal/mole; ▬, ΔH^o = 12 kcal/mole; ■, ΔH^o = 18 kcal/mole.

= 0.03. This is a 3-fold increase of the unbound drug fraction. If the enthalpy change for the drug binding reaction were ΔH^o = 18 kcal/mole, we would get more than a 5-fold increase of the unbound drug fraction at 37˚C (94.8 % protein binding). On the other hand for a drug with f_u^o = 0.03 (97% protein binding) at room temperature and ΔH^o = 1 kcal/mole there is relatively small change in protein binding at 37˚C, $f_u^o(37^\circ C)$ = 0.033. It is most likely to have the greater value of ΔH^o for the highly bound drug compared to that with low protein binding. Thus for highly bound drugs it is more likely to expect a considerable temperature decrease of f_u^o at 37˚C compared to the value measured at 20˚C.

Conclusion

The rejection of drug candidates for a particular project when a relatively high value of the unbound drug fraction is desirable, based exclusively on the high level of protein binding obtained by regularly set ultra filtration method (*i.e.* plasma incubation at 37˚C and subsequent centrifugation at room temperature), might result in missing of potentially active compounds. The actual protein binding at physiological conditions could be substantially lower than that obtained by regularly set ultrafiltration method (which eventually yields the value of protein binding at room temperature). Ultrafiltration method requires control over temperature to obtain protein binding at physiological conditions – thus the method should be performed at 37˚C until plasma ultrafiltrate, which contains the free drug fraction, is separated.

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