When Is It Important to Measure Unbound Drug in Evaluating Nanomedicine Pharmacokinetics?

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ABSTRACT

Nanof ormulations have become important tools for modifying drug disposition, be it from the perspective of enabling prolonged drug release, protecting the drug molecule from metabolism, or achieving targeted delivery. When examining the in vivo pharmacokinetic properties of these formulations, most investigations either focus on systemic concentrations of total (encapsulated plus unencapsulated) drug, or concentrations of encapsulated and unencapsulated drug. However, it is rare to find studies that differentiate between protein-bound and unbound (free) forms of the unencapsulated drug. In light of the unique attributes of these formulations, we cannot simply assume it appropriate to rely upon the protein-binding properties of the traditionally formulated or legacy drug when trying to define the pharmacokinetic or pharmacokinetic/pharmacodynamic characteristics of these nanoformulations. Therefore, this commentary explores reasons why it is important to consider not only unencapsulated drug, but also the portion of unencapsulated drug that is not bound to plasma proteins. Specifically, we highlight those situations when it may be necessary to include measurement of unencapsulated, unbound drug concentrations as part of the nanoformulation pharmacokinetic evaluation.

Introduction

Nanomedicine is described as the application of nanotechnology for medical purposes and has shown great promise in the field of drug delivery (Lobatto et al., 2011; Chow and Ho, 2013). Nanomedicines come in a variety of compositions (liposomes, emulsions, micelles, inorganic particles, and solid lipid nanoparticles), sizes (10–1000 nm), shapes, surface chemistries, and charges. These physical and chemical properties of the nanomedicine influence its biologic performance, such as toxicity, stability, and disposition.

These innovative formulations allow for the tailoring of drug absorption and distribution characteristics to address specific therapeutic objectives. In some cases, they may also alter mechanisms of drug clearance. For example, whereas some orally administered nanoformulations provide modified release properties and increased solubility in the gastrointestinal tract, others leave the gut lumen intact by a localization within gut-associated lymphoid tissue, where they act as drug-releasing depots (Jani et al., 1990; Florence and Hussain, 2001). Alternatively, they may be administered s.c. where they can be retained near the site of injection, taken up by dendritic cells, or retained by local lymph nodes where they serve as drug-releasing depots (Cheng et al., 2015). Despite the variety of formulation technologies and routes of administration applied to these products, there are many shared features. Nanomedicines are typically <350 nm, spherical, and have neutral and hydrophilic surfaces. The most common route of administration is i.v. injection, and a push has been made for utilizing this drug delivery strategy for developing creative approaches for cancer therapy (Etheridge et al., 2013).

One of the benefits associated with the use of nanoformulations is enhanced therapeutic efficacy, accomplished by targeted delivery of the active pharmaceutical ingredient (API) to the site of action, thereby minimizing systemic drug exposure and toxicity. Accordingly, most pharmacokinetic (PK) studies of these formulations focus on measuring total (encapsulated plus unencapsulated) drug, or (less frequently) the encapsulated and unencapsulated drug (Ambardekar and Stern, 2015). However, a differentiation of the total versus unbound concentrations of the unencapsulated drug (i.e., plasma, serum, or tissue protein binding) is largely ignored.

Understanding the PK mechanisms by which nanomedicines alter drug disposition is fundamental to the continued advancement and application of nanotechnology as a drug delivery science. Traditional pharmacology maintains that unbound drug is the biologically active form; thus, reliance solely on total drug has the potential to introduce significant errors into the interpretation of drug delivery mechanisms and PK/pharmacodynamic (PD) relationships. Recently, national and international regulatory bodies have introduced generic nanomedicine guidance focusing on the evaluation of encapsulated and unencapsulated PK profiles, within the context of establishing the bioequivalence (PK equivalence) (Ambardekar and Stern, 2015). For generic versions of the popular nanomedicine Abraxane, Food and Drug Administration guidance has also included the evaluation of unencapsulated, unbound drug profiles. A commentary on the need to examine the active unbound drug fraction of nanomedicines is long overdue.

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ABBREVIATIONS: API, active pharmaceutical ingredient; AUC, area under the total drug concentration versus time curve; AUCu, area under the unbound drug concentration versus time curve; Cmax, concentration maximum; CRE, cremophor; Css, clearance steady state; EPR, enhanced permeability and retention effect; fu, unbound drug fraction in plasma; MPS, mononuclear phagocyte system; MTD, maximum tolerated dose; PD, pharmacodynamics; PK, pharmacokinetics.
For reasons discussed in this commentary, we cannot simply assume that in vitro protein-binding information on the API itself (Otgarir, 2005) can be translated directly into free versus bound concentrations associated with the drug delivery platform, a concern that is only further magnified by the potential impact of disease on plasma protein-binding characteristics (Tesseromatis and Alevizou, 2008). Thus, in light of the unique attributes of these formulations and potential changes in protein binding that can occur as a function of disease, a simple reliance on the in vitro protein-binding characteristics of the API may not be appropriate for defining the PK or PK/PD relationships of nanoformulations.

With these considerations in mind, this commentary focuses on when it may be necessary to measure not only encapsulated versus unencapsulated drug, but also the concentrations of unencapsulated, unbound drug as part of the PK assessment of nanoformulations. Although plasma protein binding has been explored relative to the development of a protein corona surrounding some nanomedicines (Caracciolo, 2015), we view this as part of the PK of the encapsulated drug fraction that is carrier-dependent and, therefore, will only be considering protein binding of the unencapsulated drug in this manuscript. In addition, this article does not provide a general overview of the PK attributes of the various nanoformulations because such information has already been published in some outstanding reviews (Mukherjee et al., 2014; Onoue et al., 2014; Lucas et al., 2015).

For clarity, the following terms will be used throughout this commentary:

- Encapsulated drug: drug molecule that is physically associated with the nanoformulation.
- Unencapsulated drug: drug molecule that has been released from the nanoformulation.
- Total drug concentrations: encapsulated plus unencapsulated drug.
- Bound drug: unencapsulated drug that is bound to plasma or tissue proteins.
- Unbound drug: unencapsulated drug that is not bound to plasma or tissue proteins.

### Traditional Protein-Binding PK Paradigms

The critical PK variables for small molecules are typically the intrinsic clearance and the volume of distribution associated with the unbound (free) drug fraction ($f_u$). When measuring drug concentrations, substantial error can potentially be introduced into the PK and PK/PD data interpretation if the relationship between total (bound + unbound) and unbound drug concentrations is not considered (Zeitlinger et al., 2011).

In the majority of cases, conditions such as disease-induced changes in protein binding, drug-drug interactions, or nonlinear protein binding will not affect the extent of unbound drug exposure [expressed as area under the unbound concentration versus time profile (AUC$_u$)], even though total drug exposure may change (exception is high extraction ratio drugs administered via i.v. injection). This point was extensively discussed by Benet and Hoener (2002). Nevertheless, changes in the relationship between total versus unbound drug concentrations can still be important in disease-induced alterations in protein binding (e.g., liver and renal diseases, dehydration, infection and inflammation, and diseases such as Crohn’s and Celiac) (Smith et al., 2010), as it can result in clinically relevant changes in the shape of the tissue and/or plasma profiles of the unbound drug concentrations. Furthermore, failure to consider the relationship between bound and unbound drug concentrations can lead to substantial bias in conclusions derived from therapeutic drug monitoring when only total drug concentrations are measured. For example, the relationship between total drug exposure and therapeutic response for narrow therapeutic window drugs (e.g., intensive care patients with hypoalbuminemia or increased levels of α1-acid glycoprotein receiving sedatives and pain medications) can differ from those without similar changes in plasma protein binding (Smith et al., 2012).

Incorporating traditional PK paradigms with protein-binding considerations (e.g., due to disease) results in the following relationships, displayed in Tables 1 and 2 [based upon information in Schmidt et al. (2010)], between the extent of exposure [expressed as concentration steady state (C$_ss$)], shape of the profile [expressed as concentration maximum (C$_{max}$)] or clearance (expressed as CL), and fraction unbound in plasma ($fu = \text{unbound/total drug concentrations in plasma}$). To allow for a qualitative graphic presentation of these relationships, the effect of altered $fu$ is described in terms “increase,” “no change,” and “decrease.”

Note that with the exception of parenteral high extraction compounds, changes in protein binding do not influence C$_ss$ unbound, although changes in C$_{max}$ unbound may occur (e.g., in the case of oral drug with altered drug partitioning into peripheral tissues).

The important messages conveyed through Tables 1 and 2 are twofold:

1. Traditionally, changes in protein binding will only influence the extent of unbound drug exposure (reflected as C$_ss$ unbound) when the drug is characterized as having a high extraction ratio, low V, and is administered parenterally. In all other cases, changes in $fu$ will not influence C$_ss$ unbound. However, C$_{max}$ unbound values can differ as a consequence of altered protein binding when the drug has a low volume of distribution (oral or parenteral) or when it is administered parenterally and has a high extraction ratio. Therefore, even in situations when total exposure may not change, if C$_{max}$ unbound can influence therapeutic response or toxicity, altered protein binding may need to be considered.

2. Evaluations based upon total drug concentrations will be misleading when trying to predict PK/PD relationships as it can suggest changes in drug exposure that are without clinical relevance (based upon the absence of a corresponding change in unbound drug concentrations).

### Nanoparticle PK

Our current understanding is that encapsulation of the drug within a nanoparticle can dramatically alter the drug’s PK by altering tissue distribution and clearance. The distribution and clearance of i.v. administered nanomedicines are primarily dependent on the enhanced permeability and retention (EPR) effect and upon the mononuclear phagocyte system (MPS), respectively (Zamboni et al., 2012) (Fig. 1). The MPS also appears to be responsible for saturable clearance of some formulations (e.g., liposomes) at higher doses, and for a higher intersubject variability in the extent of drug exposure as compared with that seen with conventional formulations (Song et al., 2012). This variability can influence the clinical response (safety and effectiveness) (Schell et al., 2014).

Nanoparticles not cleared by MPS demonstrate longer circulatory half-lives and better tumor accumulation by EPR (Briley-Saebø et al., 2008; Maeda, 2012). The EPR effect is a well-known phenomenon that describes the accumulation of nanoscale drug formulations at sites of inflammation and solid tumors due to the leaky tumor vasculature and suppressed lymphatic system (Maeda, 2012). Such leaky vessels are not typically found in normal healthy tissues, thereby allowing for a more targeted drug delivery (Nakamura et al., 2015). Heterogeneity of solid tumors among patients and interpatient variability of nanoparticle PK create additional challenges in predicting the safety and efficacy of a nanomedicine formulation (Zamboni et al., 2009; Sidone et al., 2007; Caron et al., 2011; Prabhakar et al., 2013). Although a drug is encapsulated in a nanomedicine (e.g., liposome), its PK is dependent upon the physicochemical characteristics of the
carrier until the drug is released from the carrier. Conversely, the inherent PK of the drug itself dictates the PK for the unencapsulated drug fraction (Ambarekar and Stern, 2015). Therefore, the PK profiles of both the encapsulated and unencapsulated drug fractions for a given nanomedicine formulation are critical for understanding how the PK properties of the nanomedicine influence product PD and toxicodynamics. However, whereas for many kinds of nanoformulations the unencapsulated concentration/effect relationships can be derived from unbound/total drug ratio of the legacy (traditionally) formulated API, there are situations when this is not the case.

**Nonlinear Changes in Protein Binding in Which Formulation Has the Potential to Alter This Binding Behavior.** An example of where the PK of the traditionally formulated API should not have been relied upon to predict behavior of a nanoformulation is exemplified by liposomal amphotericin B (AmBisome). Bekersky et al. (2002) evaluated and compared the PK of a traditional i.v. formulation (Amphotericin B Deoxycholate) to the liposomal formulation. They used a nonlinear relationship between drug concentration and protein binding to differentiate encapsulated and unencapsulated liposomal fractions. Adapting the ultrafiltration method for fractionation of amphotericin B nanoliposome in plasma samples, the protein-bound drug fraction was interpolated from an established correlation between free (ultrafilterable) drug concentrations and protein binding. The encapsulated amphotericin B fraction was estimated by subtracting the ultrafilterable and protein-bound amphotericin B concentrations from the total amphotericin B concentrations. Thus, their estimation technique was based upon an assumption that the nanoformulation does not influence amphotericin protein-binding behavior. The error in utilizing this approach, with the assumption that protein binding was formation-independent, has since been demonstrated using stable isotope tracers to account for formulation-mediated changes in binding behavior (Skoczlen et al., 2015).

**Equilibrium Formulations.** The potential for unbound drug to associate with certain nanomedicine platforms (e.g., micelles, liposomes), thereby becoming transiently bound to the formulation in a manner similar to that of plasma protein binding, can potentially influence the rate and extent of unbound drug exposure in tissues and plasma (Sparreboom et al., 1999; van Tellingen et al., 1999; van Zuylen et al., 2000). For example, the released drug in plasma may recombine with some nanoformulations [e.g., cremophor (CRE) nanomicelle], thereby reforming into carrier-associated nanoparticles.

**Equilibrium Formulations.** From a drug release standpoint, nanomedicines can be broadly classified as solubilizing formulations, modified release formulations, and equilibrium formulations. As the name implies, solubilizing formulations are unstable, releasing the entire drug immediately upon injection. Modified release nanomedicines function as circulating depots, liposomes being an example that is capable of releasing active drug in a controlled manner. Equilibrium formulations are a unique example of a kind of formulation that provides the shared characteristics of solubilizing agents and modified release formulations. Similar to conventional modified release products, equilibrium formulation platforms are stable, remaining intact systemically. However, unlike conventional modified release formulations, the unencapsulated, unbound drug is in a dynamic equilibrium with the formulation (Brouwer et al., 2000) (Fig. 2). As a result, although the drug is immediately 100% bioavailable (similar to a simple solubilizing agent), an alteration in unbound drug fraction (due to drug binding to the equilibrium formulation) could have therapeutic implications that vary from the classic small-molecule paradigms.

The most representative example of unbound drug binding to a nanomedicine in equilibrium is the case of Taxol. Taxol is a CRE nanomicelle formulation of paclitaxel with indications for the treatment of solid tumors (de Weger et al., 2014). There are several examples in which the PK of Taxol have been described as dose dependent (i.e., nonlinear), presenting with both saturable distribution and saturable elimination (Sonnichsen et al., 1994; Gianni et al., 1995). It is important to note that this nonlinearity was identified in reference to total drug in plasma (i.e., encapsulated, protein bound, and unbound). The inclusion of unbound drug binding to CRE nanomicelles as part of various paclitaxel PK models explained not only the nonlinearity of total plasma drug, but also the linearity of whole blood and unbound plasma drug (Heningsson et al., 2001; Bulitta et al., 2009). In these models, the
nonlinearity of total plasma drug concentrations is a consequence of CRE nanomicelle binding and a decrease in the unbound drug fraction at higher Taxol doses and at decreased infusion times. Plasma unbound fraction studies in vitro, as well as blood/plasma partitioning studies ex vivo, support paclitaxel binding to CRE micelles at clinically relevant drug concentrations (Brouwer et al., 2000; van Zuylen et al., 2001). The ability of CRE micelle to bind drug in vivo is further supported by studies showing Taxol-drug interactions leading to increased total area under the total drug concentration versus time curve (AUC) for coadministered doxorubicin and irinotecan, thought to be due in part to micelle binding of drug (Vigano et al., 2001; Kasai et al., 2002).

The PK equations provided below for small-molecule binding to protein mathematically describe how decreased unbound drug fraction to micelle binding of drug (Vigano et al., 2001; Kasai et al., 2002).

\[
V = V_P + fu_P \times fu_T \times V_T
\]

\[
CL = fu_P \times CL_{int}
\]

where \( V \) = volume of distribution, \( V_P \) = volume of the central compartment (plasma), \( V_T \) = volume of the peripheral compartment (tissue), \( fu_T \) = the unbound drug fraction in tissue, \( CL \) = total systemic clearance, \( fu_P \) = the unbound drug fraction in plasma, and \( CL_{int} \) = intrinsic clearance.

Despite these changes in total drug volume and clearance, one would not anticipate that these changes would have therapeutic consequences for a low extraction drug such as paclitaxel, because the clinically relevant unbound drug exposure (AUCu) is unaffected by changes in unbound fraction (Benet and Hoener, 2002):

\[
AUC_u = fu_P \times AUC = fu_P \times Dose/CL_{int} \times fu_P
\]

These small-molecule paradigms, however, may not be appropriate for equilibrium binding of drugs to nanomedicine formulations. In fact, there is evidence that equilibrium binding of paclitaxel to the Taxol CRE micelle may actually decrease tissue distribution, negatively affecting Taxol therapeutic utility in comparison with Abraxane, a novel albumin-based nanoparticle formulation of paclitaxel. Abraxane is currently approved for treatment of pancreatic cancer, whereas Taxol is not (Goldstein et al., 2015).

In the case of Taxol, formulation components contribute substantially to maximum tolerated dose (MTD), as CRE is immunotoxic and potentially neurotoxic. Abraxane has a 1.7-fold higher MTD than Taxol due to the absence of CRE (Sparreboom et al., 2005). At comparable clinical MTD (i.e., a higher dose of Abraxane on the basis of dose calculated as mg/m\(^2\)), Abraxane has a 50% greater total drug clearance and volume of distribution than Taxol (Sparreboom et al., 2005). This difference in Abraxane and Taxol PK is also observed in animal models (Sparreboom et al., 2005). As discussed above, Taxol in vivo and in vitro PK studies support the stability of the Taxol CRE micelle in vivo, and its ability to bind paclitaxel in equilibrium with the unbound form. By contrast, the Abraxane formulation is unstable, with the nanoparticles rapidly dissociating into their component albumin molecules upon systemic administration (Svenson, 2014). The rapid dissociation of the Abraxane nanoparticles is consistent with the fact that, despite a higher administered dose of Abraxane (260 mg/m\(^2\)) versus Taxol (175 mg/m\(^2\)), the two formulations resulted in comparable total exposure estimates (expressed as the AUC of the total drug concentrations in plasma). However, at an equivalent total drug exposure as Taxol, Abraxane was associated with a greater unbound drug fraction, and at a greater unbound drug exposure, Abraxane’s higher unbound drug fraction potentially explains its greater total drug clearance (eq. 2) and volume of distribution (eq. 1). In the case of greater unbound drug fraction, terminal half-life does not change because clearance and volume of distribution increase proportionately for a moderate-high volume of distribution drug like paclitaxel, according to eq. 4 (Schmidt et al., 2010):

\[
\text{Half-life} = \frac{0.693 \times (V_P + fu_P \times CL_{int})}{fu_P \times CL_{int}}
\]

This expected similarity in terminal half-life is consistent with clinical observations for Abraxane and Taxol, further supporting changes in unbound drug fraction as being the underlying mechanism responsible for differences in their PK (Sparreboom et al., 2005; Gardner et al., 2008).

In support of the higher volume of distribution observed preclinically and clinically, greater paclitaxel tissue AUC has also been observed for the Abraxane formulation in comparison with Taxol for organs such as prostate, pancreas, and xenograft tumor in preclinical models at equal doses (Sparreboom et al., 2005; Desai et al., 2006). Apart from the increase in systemic unbound drug exposure resulting from the higher MTD, these differences in Abraxane versus Taxol tissue distribution could be clinically meaningful and a reason for the increased efficacy of Abraxane over Taxol in pancreatic cancer.

**Redefining Biologically Active Drug Fraction and Its Impact on the PK/PD of Nanoformulations**

In addition to our prior PK discussions, the biologically active drug fraction may also need to be redefined from a PK/PD perspective. This is particularly true with regard to nanoformulations in which delivery of the active agent bypasses the unbound form as a result of direct transfer.
to the target cells. In cases where the carrier delivers the active drug directly to the target cell (e.g., tumor), the significance of the unbound drug fraction with regard to efficacy is greatly diminished, although still having importance for off-target exposure-toxicity relationships. An example of such targeted delivery systems includes nanomicelles and nanoliposomes that bind to amphotropic peptide and small-molecule drugs, thereby protecting these compounds from rapid metabolic clearance, although paradoxically also increasing drug potency (Kirchherr et al., 2009; Banerjee and Onyuksel, 2012b; Nie et al., 2012). To illustrate, 1,2-distearol-sn-glycerol-3-phosphethanolamine-N-[methox (poly-ethylen glycol)]-2000 micelles of pancreatic polypeptide and vasoactive intestinal polypeptide show resistance to proteases in vitro, an increase in total drug exposure (i.e., total drug AUC), and a marked improvement in in vivo potency (Banerjee and Onyuksel, 2012a, 2013; Sethi et al., 2013). In the case of the vasoactive intestinal peptide, its systemic clearance decreased 147-fold and its volume of distribution decreased fivefold in mice (Sethi et al., 2013). In the case of the antidiabetic pancreatic polypeptide, nanomicellar formulation increased glycogen storage and insulin sensitivity 1.7- and 1.8-fold, respectively, over equivalent doses of unformulated peptide in a rat model of diabetes (Banerjee and Onyuksel, 2013). Although these are high clearance drugs, and unbound drug concentrations decreased upon formulation in both of these examples, drug therapeutic potency actually increased (which would seem to contradict the standard small-molecule paradigm of activity being a function of unbound drug concentrations). In these cases, the enhanced efficacy appears to be a result of direct distribution of active drug to the target organ by the nanoformulation, bypassing the unbound form (Yano et al., 2010; Wang et al., 2012). Thus, to accurately describe the PK/PD relationships for some nanof ormulations, we need to understand how target tissue exposure to encapsulated and unencapsulated, unbound forms of the drug relates to drug concentrations at the intracellular site of action. Indeed, for some nanomedicines, the biologically active form of the drug may be the encapsulated drug, not the unencapsulated, unbound drug, diminishing the relevance of the unbound drug fraction from a PK/PD perspective.

Debating the Question: When Unbound Fractions May Need to be Considered

Clearly, knowing the protein-binding characteristics of a drug is an essential component to translating total drug (bound plus unbound) concentration data to its clinical effects (safety or toxicity). For a nanof ormulation, the authors also express the opinion that in addition to characterizing unencapsulated and encapsulated drug, differentiating unbound and total (bound plus unbound) concentrations of the unencapsulated drug is necessary. In some situations, the protein-binding characteristics for the unencapsulated drug can be extrapolated from what is known about the drug molecule itself. However, in other situations, direct measurement of the bound and total unencapsulated drug is prudent.

On the basis of the discussions contained within this commentary, we conclude that the following are situations when extrapolation of the unbound/total drug concentration relationship of the legacy or traditionally formulated drug may not adequately describe the PK or PK/PD relationships of their nanof ormulations, and direct measurement of unbound versus total unencapsulated drug concentration is necessary:

1. Equilibrium formulations (where unbound drug fraction may change as a function of formulation).
2. When there are known nonlinear relationships between total versus unbound drug concentration that could be influenced by the nanof ormulation.
3. Oral nanomedicine formulations of high extraction drugs that are absorbed intact systemically and bypass first pass effect, thereby acting as i.v. formulations with regard to small-molecule protein-binding paradigms.
4. When the unbound drug is the same as the unencapsulated fraction, such as for protein nanoparticle formulations of small molecules, like Abraxane, and peptide drug nanomedicine formulations.
5. In the absence of an existing well-characterized relationship between total and unbound drug concentration that can be extrapolated to the nanomedicine formulation.

Ultimately, it is only when the unbound/total drug concentration relationships are understood that we can rely upon unencapsulated drug concentrations to predict changes in PK or PK/PD (safety and/or effectiveness) for a nanof ormulation. Without knowledge of the unbound drug concentrations resulting from the administration of nanof ormulations, we cannot appreciate the dose-exposure-response relationships that are integral to the safe and effective use of these formulations across a diverse patient population.

Efforts to describe the PK and PK/PD of nanoparticles are generally based on the evaluation of total (encapsulated plus unencapsulated), or encapsulated and unencapsulated drug. We have expressed our opinion that in certain instances such studies need to further define unencapsulated drug from the perspective of total versus unbound drug concentrations. Our hope is that this commentary will stimulate additional discussion and research on this important topic.

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