Importance of Relating Efficacy Measures to Unbound Drug Concentrations for Anti-Infective Agents

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SUMMARY

For the optimization of dosing regimens of anti-infective agents, it is imperative to have a good understanding of pharmacokinetics (PK) and pharmacodynamics (PD). Whenever possible, drug efficacy needs to be related to unbound concentrations at the site of action. For anti-infective drugs, the infection site is typically located outside plasma, and a drug must diffuse through capillary membranes to reach its target. Disease- and drug-related factors can contribute to differential tissue distribution. As a result, the assumption that the plasma concentration of drugs represents a suitable surrogate of tissue concentrations may lead to erroneous conclusions. Quantifying drug exposure in tissues represents an opportunity to relate the pharmacologically active concentrations to an observed pharmacodynamic parameter, such as the MIC. Selection of an appropriate specimen to sample and the advantages and limitations of the available sampling techniques require careful consideration. Ultimately, the goal will be to assess the appropriateness of a drug and dosing regimen for a specific pathogen and infection.

> **I do not care about protein binding because I always measure free, unbound concentrations.**
> —Nick H. Holford, Paul Ehrlich meeting, Nuremberg, Germany, 2004

INTRODUCTION

Most drugs bind to plasma and tissue proteins, resulting in a decrease in free, pharmacologically active concentrations. As described by the law of mass action, the binding is reversible, and equilibrium between the protein-bound and unbound drug is quickly established. The extent of binding is dependent on the structure and physicochemical properties of the drug molecule, which dictate affinity for the protein, as well as the drug and protein concentrations, experimental conditions, and the species in which the binding is studied ([1], [2]). The fraction unbound in plasma (f_U) is computed as the ratio of free, unbound (C_U) and total (C_T) drug concentrations, as shown in equation 1 ([3]). Assuming that a drug has a single binding site, f_U can also be expressed as a function of the equilibrium dissociation constant (K_D), the maximum binding capacity (B_{max}), and C_U. The relationship becomes more complex if more than one binding site is involved, but a detailed mathematical description of this scenario is outside the scope of this paper.

$$f_U = \frac{C_U}{C_T} = \frac{K_D + C_U}{B_{max} + K_D + C_U} \quad (1)$$

At commonly prescribed doses, most drugs display linear binding, whereby the fraction unbound remains unchanged as drug concentrations increase. Under these conditions, C_U is much lower than K, and the function may be simplified according to equation 2 ([3]):

$$f_U = \frac{K_D}{B_{max} + K_D} \quad (2)$$

When unbound concentrations exceed the number of available binding sites ($C_U > K_D$), this simple relationship does not hold, and protein binding becomes concentration dependent (i.e.,

saturable binding). Ceftriaxone, cefazolin, cefonicid, and ertapenem are examples of antimicrobials for which nonlinear protein binding has been reported in the literature (4–7).

Binding to protein can occur in both intra- and extravascular spaces and is an important determinant of a drug's pharmacokinetics (PK), as it will impact distribution and elimination processes. Tissue binding increases the fraction of drug outside plasma and away from systemic drug elimination organs. Similarly, plasma protein binding limits the unbound drug concentration available for drug metabolism, filtration, and/or excretion by the kidneys. For the liver, the scenario is more complex, and plasma protein binding may or may not affect drug clearance depending on the affinity of the drug for the metabolizing enzyme (i.e., intrinsic clearance) (8). Depending on the PK properties of the drug, drug elimination may be impacted. When applying the well-stirred model of hepatic elimination, low-extraction drugs will be dependent on f_U and the intrinsic clearance, whereas the hepatic blood flow is the limiting factor for high-extraction drugs.

For anti-infective drugs, the impact of protein binding is of prime interest because the free, unbound drug concentrations at the site of action/infection are responsible for the drug's effect. Frequently, however, the site of infection is not the bloodstream, and a drug's ability to cross capillary membranes to reach the site of action is critical to its efficacy. For drugs where the interstitial fluid is the site of infection, and where disease-related barriers or efflux mechanisms do not impair drug distribution, plasma concentrations often represent a reasonable surrogate for tissue concentrations due to the establishment of a rapid equilibrium between plasma and tissues (9). For drugs where these conditions do not hold, selection of an appropriate measure of drug exposure requires careful consideration.

Table 1 summarizes the extent of plasma protein binding for drugs across various anti-infective drug classes (10). The impact of protein binding on drug efficacy will depend on the extent of the binding, PK properties, and intrinsic activity of the drug (11–15). *In vitro*, the impact of protein binding on antimicrobial activity is often investigated through determination of the MIC, time-kill curves, and cell culture assays (13, 16). Numerous *in vitro* studies have been published, which evaluated the impact of protein binding on antimicrobials (17–28), antivirals (29, 30), and antifungals (31, 32) by using protein supplements and/or serum to mimic *in vivo* conditions. In the majority of studies, free drug concentrations are not measured directly in the experimental setting, and the extent of protein binding is accounted for by the using binding values reported in the literature. This approach can be misleading if the protein binding reported in the literature differs from the actual protein binding in the experimental setting (16, 33).

In vivo, animal infection models serve as one mechanism to evaluate the impact of protein binding on tissue concentrations and antimicrobial efficacy (34–37), although in most cases, unbound drug concentrations are not measured directly, but rather, total drug concentrations are corrected according to the extent of protein binding measured *in vitro*.

For anti-infective drugs where free plasma concentrations do not represent a reliable surrogate of drug exposure at the site of infection, direct measurements of free tissue concentrations offer a more meaningful approach. There are various techniques that are routinely applied to measure unbound tissue concentrations in humans. These techniques include microdialysis, tissue biopsy, imaging techniques, and saliva or blister fluid sampling (38–47).

Each of these techniques has advantages and limitations. Microdialysis is an innovative technique that is being increasingly used to determine free, unbound concentrations in the interstitial space fluid (ISF) of various tissues (Fig. 1). It is a semi-invasive sampling technique whereby a probe containing a semipermeable membrane is implanted into a tissue and infused with a solution mimicking tissue conditions at a constant rate. Movement of drug molecules via passive diffusion, and determination of the probe recovery value, allow for a direct measurement of unbound drug concentrations in tissues over time without the need for a sample purification method. In contrast to measuring whole tissue concentrations in homogenized samples, where measured concentrations represent a mixture of intra- and extracellular contents, microdialysis quantifies unbound drug concentrations in the extracellular fluid, the site of action for many antimicrobials (42).

Once free drug concentrations at or at least close to the site of infection are measured, they can be correlated to the pharmacodynamic (PD) effect to help guide the selection of an optimal dose. The goal of this review paper is to outline the benefit of measuring and relating unbound drug concentrations to anti-infective drug efficacy.

METHODS AVAILABLE TO MEASURE PROTEIN BINDING AND TISSUE DISTRIBUTION

Protein Binding Determination

Frequently, free drug concentrations in plasma are estimated by correcting total plasma concentrations for protein binding. Available techniques for measurement of protein binding include equilibrium dialysis, ultrafiltration, ultracentrifugation, microdialysis, chromatographic methods, capillary electrophoresis, fluorescence spectroscopy, and ultrafast immunoextraction. These methods have been compared extensively in published review articles, and only an overview is provided here (16, 48, 49). With the exception of microdialysis, which may be applied *in vivo* to measure unbound drug concentrations, these methods are used *in vitro* for protein binding determination. Measurement of the extent of protein binding may then be used to correct total concentrations for protein binding and to compute unbound drug concentrations *in vivo*.

The experimental setup varies between these methods. Equilibrium dialysis and ultrafiltration methods use a semipermeable membrane to separate the bound and unbound drug. In the case of ultrafiltration, separation is driven by centrifugal forces, whereas passive diffusion from plasma to a physiological buffer facilitates separation in the equilibrium dialysis setup. Equilibrium dialysis is considered to be the "gold standard" due to the reliability of the results and the robustness of the procedure. Likewise, ultrafiltration is considered to be simple and practical, as it does not require the use of a physiological buffer, the time to equilibration need not be determined *a priori*, and sample processing time is relatively short (16). A limitation of both methods is the potential of drug adsorption to the semipermeable membrane. In addition, for equilibrium dialysis, due to a greater osmotic pressure in the plasma (or serum) compartment, movement of physiological buffer into the plasma can dilute the drug concentration (16). Assuming that drug adsorption and volume shifts do not occur, the unbound drug fraction may be calculated by relating the unbound drug concentrations (C_U) in the buffer (equilibrium dialysis) or ultrafiltrate (ultrafiltration) to the drug

TABLE 1 Percentage of protein bound drug in plasma for anti-infective drug classes[a]

Drug	% PB (mean $\pm$ SD or range)
Antimicrobials	
Amoxicillin	18
Amikacin	4 $\pm$ 8
Azithromycin	7–50[b]
Cefazolin	89 $\pm$ 2
Cefdinir	89
Cefixime	67 $\pm$ 1
Ceftazidime	21 $\pm$ 6
Cefuroxime	33 $\pm$ 6
Cephalexin	14 $\pm$ 3
Cefepime	16–19
Ceftriaxone	83–96[b]
Ciprofloxacin	40
Clarithromycin	42–50
Clindamycin	93.6 $\pm$ 0.2
Dapsone	73 $\pm$ 1
Daptomycin	92
Doxycycline	88 $\pm$ 5
Ertapenem	84–96[b]
Erythromycin	84 $\pm$ 3
Fosfomycin	Negligible
Gentamicin	<10
Imipenem-cilastatin	I, <20; C, ~35
Levofloxacin	24–38
Linezolid	31
Minocycline	76
Moxifloxacin	39.4 $\pm$ 2.4
Nitrofurantoin	62 $\pm$ 4
Rifampin	60–90
Sulfamethoxazole	53 $\pm$ 5
Telithromycin	70
Tigecycline	71–89
Trimethoprim	37 $\pm$ 5
Vancomycin	30 $\pm$ 11
Antivirals	
Acyclovir	15 $\pm$ 4
Atazanavir	86
Cidofovir	<6
Darunavir	95
Didanosine	<5
Efavirenz	99.5–99.75
Ganciclovir	1–2
Emtricitabine	<4
Foscarnet	14–17
Lopinavir	98–99
Maraviroc	76
Raltegravir	83
Ribavirin	0
Ritonavir	98–99
Tenofovir	<1
Valacyclovir	13.5–17.9
Zidovudine	<25
Antifungals	
Amphotericin B	>90
Caspofungin	96.5
Fluconazole	11 $\pm$ 1
Itraconazole	99.8
Micafungin	99
Posaconazole	98
Voriconazole	58

TABLE 1 (Continued)

Drug	% PB (mean $\pm$ SD or range)
Atypical	
Chloroquine	S, 66.6 $\pm$ 3.3; R, 42.7 $\pm$ 2.1
Ethambutol	6–30
Albendazole	70
Hydroxychloroquine	45 $\pm$ 3
Isoniazid	Negligible
Mefloquine	98.2
Metronidazole	11 $\pm$ 3
Pyrazinamide	10

[a] Protein binding values reported are taken from the same reference (10). % PB, percentage of protein-bound drug in plasma for anti-infective drug classes; I, imipenem; C, cilastatin; S, S isomer; R, R isomer.
[b] Concentration-dependent binding.

concentration in plasma (or serum) at the start of the experiment (C_T), according to equation 1.

For ultracentrifugation, bound and unbound drug concentrations are separated by centrifugal forces only. As a result, membrane binding and fluid shifts need not be considered. Following centrifugation, a sample partitions into three layers: a top layer containing very-low-density lipoproteins and chylomicrons; a middle aqueous layer, which is protein free; and a bottom layer, which contains larger plasma proteins and lipoproteins (50). f_U can be calculated by comparing the C_T obtained prior to centrifugation and the C_U obtained from the aqueous, middle layer. A major disadvantage of the method is that it is relatively low throughput, limiting how many samples may be processed at once (50).

Microdialysis uses a semipermeable membrane at the tip of a flexible probe to measure C_U. The probes differ in size, geometry, and molecular weight cutoff, allowing for customization based on individual research needs. The microdialysis probe is perfused with a fluid (i.e., the "perfusate") that resembles the composition of body fluids in the tissue of interest at a constant flow rate. Drug present in the sample matrix diffuses across the semipermeable membrane and into the perfusate. The collected sample (i.e., the "dialysate") is then analyzed, and the drug concentration is then
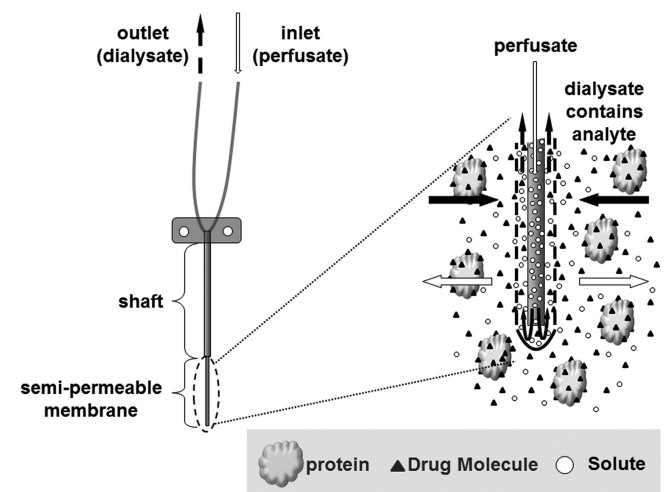

FIG 1 Pictorial representation of a microdialysis probe inserted into a medium containing an analyte of interest and a drug binding protein.

determined. At typical flow rates (1 to 10 μl/min), total equilibrium is not established between the tissue and perfusate. However, the constant flow of solute and unbound analyte across the semipermeable membrane results in the establishment of a pseudo-equilibrium, which can be used to determine a calibration factor, i.e., the recovery. The flow of drug is assumed to be equal in both directions, resulting in identical recovery values. This assumption should always be tested *in vitro*, for example, by loss-of-drug (retrodialysis) and gain-of-drug (extraction efficiency) calibration experiments, before performing a clinical study. There is a plethora of calibration techniques available to experimentally determine the recovery value. These techniques include retrodialysis, extraction efficiency, no-net-flux, and internal standard calibration methods (51, 52). In a retrodialysis setup, a known drug concentration is added to the perfusate, and the relative recovery is calculated by relating the concentrations in the perfusate ($C_{\text{perfusate}}$) and dialysate ($C_{\text{dialysate}}$) (equation 3). The retrodialysis method is frequently applied *in vivo* for recovery determination.

$$\text{Recovery (\%)} = 100 - \left(100 \times \frac{C_{\text{dialysate}}}{C_{\text{perfusate}}}\right) \quad (3)$$

For the extraction efficiency method, the ability of drug present in the matrix to cross the membrane and appear in the dialysate is evaluated by comparing the known concentration in the surrounding fluid (C_{fluid}) with $C_{\text{dialysate}}$ (equation 4).

$$\text{Recovery (\%)} = \frac{C_{\text{dialysate}}}{C_{\text{fluid}}} \times 100 \quad (4)$$

In theory, the relative recoveries determined by both methods should be identical and can be used to compute the true unbound drug concentrations in the ISF of the tissue by correcting $C_{\text{dialysate}}$ by the experimentally determined recovery value (equation 5).

$$C_{\text{tissue}} = \frac{C_{dialysate}}{in\ vivo\ \text{probe recovery}} \times 100 \quad (5)$$

Improvements in technology have led to recent advances in the methods available to study drug-protein interactions. For example, high-performance affinity chromatography (HPAC), a technique coupling high-performance liquid chromatography (HPLC) with affinity columns containing proteins of interest, has been applied to measure the extent of protein binding in blood and characterize the binding process through estimation of equilibrium constants (53–57). Moreover, immunoaffinity chromatography, a chromatographic method that uses drug binding antibodies, has been applied to measure the free drug fraction of warfarin in a sample containing human serum albumin (58). One major advantage of these techniques is the ability to analyze a large number of samples in a short period of time. Fluorescence and UV absorption spectroscopy methods can also be used to characterize binding characteristics with plasma proteins (59). These techniques are useful to systematically study the binding mechanism and the extent of drug binding.

Target Site Exposure

If complete equilibration between unbound plasma and tissue concentrations is not achieved, evaluating a drug's tissue PK may offer considerable insight into its distribution properties. For anti-infective drugs, disease-specific factors may contribute to poor distribution to the site of infection. Direct measurements of tissue drug exposure may aid in accounting for differences in differential

tissue penetration and facilitate rational dose selection for clinical drug development. Linking these measured target site concentrations to a PD effect provides a more meaningful approach for determining safe and effective dosing regimens.

Of special note, once a drug reaches the extracellular fluid, there may be an additional barrier to reaching the site of action, the cell membrane. This last step is critical for intracellular infections, where a pathogen may replicate and hide from a host's immune system. As with plasma and extracellular fluid concentrations, drug exposures inside and outside the cell may be different. Once within the cell, a drug must be able to reach its target, whose location will depend on the specific pathogen involved. For example, some species reside primarily in the cytosol (e.g., *Listeria*, *Shigella*, and *Rickettsia*), whereas others remain in phagosomes that may or may not fuse with endosomes (e.g., *Mycobacterium*) or lysosomes (60). Using antibiotics as an example, the extent of intracellular accumulation varies considerably between drug classes, and factors such as physicochemical properties, binding to cellular structures (e.g., phospholipids), and the presence of antibiotic efflux pumps play a key role in determining the extent of accumulation within the cell (60–62). Although within a drug class there are differences in the extent of intra- versus extracellular distribution, macrolides and fluoroquinolones accumulate extensively within cells, whereas β-lactams distribute only into the extracellular body water (60, 63).

The ability of an antibiotic to accumulate intracellularly is studied *in vitro* by using specific target cells (e.g., macrophages, polymorphonuclear neutrophils, and lung parenchyma cells) and/or *in vivo*, for example, by using a murine peritonitis infection model (64–67). *In vivo* studies provide an opportunity to evaluate drug distribution to the target site and the immune system's response to an infection. In human subjects, collection of plasma and microdialysis samples, coupled with isolation of white blood cells and subsequent determination of intracellular drug concentrations, provides an opportunity to assess tissue distribution in various compartments (68).

Tissue homogenates are frequently used to determine drug concentrations within a specific organ. However, during the homogenization process, intra- and extracellular components are mixed. Determined concentrations therefore represent average values for this particular tissue, which are not reflective of specific target site concentrations, such as concentrations within the ISF, the cell, and organelles, etc. (42). In fact, this approach underestimates concentrations of drugs that equilibrate exclusively with the interstitial fluid (e.g., β-lactams and aminoglycosides) and overestimates the concentrations of those that accumulate within cells (e.g., fluoroquinolones and macrolides) (9, 40, 69). Alternative methods for assessment of drug tissue distribution include microdialysis, tissue biopsy, skin blister fluid sampling, saliva sampling, and imaging techniques such as positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). Major differences between these methods include the matrix sampled (blood, plasma, ISF, and saliva, etc.), invasive nature, collection period (continuous versus single time point), and direct measurement of unbound drug concentrations (40). However, a routine measurement of free target site concentrations may not always be feasible under certain conditions due to the accessibility of the tissue (e.g., the brain) or the health status of the patient (e.g., in critically ill patients).

A plethora of clinical microdialysis studies have been performed to

TABLE 2 Summary of clinical microdialysis studies performed with antimicrobials

Drug(s)	Subject group	Tissue(s)	No. of subjects	Collection period (h)	Dosage(s)[a]	Reference
β-Lactams						
Cefaclor	Healthy volunteers	Muscle	12	5.5	Single dose, various doses*	70
Cefazolin	Aortic valve replacement	Muscle, subcutaneous	7	12	2 doses, 4 and 2 g	71
Cefpirome	Healthy volunteers	Muscle, subcutaneous	12	8	Single dose, 2 g	72
Cefpirome	Sepsis and healthy controls	Subcutaneous	20	4	Single dose, 2 g	73
Cefpirome, cefodizime	Healthy volunteers	Muscle, subcutaneous	6	5	Single dose, 2 g	74
Cefpodoxime, cefixime	Healthy volunteers	Muscle	6	8	Single doses, 400 mg each	75
Cefuroxime	Morbidly obese, abdominal surgery	Muscle, subcutaneous	6	6	Single dose, 1.5 g	76
Cefuroxime	Cardiac surgery	Muscle	9	6	Multiple dosing, various doses**	77
Ceftobiprole	Healthy volunteers	Muscle, subcutaneous	12	0–12, 16, 24	Single dose, 0.5 g	78
Doripenem	Healthy volunteers	Muscle, subcutaneous	6	8	Single dose, 0.5 g	79
Ertapenem	Healthy volunteers	Muscle, subcutaneous	6	12	Single dose, 1 g	7
Imipenem	Critically ill	Muscle, subcutaneous	11	8	Single and multiple dosing, 500 mg 3 or 4 times daily	80
Meropenem	Acute brain injury	Brain	2	7	Multiple doses, 1 g 3 times daily	81
Meropenem	Septic shock	Peritoneal fluid	6	7	2 1-g doses	82
Meropenem	Pneumonia	Lung	7	8	Single dose, 1 g	83
Piperacillin	Aortic valve replacement	Muscle, subcutaneous	6	4	Single dose, 4 g	84
Piperacillin	Pneumonia	Lung	5	8	Single dose, 4 g	85
Aminoglycosides						
Gentamicin	Healthy volunteers	Subcutaneous	7	6	Single dose, 240 mg	86
Macrolides-ketolides						
Clarithromycin	Healthy volunteers	Muscle, subcutaneous	6	8	250 mg (single dose) and 500 mg twice daily	87
Telithromcyin	Healthy volunteers	Muscle, subcutaneous	10	8	Single dose, 800 mg orally	88
Fluoroquinolones						
Ciprofloxacin	Healthy volunteers	Muscle, subcutaneous	8	8	Single dose, 200 mg	89
Ciprofloxacin	Healthy volunteers	Muscle, subcutaneous	8	12	Single doses, 400 mg (i.v.) or 500 mg (oral)	90
Ciprofloxacin	Obese and lean subjects	Muscle, subcutaneous	24	6	Single dose, 2.85 mg/kg of body wt	91
Ciprofloxacin	Diabetics	Foot lesion, subcutaneous	6	5	Single dose, 200 mg	92
Gemifloxacin	Healthy volunteers	Muscle, subcutaneous	12	10	Single dose, 320 mg	93
Levofloxacin	Cardiac surgery	Lung	10	8	Single dose, 500 mg	94
Levofloxacin	Cardiac surgery	Lung	6	8	Single dose, 500 mg	95
Moxifloxacin	Healthy volunteers	Muscle, subcutaneous	13	12	Single dose, 400 mg	46
Oxazolidinones						
Linezolid	Sepsis or septic shock	Muscle, subcutaneous	12	8	Single and multiple doses, 600 mg every 12 h	96
Linezolid	Healthy volunteers	Muscle, subcutaneous	10	8	Single and multiple doses, 600 mg twice daily	97
Linezolid	Healthy volunteers	Muscle, subcutaneous	9	8	Multiple doses, 600 mg twice daily	98
Tedizolid	Healthy volunteers	Muscle, subcutaneous	12	12	Single dose, 600 mg	99
Lipopeptides						
Daptomycin	Diabetic and healthy controls	Subcutaneous	12	24	Single, 4 mg/kg	100
Miscellaneous						
Fosfomycin	Healthy volunteers	Muscle, subcutaneous	6	8	Single, 4 or 8 g	101
Metronidazole	Gynecological	Muscle	6	10	Single, 500 mg	102

[a] i.v., intravenous. *, modified release, 500 and 750 mg; immediate release, 500 mg. **, cefuroxime, 3 g i.v. with anesthesia induction, then 1.5 g i.v. after cardiopulmonary bypass with protamine sulfate, and 1.5 g i.v. 8 h after surgery.

study the extent to which antimicrobials reach the extracellular fluid of specific tissues (Table 2) (7, 46, 70–102). Although most have been performed with healthy volunteers, this technique has also been applied to measure unbound drug concentrations of the ISF of critically ill patients. Frequently, these studies are of small sample size and focus on soft tissue penetration.

Similar to microdialysis, the skin blister technique attempts to evaluate tissue distribution through measurement of interstitial drug concentrations. First reported 40 years ago (103), the basic principle of the technique involves the separation of the dermis and epidermis through applied suction on the skin surface (104). The resulting fluid-filled blisters serve as a surrogate of interstitial fluid. In addition to drug sampling, the technique has also been used to quantify concentrations of endogenous, inflammatory mediators (105–107). Limitations of this technique include the discomfort resulting from skin blister formation, limited sampling times, difficulties related to standardization, and the presence of inflammatory proteins and mediators in the blister fluid (40, 104, 105). One study sought to compare the skin blister and microdialysis techniques to evaluate the subcutaneous penetration of fluconazole following single-dose, oral administration. Microdialysis concentrations measured in the subcutaneous tissue were similar to unbound concentrations in plasma, although a lag time of 0.5 h was observed (108). On the other hand, flucona-

zole concentrations measured in blister fluid were significantly low and delayed compared to plasma concentrations. In this study, microdialysis appeared to more appropriately capture the time course of drug concentrations in subcutaneous tissue. In separate studies, less stark differences were observed between these methods when evaluating dermal PK for famciclovir and acetylsalicylic acid (104, 109). To date, there is no widely accepted gold-standard technique, and study-specific factors must be considered in the design of a clinical study and the interpretation of its results.

Saliva drug sampling represents another potential measure of extravascular drug penetration. Drug-related factors impacting salivary drug concentrations include molecular size, lipophilicity, pK_a, and protein binding, whereas salivary flow and clearance mechanisms, salivary pH, and pathophysiological factors will also dictate drug distribution in saliva (47, 110). Two studies comparing the microdialysis, skin blister, and salivary drug sampling techniques using theophylline and paracetamol showed that salivary concentrations were poorly predictive of unbound plasma concentrations (111, 112). Moreover, with theophylline, microdialysis proved to be the more reliable technique, with unbound drug concentrations in plasma and tissue being highly correlated after accounting for protein binding. In the case of paracetamol, a drug with negligible protein binding, drug levels determined by both microdialysis and the skin blister technique closely mirrored serum drug levels. However, there are also examples in the literature that suggest method-specific differences in determined free drug concentrations. For example, it was shown that following oral administration, ciprofloxacin penetrates preferentially into inflamed lesions, such as cantharis-induced skin blisters (area under the concentration-time curve for blister fluid [$AUC_{blister}$]/ AUC_{plasma} = 1.44 ± 0.16), a fact that would have been missed based on measurements from different sampling sites, such as saliva ($AUC_{saliva}/AUC_{plasma}$ = 0.33 ± 0.01) or capillary blood ($AUC_{capillary blood}/AUC_{plasma}$ = 0.88 ± 0.07) (90).

Imaging techniques, namely, MRS and PET, provide an opportunity to visualize patterns of drug distribution in key organs. MRS, a technique based on nuclear magnetic resonance (NMR), uses radiofrequency pulses and magnetic fields to capture resonance signals emitted by specific nuclei (e.g., ^1H and ^{13}C) on molecules of interest (40, 45). MRS can provide continuous monitoring of both the parent drug and metabolites (40, 113–116). PET is a noninvasive imaging technique that uses a radionuclide attached to the molecule of interest to elucidate distribution patterns and drug target binding characteristics and expression patterns (43, 44). Although PET can also provide continuous monitoring, the length of time depends on the half-life of the radioisotope. Also, because PET records nuclear decay events altogether, it cannot exclusively quantify unbound drug concentrations (40). However, one study combined microdialysis and PET imaging to study intracellular ciprofloxacin concentrations (117). The authors of that study were able to relate unbound extracellular concentrations measured by using microdialysis to total concentrations quantified by using PET to assess the extent of intracellular uptake and retention.

APPROACHES FOR ESTABLISHING PK/PD RELATIONSHIPS

In Vitro Studies

In vitro studies of anti-infective drugs are often designed to mimic conditions observed *in vivo*. Of the various factors that must be taken into consideration (e.g., pH, growth media, electrolytes, and fatty acids), the appropriate resemblance of free, active drug concentrations in these *in vitro* settings is of utmost importance.

To do so, two general approaches have been established. First, protein supplements, such as human or bovine serum albumin or human serum, may be added to the growth media to mimic physiological binding conditions in *in vitro* settings. Although there is no general consensus on the amount of protein to be added, 4 g/dl is typically regarded as the target concentration, as it resembles normal physiological conditions (16, 17, 20, 21, 26, 27, 118–122). Selection of the serum concentration is even more difficult, as bacterial growth is often inhibited once the serum content exceeds 70% of the growth medium (16). Moreover, variability in the extent of drug binding between protein supplements, binding characteristics dissimilar from those *in vivo*, differences in experimental conditions, and application of literature protein binding values have led to various conclusions related to the significance of protein binding for antimicrobials (16, 25, 33). A second approach is to account for protein binding through simulation of unbound concentrations *in vivo*, circumventing the need to add a protein supplement (26, 123, 124).

An *in vitro* study compared the antimicrobial activity of cefditoren against penicillin-resistant *Streptococcus pneumoniae* in the presence of Mueller-Hinton broth plus 5% lysed horse blood (MH), MH plus 90% human serum, MH plus human serum albumin (4 g/dl), and a kill curve assuming a final drug concentration corresponding to 88% protein binding (26). Three separate strains with increasing MIC values (0.12, 0.25, and 0.5 mg/liter) were tested (the MIC corresponded to the lowest concentration inhibiting visible bacterial growth after 18 to 24 h). For the strain with the lowest MIC, no significant differences were observed between the four scenarios. In contrast, for the other two strains, significant reductions in bacterial counts at 24 h were observed with human serum compared to the study arms containing broth alone, human serum albumin, or a simulated unbound concentration. This may be explained partly by the presence of other serum constituents (e.g., gammaglobulins) which can enhance bactericidal activity. Moreover, as noted by the authors of that study, the fact that only in the presence of human serum was cefditoren's bactericidal activity unaffected may suggest that accounting for protein binding effects through the use of reported protein binding values can result in poor conclusions based on the observed data.

A similar study performed by using the antifungals voriconazole and anidulafungin sought to compare antifungal activities against *Aspergillus fumigatus* and *Aspergillus flavus* in the presence of RPMI broth alone, human serum, human serum albumin, and expected unbound drug concentrations based on theoretical protein binding (voriconazole, 58%; anidulafungin, 99%) (32). By using the XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide} assay to assess metabolic activity, anidulafungin activity was reduced in the presence of human serum and human serum albumin, while the pattern of voriconazole's activity remained unchanged following 48 h of incubation. A few additional points are worth noting. First, as in the above-described example, the impact of protein binding was dependent on the culture medium. Anidulafungin exhibited greater activity in the presence of human serum than in the presence of human serum albumin. This difference was still apparent despite the fact that heat-inactivated serum was used,

suggesting that perhaps some additional non-protein-mediated mechanism may help explain this phenomenon. Second, for both drugs, greater bactericidal activity was observed with human serum than with the use of a theoretical protein binding value. This may suggest that simply accounting for protein binding through a theoretical fraction that is unavailable to exert a drug effect may be too simplistic. Finally, the impact of protein binding may be species dependent in some cases. Anidulafungin's increased activity in the presence of serum was particularly evident for *A. flavus*. All these factors explain why it may be difficult to account for protein binding in *in vitro* experimental settings.

Although greater protein binding can reduce unbound drug concentrations at the site of action, this may not always translate into an impact on drug efficacy. For example, telavancin and daptomycin display bactericidal activity against Gram-positive organisms despite being highly protein bound, 93 and 92%, respectively (21, 23, 125). Various factors have been proposed to explain these observations. For example, weak binding to plasma proteins relative to the drug target may play a role (21, 27). In the case of telavancin, the molecule's ability to disrupt bacterial plasma membrane function, in addition to inhibiting peptidoglycan synthesis, was proposed to partly explain its efficacy despite significant binding to plasma proteins (126).

Regardless of the approach used to account for the impact of protein binding, free drug concentrations should be measured whenever feasible. For example, one study sought to simulate total and unbound serum concentrations by using a two-compartment, *in vitro* dynamic model for two compounds which differ in the extent of protein binding (cefpodoxime, 21%; cefditoren, 88%) (123). In the experimental setup, no protein supplements were added, and target drug concentrations were simulated by using estimates of the extent of drug binding reported in the literature. Target drug concentrations were compared with experimental total and unbound concentrations quantified by taking aliquots from the peripheral compartment at predefined time points and analyzing the samples by using a bioassay. The measured drug concentrations were then compared directly with the reduction in bacterial counts over time for two strains of *Streptococcus pneumoniae*.

In Vivo Studies

Animal models. Animal infection models provide an opportunity to test the efficacy of anti-infective drugs *in vivo* across a range of scenarios, including multiple pathogens, various drugs and dosing regimens, and different types of infection. Lung and thigh infection models are often used to study cyclophosphamide-induced neutropenia (127). When evaluating the PK in these animal models, frequently, total plasma concentrations are determined and corrected by using protein binding values reported in the literature. A limited number of studies that evaluated unbound tissue concentrations in animal infection models are available. For example, two separate studies sought to compare unbound plasma and tissue concentrations of voriconazole and fluconazole in healthy and *Candida* species-infected rats (128, 129). In both cases, unbound plasma levels could serve as a good surrogate for unbound drug levels in the kidneys. In contrast, a separate study that compared azithromycin tissue concentrations in uninfected and infected tissue by using a rat thigh infection model (*Staphylococcus aureus*) noted greater drug exposure in the infected tissue (Fig. 2) (130). The azithromycin example clearly demonstrates the
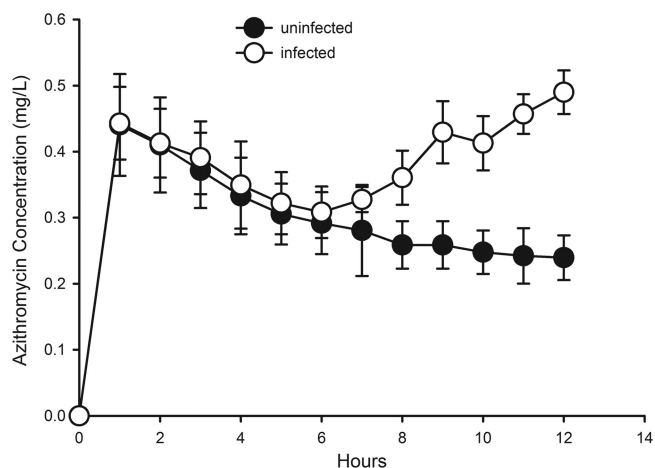

FIG 2 Azithromycin concentration versus time in uninfected and infected (*S. aureus*) tissue in a rat thigh infection model. (Reprinted from reference 130.)

potential impact of disease on free, active concentrations at the site of action/infection. Extrapolation of findings from healthy animals directly to diseased conditions can consequently be misleading, and the underlying assumptions warrant further investigation.

Clinical studies. Clinical studies evaluating drug distribution are frequently conducted with healthy subjects. Although there are several practical advantages to performing these studies with healthy subjects, disease-related changes can result in significant differences between healthy subjects and the intended patient population. It may consequently be misleading to translate findings from healthy subjects directly to patients without considering the underlying pathophysiological changes. For example, antibiotics, such as beta-lactams or aminoglycosides, that freely distribute in extracellular water can be "dragged" into the interstitial space of a critically ill patient with "leaky capillaries" by extravascular fluid movement, resulting in an increase of the distribution volume (131). As a result, plasma concentrations will decrease for a given dose, requiring an increased loading dose to compensate for the drug "lost" to the tissue. Once the patient's condition improves, the distribution volume will slowly return to its original value. The time course of changes in the PK of these drugs therefore mirrors that of the patient's pathophysiology (131), a phenomenon observed for vancomycin (132, 133), amikacin (134), and beta-lactams (135), to name a few. On the other hand, for drugs that distribute into extra- and intracellular spaces, such as quinolones (136), moderate changes in the volume of distribution as a result of disease are less important and are likely to require no adjustment of the loading dose.

In addition to fluid-shift-related phenomena, changes in the rate at which the primary eliminating organs (i.e., the liver and the kidneys) are perfused may alter the PK of antibiotics in patients (131). For example, altered cardiac output due to position changes during surgery and/or anesthesia or due to the administration of large fluid volumes may substantially alter drug clearance. It was shown for the kidneys that an augmented clearance was not appropriately reflected by measured serum creatinine concentrations, the clinical surrogate for the glomerular filtration rate. While serum creatinine concentrations were in some cases within the normal range, the renal clearance rate was significantly ele-
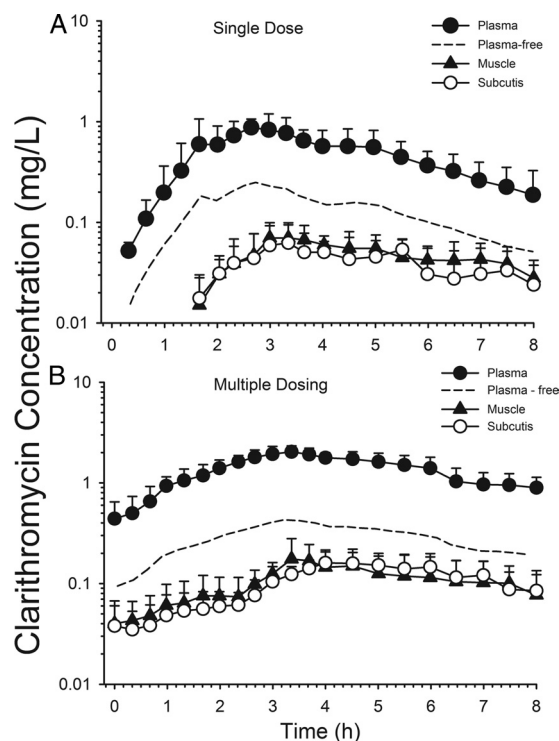
FIG 3 Clarithromycin concentration versus time in plasma, subcutis, and skeletal muscle after a 250-mg single dose (A) and 500 mg twice daily for 3 to 5 days (B). (Reprinted from reference 87.)

vated, making more frequent dosing necessary (137). The situation becomes even more complex if patients require additional hemodialysis, as the anti-infective agent itself may also be removed during the process. In addition to these distribution- and clearance-related factors, pH changes, tissue and/or plasma protein binding alterations, as well as drug delivery via macrophages or neutrophils may impact target site concentrations (138, 139).

Microdialysis has been applied to study the differential tissue penetration of anti-infective drugs in humans, frequently in healthy volunteers but also in patients. For example, a clinical microdialysis study performed with six healthy volunteers evaluated the distribution of clarithromycin in muscle and subcutaneous tissue and compared it with unbound plasma concentrations (87). Following a single dose of clarithromycin (250 mg), the ratios of the area under the unbound drug concentration-time curve from 0 to 24 h ($fAUC_{0-24}$) in subcutaneous tissue and skeletal muscle versus plasma were 0.29 ± 0.17 and 0.42 ± 0.18, respectively (Fig. 3A). At steady state, similar ratios were observed, although less stark differences between subcutaneous tissue and muscle were noted (subcutis, 0.39 ± 0.04; skeletal muscle, 0.41 ± 0.19) (Fig. 3B). As noted by the authors of that study, these findings are at odds with measurements of clarithromycin in biopsy specimen homogenates, which supported greater exposure in tissue than in plasma (140, 141). This example nicely illustrates the fact that free, unbound concentrations in plasma (although not directly measured in that study) may not always be an appropriate surrogate for unbound concentrations in the interstitial space fluid of various tissues. The use of free, unbound plasma concentrations for predicting outcome would have resulted in an over-

prediction of clarithromycin's free tissue concentrations, which can result in therapeutic failures and resistance development.

Whenever possible, drug concentrations should be measured directly at the site of infection and in the patient population of interest. Doing so would prevent erroneous conclusions based on differences in penetration between tissues and due to disease-related changes. For example, one study which evaluated levofloxacin concentrations in peripheral soft tissues and the lung found 2-fold-lower concentrations in the latter (142). Soft tissue concentrations are consequently a poor predictor of levofloxacin's exposure in the lung, and the respective dosing recommendations may result in therapeutic failures.

The type of infection generally determines the site at which target concentrations should be sampled (9). For lung infections, epithelial lining fluid (ELF) and lung interstitial fluid have been studied, and the respective concentrations have been used to predict the probability of success or failure for a given therapy. For example, measurement of dapsone concentrations in ELF of patients infected with human immunodeficiency virus type 1 (HIV-1) showed that appropriate tissue penetration is achieved, and a twice-daily prophylactic treatment regimen resulted in sufficient drug exposure (143). Improvements in the methodology used to sample ELF drug concentrations have addressed known limitations of the technique (144), namely, the inaccuracies associated with quantifying the ELF volume and the inability to sample the same site multiple times (145). Microdialysis has also been applied as a means to quantify lung concentrations of antimicrobials in human subjects (83, 85, 94, 142, 146, 147). However, a limitation of the technique in this setting is that pulmonary infections may be located in different compartments (e.g., alveoli, bronchioles, and intracellularly) (148). Consequently, it is important to choose a sampling technique that allows the determination of concentrations at or at least as close as possible to the infection site. Failure to do so may result in treatment failures due to insufficient knowledge about target site exposure.

Even when unbound drug concentrations are measured at or near the site of infection, data need to be interpreted cautiously. One contributor of differential tissue penetration is a difference in pH between milieus, as may be caused by disease states. Depending on the physicochemical properties of a drug, a pH gradient can facilitate movement of neutral molecules across membranes, where ionization then restricts further diffusion, a phenomenon known as "ion trapping" (149, 150). Although higher unbound drug concentrations may be measured at the site of infection, the charged species may not be able to diffuse across the bacterial cell wall, and thus, no additional therapeutic benefit would be obtained (151). The potential role of ion trapping may be considered not only by relating drug concentrations in plasma and tissue but also by taking into account potential differences in pH between plasma and tissue, under the given set of circumstances. For example, moxifloxacin has been shown to accumulate in prostatic secretions in healthy subjects, with a prostatic secretion-to-plasma ratio of 1.57 (152). In this scenario, as minimal differences have been observed between plasma and prostatic secretion pH in healthy subjects (153), it is unlikely that ion trapping explains the observations, but rather, differences in lipophilicty, binding to cellular matrices, and/or rapid cellular uptake/release kinetics may play a role (154).
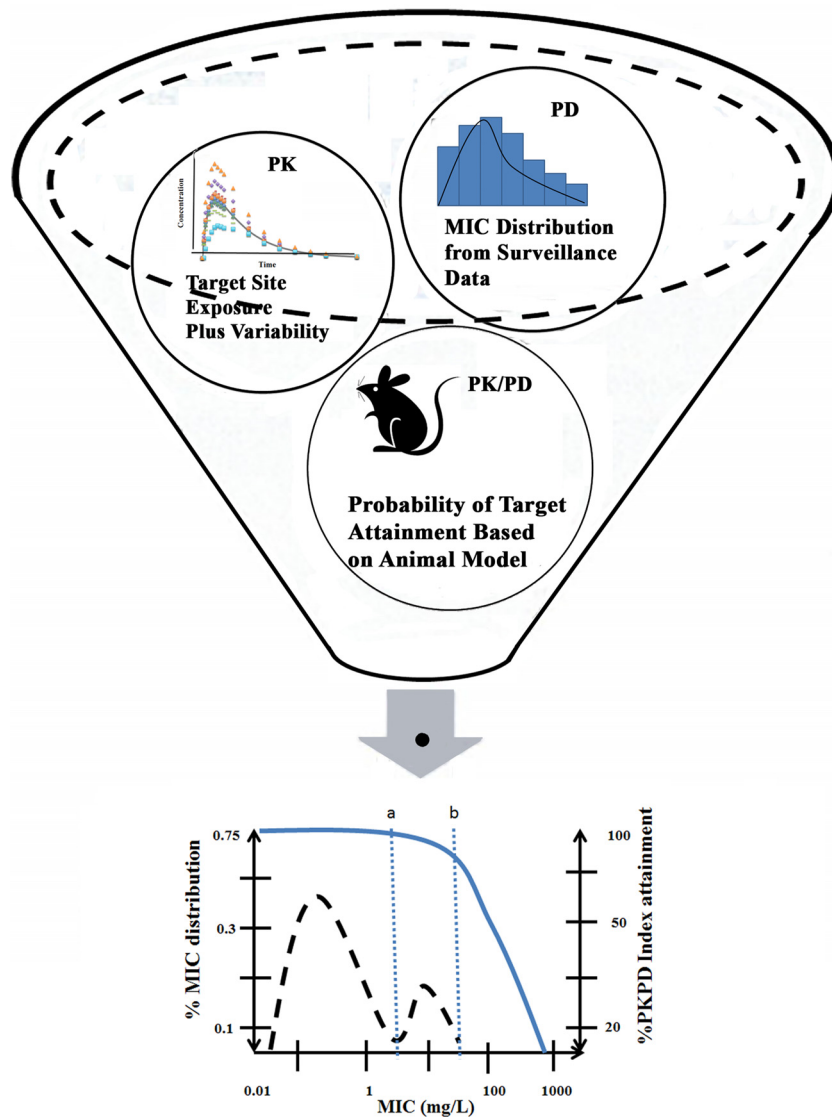
FIG 4 Application of PK/PD modeling and simulation to integrate *in vitro* and *in vivo* data and guide drug and dosage selection. Dosage recommendations are further supported by determination of inflection points (a and b) in the MIC distribution obtained from clinical surveillance data and estimates of target drug exposure. (Adapted from reference 160 [Fig. 12.5] with kind permission from Springer Science+Business Media B.V.)

PK/PD RELATIONSHIPS

In isolation, information on the PK of a drug is of limited meaningfulness. Only the link between a drug's PK and the corresponding drug effect provides a meaningful rationale for the selection of a safe and effective dose. For antimicrobials, the drug effect is typically determined *in vitro* by using MIC or time-kill curve experiments. The determined PD/susceptibility breakpoints for a specific pathogen or group of pathogens are then correlated to the PK parameters of a certain anti-infective agent. To date, the majority of these correlations are MIC based due to the relative simplicity of its experimental determination, resulting in three main indices: $fT_{>MIC}$, $fAUC/MIC$, and fC_{max}/MIC. In general, for β-lactams, a drug class associated with time-dependent killing, it is the time that drug concentrations stay above the MIC ($T_{>MIC}$) that correlates best with drug efficacy. In the case of aminoglycosides and fluoroquinolones, where bacterial killing is concentra-

tion dependent, it is the maximum concentration of the drug in serum divided by the MIC (C_{max}/MIC) and AUC/MIC ratio that are important (155, 156). Since only the unbound drug is pharmacologically active, these indices should be expressed in terms of unbound drug exposure ($fT_{>MIC}$, fC_{max}/MIC, and $fAUC/MIC$) (157). These same three PK/PD indices may also be applied to antifungals (158). Similar principles have also been applied to antiviral agents, but the respective PK/PD correlations oftentimes use more stringent PD endpoints (e.g., 95% effective dose [ED_{95}]or ED_{99}) than antibacterials or antifungals to achieve a faster elimination of the virus from the body. In many cases, the relationship between host, virus, and antiviral agent is somewhat more complex, requiring more sophisticated modeling approaches.

Although MIC is the most widely used PD parameter, there are limitations to its use (159). It is a single-point estimate after 18 to

24 h of incubation, which does not provide information on the time course of the drug effect or the presence of a postantibiotic effect. MIC values determined *in vitro* may differ from the actual susceptibility breakpoints *in vivo* due to faster growth of bacteria in nutrition-rich growth media and the absence of immune factors. The static nature also allows for only a direct comparison of drug effects at a limited concentration range. It should also be noted that the concentrations of the starting inoculum routinely employed for MIC testing ($\sim 5 \times 10^5$ to 5×10^6 CFU/ml) can be quite different from the bacterial burden observed *in vivo*, depending on the type of infection (e.g., $>10^{10}$ in pneumonic lung). As a result, PK/PD indices based on standardized *in vitro* values may not accurately reflect the *in vivo* situation. Time-kill curve experiments and particularly hollow-fiber models have been proposed as more robust approaches to determine the concentration-effect relationship over time. Due to the time-consuming and labor-intensive nature of these experiments, they have so far been used primarily in drug development and research settings, yet technical advancement and automation may make this approach more attractive for routine application in the future.

PD parameters, such as the concentration necessary to produce 50% of the maximum effect (EC_{50}), determined from these *in vitro* experiments can then be linked to *in vivo* drug exposure to predict the concentration-effect relationship at the site of infection and account for between-subject variability in treatment response (Fig. 4) (160). Through a simulation-based approach, the developed models may then be applied to evaluate "what-if" scenarios, which may help to gauge the appropriateness of a particular drug or dosing regimen against a pathogen (161). For example, one study applied a population PK/PD model developed in healthy subjects and cystic fibrosis patients to guide dosage selection for piperacillin (162). The model predicted that continuous (9 g/day) or 4-h (3 g every 8 h) infusions resulted in a greater or similar target attainment ($fT_{>MIC} \geq 50\%$) compared to a higher daily dose (3 g every 4 h) administered as a 30-min infusion.

CONCLUSIONS

Free, unbound drug concentrations are responsible for the PD effect of anti-infective agents. Although plasma protein binding is an important PK parameter, it is only a surrogate for the free, unbound concentrations at the site of action causing the drug effect. As a consequence, free drug concentrations should be experimentally determined at or at least close to the site of action whenever possible. For anti-infective agents, the site of infection is typically located outside plasma, and thus, a drug must diffuse to reach its target. There are many available techniques to quantify tissue concentrations. Selection of an appropriate sampling technique requires careful consideration of the infection site and the advantages and limitations of each approach.

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