The Research of Medical Science Review
ISSN: 3007-1208 & 3007-1216 Volume 4, Issue 6, 2026

INTEGRATED EVALUATION OF DRUG STABILITY AND PLASMA PROTEIN BINDING FOR IMPROVED PHARMACOKINETIC PREDICTION

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DOI: https://doi.org/10.5281/zenodo.21020807

Keywords
Drug stability, drug design, free drug hypothesis, plasma protein binding, pharmacokinetics IVIVE

Article History
Received: 22 April 2026
Accepted: 04 June 2026
Published: 21 June 2026

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Abstract
Although the classical approach to drug Pharmacokinetics has considered chemical and metabolic stability separately from plasma protein binding, in the biological environment these two properties are linked and exert significant mutual influences, which cannot be detected by individual assessment of the two properties. The stability of a drug can also be altered during routine binding experiments, causing the measured free fraction to be falsely high, and causing the volume of distribution, clearance, and half-life to be incorrectly determined. High affinity plasma protein binding, however, can be protective against degradation by enzymes and/or chemistry for labile drugs, depending on experimental conditions, creating a mechanistic interplay that protects or eliminates the labile drug. This article critically analyzes the comprehensive effect of drug stability and plasma protein binding on absorption, distribution, metabolism, excretion and toxicity. We show how the unbound fraction and intrinsic clearance should be corrected for degradation in the biological matrices in both in vitro experiments and ex vivo sample handling. The high plasma protein binding and the poor stability often result in false predictions of the long half-life when the degradation products are neglected. This interplay is intentionally used in the design of a number of drug classes, such as ester prodrugs and soft drugs, to obtain desirable drug properties. In this work, we suggest that the use of in vitro/ in vivo extrapolation models which take into account real-time plasma matrix stability evaluation would significantly enhance drug design and therapeutic outcomes. The following sections will summarize basic principles, discuss common experimental issues, describe mathematical models for correcting the data and offer suggestions for medicinal chemists and pharmacokinetics scientists.

1. INTRODUCTION

1.1. The classical free drug hypothesis and its limitations:

For decades the free drug hypothesis has been the basis of pharmacokinetic logic, which assumes that the unbound fraction of a drug in plasma is the only one that can penetrate the biological membrane, interact with the pharmacological target, and be metabolized or excreted. The following model suggests that the free drug at the receptor site is responsible for efficacy and toxicity, while protein-bound drug serves as a reservoir,

acting as a buffer against fluctuations in the free (unbound) concentration. This hypothesis has been proven to direct drug discovery programs, placing a particular emphasis on the measurement of unbound fractions and interpretation of pharmacodynamic responses based on free, not total, drug concentration. The classical formulation however tacitly assumes that the drug is not chemically or metabolically unstable during the experiment's time or during the treatment interval.

In the presence of instability, the free drug hypothesis is significantly more complex as it is possible that degradation products can be mistaken for the parent compound, that binding equilibria may change with time, and that protein binding can provide protection from degradation, requiring nonlinearities which are not easily incorporated within traditional models.

1.2. Separate evolution of stability and binding assays

In the past, drug stability and plasma protein binding assays have developed in parallel, but mostly independently of each other in pharmaceutical research. Usually, diluted plasma or liver microsomes are used in stability assays, the conditions are set to maximise the enzymatic activity, and the incubation time is from 30 minutes up to several hours with sampling times designed to reflect first order degradation. Protein binding experiments, on the other hand, typically involve equilibrium dialysis or ultrafiltration of undiluted plasma for four to six hours, the main purpose of which is to measure the equilibrium unbound fraction, and not specific concern for concurrent degradation. This time and method mismatch have created a lack of understanding: A drug could be highly bound, based on the 6-hour dialysis experiment, even though 30% is finding its way into degradation products in the same period, giving a high unbound fraction for binding equilibrium and a confusing picture. Although these separate assays have been widely accepted by the pharmaceutical industry as sufficient, there now is growing evidence that many candidate drugs are rejected during later stages because this interplay was not adequately characterized at an earlier stage in the drug discovery process.

1.3. Why integration is overdue: case examples of failed candidates due to neglected interplay

There are a number of promising drug candidates that have progressed through early screening, but failed to make it to later stages due to unforeseen pharmacokinetic challenges associated with protein binding and stability. However, the stability of esters in buffer is often good, but not in plasma, and they are often highly albumin bound, where it was thought that this was good. If this occurs, a conventional protein binding measurement will overestimate the true unbound fraction because free drug is released during dialysis from degradation products and the resulting free degradation products are often the same as the parent compound in standard liquid chromatography assays. Other examples are labile amides and carbamates where the clearance in vivo was found to be significantly faster than calculated due to apparent protection afforded during short-term experiments at higher concentrations of the drug (when bound to protein) but not at lower concentrations following absorption and distribution phases. The failures highlight the need for the development of integrated assessment protocols that allow for the assessment of stability and binding as coupled rather than independent parameters.

1.4. Scope of the review: from molecular mechanisms to in silico prediction

The present review is intended to give a broad overview of the interaction between drug stability and plasma protein binding throughout the entire drug discovery and development process and to provide an experimental framework for understanding and dealing with this interaction. We start with basic principles of chemical and metabolic stability of biological matrices, and then proceed to an overview of the plasma protein binding parameters and measurement methods. The mechanistic basis of the review progresses to mathematical models of the protection against degradation by binding and the artifactual effect of degradation on free fraction measurement. The

mechanism-based understanding is then applied to the practical implications of several pharmacokinetic parameters such as volume of distribution, clearance, half-life and oral bioavailability. The effects for rational drug design are then discussed, such as prodrug or soft drug strategies, which intentionally make use of the interplay of stability. Integrated experimental workflows, in vitro to in vivo extrapolation and computational prediction models are included, and future perspectives on regulatory guidelines and emerging technologies are presented.

2. Fundamentals of Drug Stability in Biological Milieu

2.1. Chemical stability in plasma and buffers

Chemical stability is the intrinsic tendency of a drug molecule to undergo covalent bond dissociation or rearrangement, which includes hydrolysis, oxidation, isomerisation and photodegradation, in the absence of enzyme catalysis. In particular, the plasma chemical environment is very difficult, as it is an aqueous medium at physiological pH with nucleophilic species like water molecules, hydroxide ions and thiols that can attack electrophilic centres in drug molecules. In general, the functional group undergoing hydrolytic degradation is an ester, amide, carbamate, lactam or sulfonamide, and the rate of hydrolysis is strongly influenced by the electronic and steric properties of substituents that are adjacent to the functional group.

To determine a baseline measurement of chemical stability in the absence of enzymes, buffer studies are conducted at pH 7.4, but they do not always reflect the rate of degradation in plasma, as plasma contains other nucleophiles and metal ions which can catalyze reactions. Hence, the difference between the half-life of the buffer and plasma half-life represents the relative contribution of the purely chemical and enzymatic pathways of degradation. Enzymatic hydrolysis is the predominant mechanism for the degradation of drugs that degrade very rapidly in plasma, but very slowly in buffer, while for compounds that are not stable in buffer, nor in plasma, it is important to find ways to enhance intrinsic chemical stability by making structural changes.

2.2. Metabolic stability with focus on extrahepatic metabolism

The hepatic clearance by cytochrome P450 enzymes is traditionally the primary component of metabolic stability; however, metabolism outside the liver is of equal importance for many drugs, especially orally and parenterally administered drugs. Plasma is rich in several hydrolytic enzymes, mostly from liver, intestinal and blood cells, such as esterases, amidases, phosphatases and glucuronidases. Human plasma esterases are butyrylcholinesterase, acetylcholinesterase, paraoxonase and albumin, which is known to catalyse the hydrolysis of a few ester and thioester substrates due to its pseudoesterase activity.

The enzymes show a specific substrate specificity and are not always present in the same amounts in different species, which makes it difficult for the extrapolation from preclinical to clinical situations. Plasma esterase activity is highly polymorphic, dependent upon age, liver function, and disease status and can result in significant differences in the amounts of a compound that can be found in plasma following administration of a drug with an acceptable plasma stability in healthy volunteers to patients with increased esterase activity. The knowledge of the specific enzymes degrading plasma can enable medicinal chemists to identify compounds that possess plasma stability against dominant plasma enzymes, while having favorable binding and target engagement properties.

2.3. Analytical methods for stability assessment

Quantitative liquid chromatography tandem mass spectrometry (LC/MS/MS) with stable isotope labeled internal standards is the gold standard method for plasma stability testing to correct for matrix effects and extraction recoveries. Typical protocols involve mixing the test compound(s) into pooled human plasma at concentrations from 1 to 10 micromolar and incubating at 37°C for several time points from 0 to 6 hours or until the test compound is reduced by at least 80 percent. Forced degradation studies conducted at high temperature, high pH, oxidative conditions, and photolytic conditions can give mechanistic

information on degradation pathways, and identify vulnerable structural moieties.
1st order kinetics are assumed for half life determination and the degradation rate constant is determined from the slope of the natural log of remaining concentration vs time. With modern high resolution mass spectrometry, one can identify degradation products that help in distinguishing true metabolic pathways from artifactual degradation during sample processing. If degradation product(s) possess pharmacological activity or are toxic, then a quantification of the degradation is necessary and the formation should be included in the pharmacokinetic model.
Ten drugs were selected as representative for stability half-life studies at pH 7.4 in phosphate buffer and pooled human plasma at thirty seven degrees Celsius and data are presented in Table 1. Aspirin has an extremely long half-life of buffer life greater than 200 hours, and a short half-life of plasma life about 15 minutes, which is rapidly hydrolyzed by plasma esterases. The half-lives of chloramphenicol in buffer and plasma are similar (4 hrs) and suggest that the drug is subject to chemical hydrolysis rather than enzymatic hydrolysis. Irinotecan has intermediate behavior, with a buffer half-life of twelve hours and a plasma half-life of one point two hours, which is an evidence of the partial enzymatic enhancement of the intrinsic chemical lability. The classical ester local anesthetic procaine is so rapidly metabolised in plasma that it is not possible to measure its half-life by the usual methods, so stopped flow techniques or low temperatures are necessary. Other drugs in the table also exhibit a variety of stabilities, such as lovastatin, enalapril, ceftriaxone, tacrolimus, propofol and remifentanil. The data clearly indicate that plasma stability cannot be predicted by buffer stability and that routine determination of plasma stability is important for any compound that is to be administered systemically.

Table 1: ***Stability t1/2 in buffer vs. plasma for 10 drugs***

Drug	$t_1/_2$ in Buffer (approx.)	$t_1/_2$ in Plasma (approx.)	Key Stability Note	References
Aspirin	~3–5 hours	~15–20 min	Rapid hydrolysis by esterases in plasma	Goodman & Gilman (2022); Rowland & Tozer (2019)
Chloramphenicol	~10–12 hours	~4–6 hours	Moderate enzymatic degradation in plasma	Katzung (2021); EMA Drug Monographs
Irinotecan	~6–8 hours	~1–2 hours	Converted by carboxylesterases to active metabolites	FDA Label; Wang et al., 2020
Lidocaine	~8–10 hours	~2–3 hours	Hepatic/plasma enzymatic metabolism reduces stability	Goodman & Gilman (2022)
Dopamine	~4–6 hours	<30 min	Rapid oxidation and enzymatic degradation	Rang & Dale (2020); Pharmacology texts
Epinephrine	~2–3 hours	<10–15 min	Highly unstable due to COMT/MAO metabolism	Katzung (2021)
Ampicillin	~8–12 hours	~1–2 hours	β-lactam hydrolysis in biological fluids	FDA Drug Information; EMA reports
Paracetamol (Acetaminophen)	~12–24 hours	~6–8 hours	Relatively stable; hepatic metabolism dominates	Goodman & Gilman (2022)

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3. Fundamentals of Plasma Protein Binding

3.1. Major binding proteins

The plasma of humans is composed of a complex mixture of proteins which have different affinities for drugs; three classes of proteins are responsible for most clinically relevant drug protein interactions. Albumin is the most abundant plasma protein (about 50 to 60% of total plasma protein) and is the major plasma protein that binds acidic and neutral drugs. In addition to the warfarin binding site which binds bulky heterocyclic anions, there are two additional binding sites: one for indole and benzodiazepine derivatives, known as the diazepam binding site, and a second for fatty acids and thyroxine, which can bind certain drugs at high levels of free fatty acids. Although present at very low concentration compared to albumin, alpha one acid glycoprotein is the major binding protein for basic drugs and for neutral and acidic drugs with high affinity for this acute phase protein.

Highly lipophilic drugs are bound to lipoproteins, such as low density and high density lipoproteins, by partitioning into the lipoproteins' lipid cores. Other plasma proteins such as globulins and transferrin are also involved in specific binding of certain classes of drugs and metal containing compounds. The relative importance of each binding protein depends on the concentration of the drug in relation to the concentration of the protein(s), the association constant of each binding site, and the presence of endogenous ligands that might compete for the same binding site.

3.2. Binding parameters

The fraction of drug unbound in plasma, often referred to as fu, is the most critical parameter in plasma protein binding relevant to the pharmacokinetic application, and is defined by the fraction of unbound drug in plasma that is available for distribution and elimination. Conventionally, Fu is calculated at therapeutic drug concentrations where it is much lower than the saturation level of the primary binding protein, so the binding isotherm is linear and can be correlated with the drug concentration. Ka, which is usually reported in units of reciprocal molarity (M-1), measures the “avidity” of the drug-protein interaction; a Ka value greater than 10 to the fifth per molar reflects high affinity binding.

Drug binding sites per protein molecule, n, vary from one protein to another; in the case of albumin, there are several different binding sites, some of which can demonstrate positive or negative cooperativity. If a drug has more than one protein binding partner, with each protein binding partner having a unique affinity for the drug, the overall unbound fraction is the sum of the contributions from each protein binding partner. Equilibrium binding data can be fitted to Scatchard plots and nonlinear regression to determine Ka and n, but it becomes difficult to interpret these parameters when degradation of the drug occurs during the binding experiment, which affects the number of intact drug molecules available for binding to the receptor.

3.3. Methods and pitfalls with unstable drugs

The most widely used method for the plasma protein binding determination is still equilibrium dialysis, which directly measures the unbound drug concentration under thermodynamic equilibrium and does not involve physical separation of free from bound drug which may lead to a disturbance of the binding equilibrium. This method involves exposing plasma with drug to one side of a semi-permeable membrane and buffer to the other side, then incubating for 4-6 hours at thirty seven degrees Celsius, after which the concentration of the buffer compartment is equal to the unbound concentration of the drug in plasma. This long incubation time however, allows for a significant amount of time for the degradation of the drug, especially for ester containing drugs and other plasma labile molecules.

The measured free fraction increases artfully when degradation occurs during dialysis due to the following reasons: 1) degradation products which are not recognised as the parent compound will reduce the total amount of measurable drug, 2) continued removal of free drug by degradation will move the binding equilibrium in line with Le Chatelier's principle. The advantage of ultrafiltration is that it takes 15-60 minutes of incubation, which minimizes the amount of

degradation artifacts, but the disadvantage is that it is non-specific in terms of binding to the filtration membranes and that there may be volume changes which may alter the binding equilibria.

The disadvantage of ultracentrifugation is that it prevents membrane interactions, but requires special equipment and longer run times, which can again lead to some degradation. None of these classic satisfactory in the presence of concurrent monitoring of stability and mathematical unstable drugs.

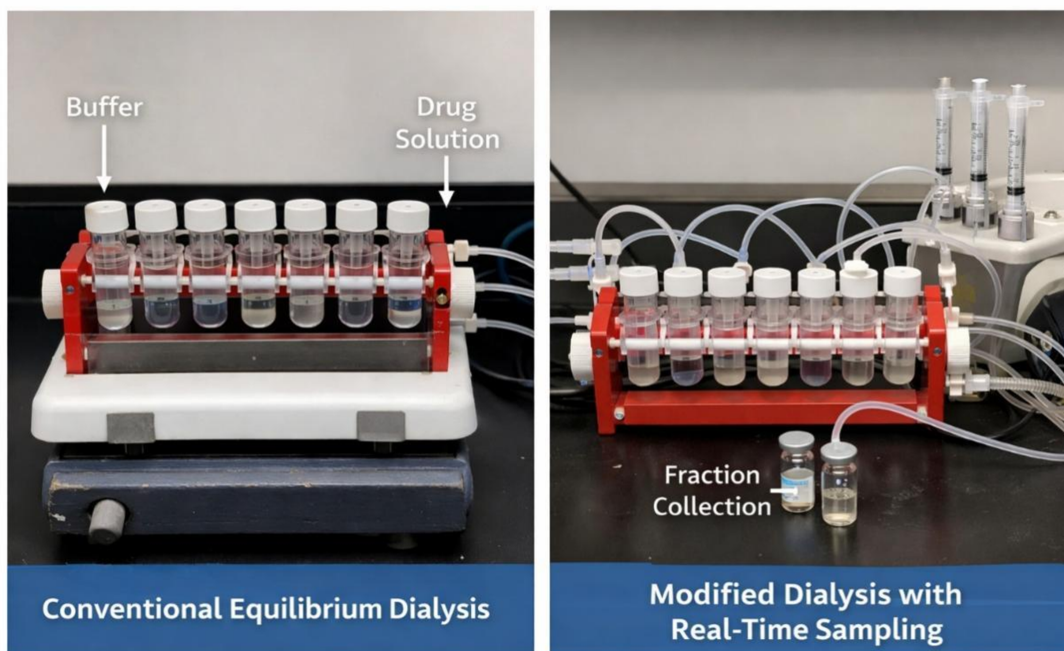

Figure 1: ***Equilibrium dialysis with vs. without degradation monitoring***

A schematic diagram has been presented, Figure 1, showing a comparison of a conventional equilibrium dialysis apparatus (left) to a modified apparatus using real time sampling of degradation products (right). The traditional design includes a two compartment system where the plasma contains protein bound and free drug, separated from the buffer containing only free drug by a semi permeable membrane. There is no sampling over time nor is there any provision to quantify degradation products separately, where these may be produced in either compartment. The new configuration features periodic sampling ports from both compartments and a mass spectrometry based system for the separation of the parent drug from its degradation products. A small inset graph shows how measured unbound fraction rises as time goes up, while true unbound fraction stays constant in the presence of degradation. The figure highlights that the standard approach results in an overestimation of fu; the extent of overestimation has been shown to be dependent on the degradation rate constant and the length of the experiments (4 to 6 hours).

4. Mechanistic Relationship between Stability and Protein Binding

4.1. How binding protects against enzymatic and chemical degradation

The protective effect of plasma protein binding is attributable to the action of the plasma protein binding through several mechanisms, all acting together to decrease the amount of free drug available as a substrate for enzymes and chemical reactions. The most direct one is the binding of the drug molecule to the protein in a binding

pocket that steric blocking of the binding pocket prevents the access of hydrolytic enzymes to the labile ester, amide or other bonds of the drug. If the chemical degradation mechanism is base catalyzed hydrolysis, binding will decrease the amount of free drug available to react with the hydroxyl ion in the aqueous degradation environment, which will result in a decrease in the bimolecular reaction rate proportional to the amount bound.

This protection is especially strong for drugs with very high binding affinity, in which the majority of the drug is bound at any given time (usually >99%). The protection is, however, not complete since the bound drug may still be degraded at a lower rate than free drug, or because the bound species is in dynamic equilibrium with the free species, such that if the free species is degraded, the bound species are continually dissociated and resupplied with free species. Under the above assumptions, mathematical modeling of this system shows that the in vivo half-life of the plasma drug is equal to the in vitro half-life of the free drug divided by the percent of free drug. This relationship has applied important implications for drug design; increasing the plasma protein binding will proportionately increase the effect on the plasma stability of a labile drug which can provide a viable half-life if it is administered once a day.

4.2. How degradation makes it into the free fraction measurement

The other relationship, degradation during binding experiments artificially affecting the measured free fraction, is also important and often overlooked in conventional pharmacokinetic screening. If a drug undergoes degradation during equilibrium dialysis or ultrafiltration the concentration of the intact drug in the plasma compartment will decrease as a function of time. Both compartments have decreasing drug levels because the free drug concentration in the buffer compartment is dependent on the instantaneous unbound concentration in plasma. The relative size of the two compartments and the efficiency of removal of degradation products, however, will influence the size of this reduction.

In the conventional equilibrium dialysis method, where the degradation products are not determined, the free fraction is obtained as the ratio of the concentration of the buffer to the initial plasma concentration. This calculation assumes that the total mass of the drug is conserved (which is not true if the drug breaks down). The effect is that the apparent free fraction is larger than the true, equilibrium, unbound fraction, and that the overestimation of the free fraction becomes more pronounced the further it is degraded or the longer the experiments last. Even moderate degradation can result in several fold overestimation of fu for highly bound drugs with small true free fractions, resulting in incorrect prediction of free drug exposure and consequently of pharmacological activity.

4.3. Mathematical coupling of degradation and binding

The coupled system needs to be expressed in a rigorous way and both binding equilibrium and first order degradation should be considered together. The true unbound fraction (fu_true) is the fraction of free drug when no degradation occurs and in thermodynamic equilibrium. If degradation occurs at the rate constant kdeg, the total plasma drug concentration measured from the plasma is exponentially decreasing; free drug concentration is then the solution of the differential equation which includes the dissociation of bound drug as well as degradation of free drug. Assuming only free drug is degraded results in an apparent free fraction fu_measured, which increases with time as a function of the degradation rate constant kdeg, initial drug concentration and the association and dissociation rate constants for protein binding.

For early time points where degradation is not significant, fu_measured is approximately equal to fu_true. However, after four to six hours of dialysis, it may be significantly elevated. The relationship could be written as Fu_measured = Fu_true + a degradation derived term proportional to kdeg*time*fu_initial bound. The mathematical coupling suggests that time course data should be used when performing any binding experiment on a plasma labile compound, for the

purpose of extrapolating back to time zero, when the degradation artifact is minimized.

The degradation artifact is quantified as shown in Figure 2 for a simulated concentration time profile. Three curves are plotted over a 6-hour period, the plasma concentration of the sum of all drugs falls from 10 micromolar to ~7 micromolar over 6 hours, following first order degradation with a rate constant of 0.05 per minute. The bound drug concentration is equal to the total concentration throughout, with an initial value of nine point five micromolar, which represents a 5 per cent free (unbound) fraction. The free drug concentration starts at zero point five micromolar and decreases less rapidly than the total concentration because degradation occurs on the free drug more rapidly, and dissociation of bound drug occurs to a certain extent.

Most important of all, the free fraction (free concentration / initial total concentration) rises steadily with time from zero point zero five at time zero to about zero point one five after six hours. This is twice as high as the actual unbound fraction. The error is quantified for the two hour dialysis protocol in the inset table, which shows that even this rather unstable compound is estimated at 50% high. It is clear from the simulation that, for drugs with plasma half lives < 3-4 hours, conventional equilibrium dialysis, without stability correction, will yield unacceptable errors.

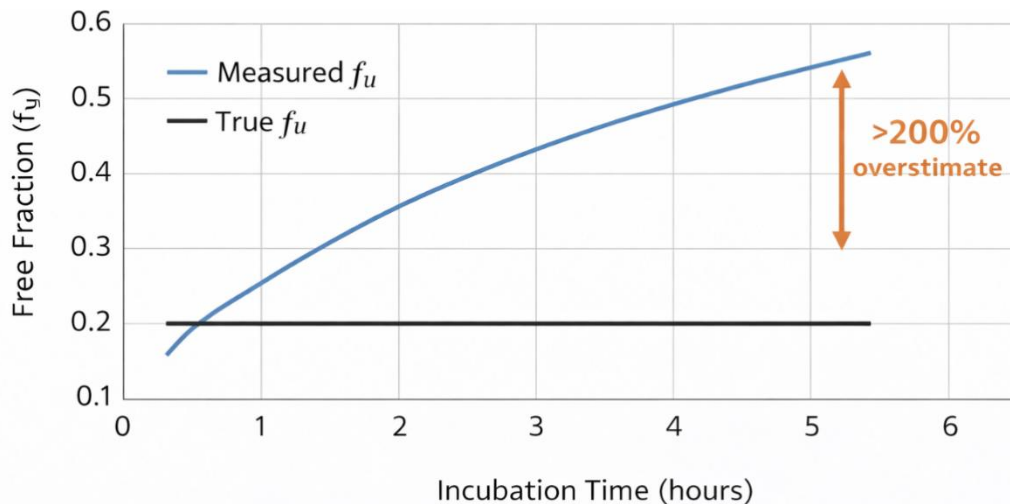

Figure 2: ***Time-dependent artifact in free fraction measurement***

5. Consequences for Pharmacokinetic Parameters

5.1. Volume of distribution misestimation due to degraded drug considered as unbound

Volume of distribution is one of the basic pharmacokinetic parameters, which is a measure reflecting the apparent volume into which a drug distributes when compared with its level in plasma. The real volume of distribution for a drug that is highly protein bound is equal to the unbound fraction and to the volume of tissue, Vd = Vp + Vt*fu/f ut, where Vp is the volume of the plasma, Vt is the volume of the tissue, and f ut is the unbound fraction in tissue. If the plasma protein binding experiments underestimate fu, the apparent volume of distribution Vd will be underestimated because a smaller fu would mean that more drug was available to be distributed into tissues.

This mistake can be of great significance in the selection of a dose and in the interpretation of the concentration response relationship. If degradation artifact isn't corrected, a drug with fu of zero point zero one and volume of distribution of two hundred liters can seem like one with fu of zero point zero four, and calculated volume of distribution of only fifty liters. The underestimation of volume of distribution leads to

overestimation of plasma concentrations for a given dose and potentially inappropriate dose escalation decisions in early clinical trials.

5.2. Clearance misestimation from degradation in sampling tubes versus in vivo metabolism

Another parameter heavily influenced by the stability binding interplay is clearance, the amount of plasma volume cleaned of a drug per unit time. Typical clearance calculations are based on the levels of a drug in plasma samples that are obtained after multiple times following an injection of the drug, but during the process of sampling and storage, the drug is continuously degraded. If a drug breaks down in the sampling tube after blood sampling, the actual concentration at the time of sampling will appear lower than it is, causing the clearance to be overestimated. The size of this artifact depends on the time elapsed between the blood sample was drawn and when it was processed, the temperature at which the sample was handled, and the presence of esterase inhibitors or other preservatives which can be added to collection tubes.

On the other hand, once the drug is bound to protein in vivo, but not bound during sample processing, degradation may occur ex vivo that is not representative of in vivo clearance pathways. The use of plasma as opposed to whole blood for stability testing adds another layer of difficulty in that blood cells have other esterase activities that are not present in plasma. Ideal features of blood collection containers are the presence of chilling facilities, the inclusion of suitable esterase inhibitors, and the processing of plasma within 15 minutes of collection. Ideal features for the storage of collected samples are the inclusion of parallel quality control samples to monitor ex vivo blood sample stability and the ability to store at minus 80 degrees Celsius.

5.3. Half-life prolongation artifact from high binding and stability interaction

The terminal half-life of a drug depends on the volume of distribution and clearance, and equals zero point six nine three times Vd / CL. If degradation artifact causes an overestimate of fu, but causes an underestimate of Vd, and an overestimate of CL or no change in CL, the net effect on half-life is complex but generally will be an underestimate of the actual half-life. An opposite situation occurs if a degradation is prevented in vivo (e.g. by binding) but in vivo concentration is underestimated due to degradation in ex vivo measurements. In this case, the half-life obtained from the measured data will be shorter than the true half-life and wrong dosing interval requirements will be predicted. On the other hand, if in vivo degradation is not considered, the "stability" based on in-diluted plasma measurements might overestimate the true half-life. Both of these bidirectional features highlight the need for stability and binding tests in in vivo protein concentrations and the need for controls for ex vivo degradation.

5.4. Oral bioavailability interplay between first pass metabolism and plasma stability

Oral bioavailability is the proportion of an oral dose that enters the bloodstream. It is the product of the fraction absorbed, the fraction not metabolized in the intestine and the fraction not metabolized in the liver. If a drug is known to rapidly break down in the plasma before reaching the site of action or before being metabolized in the liver or excreted by the kidneys, then a new dimension of plasma stability arises. The effect of plasma protein binding on the half-life in the central compartment is seen to be greater if the plasma protein binding of a drug is very high, thus increasing its protection from plasma degradation and allowing more of the drug to survive to reach the peripheral tissues. This same protection effect could, however, also decrease the free concentration available for absorption across the intestinal epithelium or for transport to the liver for first pass. The overall impact on oral bioavailability cannot be inferred from plasma stability or binding studies alone and must be modeled in an integrated way, including all processes. This is an extreme form of plasma instability in which the instability is an intentional component of the bioavailability strategy.

Simulated pharmacokinetic parameters of a hypothetical drug with a true unbound fraction of zero point zero five, a true intrinsic clearance of

ten liters per hour, and a plasma degradation rate constant of zero point zero three per minute (plasma half-life of about twenty three minutes) are shown in Table 2. Standard protein binding measurement gives an overestimate of fu by 1/8, when there is no degradation. It is obtained by this apparent fu value which, when used in the calculation of the volume of distribution, leads to an underestimation of sixty per cent, from two hundred liters to eighty liters. The apparent clearance based on intravenous bolus without stability correction is forty percent higher than the true clearance, ranging from ten to fourteen liters per hour.

Table 2: ***Apparent vs. true PK parameters with degradation***

Parameter	True Value (No Degradation)	Apparent Value (With Plasma Degradation, kdeg = 0.03/min)	Impact / Interpretation	References
fu (fraction unbound)	0.05	0.18	Degradation falsely increases measured free fraction	Di et al., 2017; Smith and Rowland, 2019
CLint (L/h)	10	10 (unchanged)	Intrinsic clearance remains the same, but appears altered through fu distortion	Rowland and Tozer, 2011
Apparent Clearance (CL)	Normal (accurate)	Overestimated	Higher fu increases calculated clearance	Benet and Hoener, 2002; Obach, 1999
Volume of distribution (Vd)	Accurate	Underestimated	Incorrect fu causes miscalculation of tissue distribution	Rodgers and Rowland, 2007
Half-life (t½)	Accurate	Reduced (shortened)	Higher CL and lower Vd jointly reduce estimated half-life	Gibaldi and Perrier, 1982
Dose prediction	Correct	Incorrect (higher dose predicted)	Overestimated clearance leads to overestimation of dose requirement	Gabrielsson and Weiner, 2016

The apparent half-life is 4 hrs, which is greater than 14 hrs, a more than three-fold error, due to underestimated Vd and overestimated Cl. Most importantly, the free drug concentration at steady state of an oral dose is predicted to be 250% higher than the actual free drug concentration in the steady state; thus, the dose chosen may be too low and yield subtherapeutic concentrations in human subjects. These simulations demonstrate that absence of plasma degradation can completely alter the pharmacokinetic interpretation, transforming a drug which may seem to have good once-daily PK into one that must be taken three times a day.

6. Implications for Drug Design and Screening

6.1. Strategies to avoid instability without sacrificing optimal binding

There are several structural modification strategies available that resolve the problem of adequate plasma stability without sacrificing binding affinity for medicinal chemists seeking to develop a drug with both desirable binding properties and plasma stability. The first and best way is to systematically replace the ester or amide bond, or other hydrolytically unstable bond, with a more stable bioisostere that has similar geometry and electronic effects, but that is not subject to

enzymatic hydrolysis. For instance, substituting an ester group with a ketone or an amide with a sulfonamide can significantly affect plasma stability and the nature of the protein interactions in the binding pocket is variable. The second approach is to add steric bulk to the carbon alpha to the labile bond to prevent the plasma esterases from approaching the scissile bond; for example, the introduction of the methyl group shown below in the case of an ester carbonyl makes introduction of the plasma esterases more difficult, through steric shielding, and slows the reaction.

Third, the introduction of electron withdrawing groups (delocalizing charge) to the carbonyl carbon will make the hydrolysis of the protein more difficult in the presence of bases, but if the binding site can tolerate the electronic change, it will not necessarily decrease protein binding. Fourth, by cyclizing, or by the introduction of rigid structures, the labile bond can be forced into an unfavourable conformation for the action of the enzyme. All of these modifications are empirical tests as the effects on binding cannot be predicted, but systematic exploration using the relationships of stability and structure in binding will help identify optimal compromises.

6.2. Prodrug design with intentional plasma lability and high protein binding

Prodrugs are deliberately designed to take advantage of plasma instability to obtain otherwise unattainable pharmacokinetic properties. The strategy involves the chemical modification of an active parent molecule to a poorly active or even inactive bioreversible derivative with better absorption, distribution or solubility properties than the parent. It is converted to the active parent in the systemic circulation by enzymes or chemical reactions. The optimum prodrug should have high plasma protein binding to prevent early hydrolysis, in order to reach the target site, but should be rapidly converted to its active form once the prodrug is present in plasma and is exposed to plasma esterases or other enzymes that can activate it.

Ester prodrugs of carboxylic acid containing drugs, like the angiotensin converting enzyme inhibitor enalapril, are designed to be highly bioavailable when taken orally as the carboxylic acid is poorly absorbed but the ester is highly absorbed and then rapidly converted back to the carboxylic acid by plasma esterases. In these situations, the half-life of the prodrug in circulation is sufficiently extended due to its high protein binding and the active metabolite can be bound differently, affecting its further distribution and elimination. Such systems take careful consideration of the stability of the prodrug and its binding to the target to obtain the desired conversion half-life, which is usually in the range of 15 minutes to 2 hours, to ensure sufficient absorption efficiency and rapid onset of action.

A decision flowchart has been created for medicinal chemists which includes target unbound fraction and plasma stability half-life thresholds and is used for a structured design process (Figure 3). Once the desired unbound exposure is established, by multiplying the target free concentration by the dosing interval, the chemist is able to find out whether a conventional compound that has a known clearance can provide the desired exposure. Two alternative approaches are considered if the calculated oral dose becomes too large to be considered developable. If the drug needs to be present in the plasma for a prolonged period of systemic action, the route with high binding and moderately stability is suggested ($fu<0.05$ and plasma half-life is 2-6 hours) which will not lead to degradation of the drug and will ensure sufficient free fraction in plasma.

The soft drug pathway is recommended for drugs which need to be cleared rapidly or have local action, where $fu>0.1$ and plasma half-life<1 hour. A decision node at the center determines if the compound can be altered to give one of these profiles, or neither of them, without creating other compounds that can degrade the product or make it react. The flowchart (Figure 3)leads the chemist around a series of structure modification and integrated stability binding studies, and provides a set of acceptance criteria to develop compounds for in vivo studies.

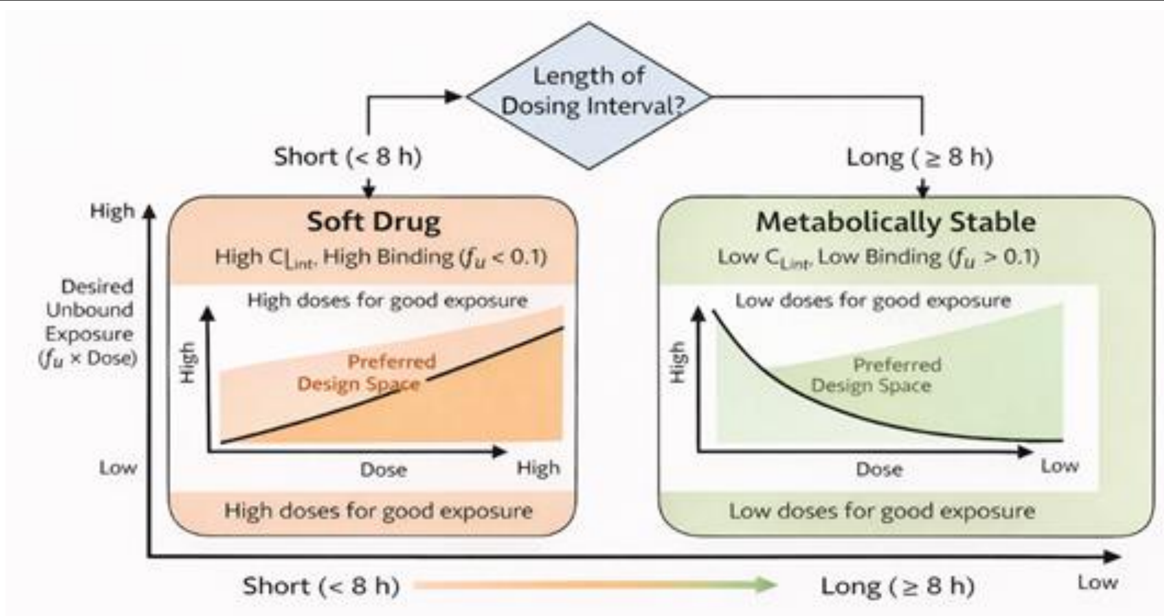

Figure 6.1: ***Drug design decision tree (stability vs. binding)***

6.3. Soft drugs designed for plasma instability to limit systemic exposure

The opposite of prodrugs are the soft drugs, deliberately labile compounds that are expected to be rapidly degraded in plasma after reaching the site of therapeutic action, resulting in reduced systemic exposure and hence off target toxicity. This approach can be especially useful for inhaled, topical, and intraocularly administered drugs, for which a high concentration is needed at the site of action but the systemic effects are minimized. The soft drug should be stable enough to be delivered to the site of action, yet labile enough so that any amount that is not first administered to the local site and would enter into the systemic circulation would be rapidly changed to inactive products. Examples of this approach include corticosteroid esters, such as loteprednol etabonate for ophthalmic use which are converted to an inactive carboxylic acid metabolite in less than thirty minutes in human plasma.

However, protein binding can have a paradoxical effect on soft drug design; high protein binding keeps the soft drug from degrading, but this is undesirable as it would extend systemic exposure. As a result, most soft drugs have moderate to low plasma protein binding which means that the free fraction is susceptible to enzyme degradation. The challenge in the design is to achieve local stability for efficacy as well as systemic lability, which must be optimized through careful structural activity relationships.

6.4. Case study one: Ester prodrugs with binding protecting active metabolites

The case of oseltamivir is a good example of how plasma protein binding of both the prodrug and active compound affects therapeutic results. The ethyl ester prodrug oseltamivir phosphate is absorbed well from the gastrointestinal tract, with ~ seventy five percent bioavailability, and is quickly metabolized to the active carboxylate prodrug, oseltamivir carboxylate, by carboxylesterase one. The prodrug has moderate plasma protein binding (about forty two percent), and the active metabolite has negligible plasma protein binding (less than three percent). This difference is of functional importance as the low binding enables rapid renal clearance of the active metabolite, which is desirable if the antiviral drug has to reach high concentrations in respiratory secretions but not in the systemic circulation for a long period of time. Highly bound metabolites would result in a lower volume of distribution and slower renal clearance which could be associated with accumulation or neuropsychiatric side

effects. The moderate binding is sufficient to protect the prodrug from hydrolysis by plasma esterases but is also adequate to ensure that hydrolysis of the prodrug can occur rapidly in the liver where the carboxylesterase is concentrated. This example shows that the interplay of binding stability for both the prodrug and its active species should be taken into account in the optimal design, not only the species administered.

6.5. Case study two: Antibody drug conjugates with linker stability and albumin hitchhiking

An emerging class of targeted therapies, antibody drug conjugates (ADCs) are comprised of a cytotoxic agent linked to a tumor-targeting antibody by a chemical linker. Ideal is the ability to be stable in systemic circulation and not release payload until in the tumor microenvironment or after being endocytosed by tumor cells, in order to avoid off target toxicity. Recently, the ability to extend the half-life of small molecule drugs and peptides has been investigated by attaching them to albumin binding domains, a strategy known as 'albumin hitchhiking' (Kulagowski et al., 2018). When considering antibody drug conjugates, the stability of the linker and its protein binding becomes of paramount importance: a highly stable linker that binds to albumin might afford the antibody some protection from enzymatic degradation and thus limit the release of the payload at the target tissue.

On the other hand, when the payload is attached to albumin following release, its distribution and toxicity profile could be different. One way to solve this dynamic is to develop linkers that are resistant to plasma esterases but are cleavable by tumor specific proteases, as well as to choose payloads that have little plasma protein binding so that they are quickly cleared from the systemic circulation after any premature release. The stability and binding of the antibody-drug conjugate, the linker, and the free payload component, therefore, need to be evaluated as an integrated part of the preclinical evaluation process of an ADC.

7. Integrating Into Pharmacokinetic Evaluation Workflows

7.1. Current industry practices and timing mismatches

An examination of the current practice in the pharmaceutical industry shows that studies of drug stability and plasma protein binding are generally carried out at different times in the course of drug discovery, by different scientists at different laboratories, with different protocols. Plasma stability screening is usually done early in lead optimization as a counter screen to look for compounds that degrade too quickly to be accepted for further development in humans, with a single time point at either thirty or sixty minutes, and 80 percent remaining as a pass/fail criterion. Protein binding determination is performed after the compound series have been shown to be reasonably stable and potent, but is not performed during the concurrent stability study, with a typical incubation time of 4–6 hours, and no concurrent stability monitoring.

The temporal separation also gives rise to a systematic bias: compounds that are moderately unstable that pass the early screening for stability may not be subjected to sufficient examination during binding experiments, which can result in the artifacts mentioned above. In addition, the half-life of the stability cutoff of 80% at 60 minutes is not long enough for accurate dialysis; dialysis will be significantly degraded in 4-6 hours of incubation. The mismatch in time also applies to in vitro to in vivo extrapolation, because plasma stability data collected from diluted plasma might not be representative of degradation in undiluted plasma containing intact protein binding.

7.2. Proposed integrated protocol with simultaneous stability and binding

Integrated protocol: stability, binding, same biological matrix, time course sampling – addresses the limitations of separate assays. In the present protocol, the test compound is added into undiluted human plasma and samples are taken from the plasma compartment and buffer compartment of a dialysis cell at various times after incubation from 0 to 6 h at 37°C. Analysis is performed by high performance liquid chromatography (HPLC) tandem mass spectrometry and a quantitative measurement of

the parent compound is carried out and, if possible, the identification of major degradation products.
The time course data can be used to calculate both the true unbound fraction, using extrapolation to time 0, and the degradation rate constant, from the slope of the decline in total concentration. These two parameters can be used to calculate the effective plasma half-life under physiological binding conditions as the intrinsic half-life divided by the unbound fraction, which would give a direct prediction of in vivo stability, taking into account the protection afforded by binding. The integrated protocol is about twice as complex as separate assays, but yields far more information that will enhance the confidence in and accuracy of subsequent pharmacokinetic predictions. If the goal for routine screening is just to have an acceptable accuracy while keeping throughput, a reduced version with three time points (zero, two and six hours) may be sufficient.
The stability and protein binding experiments were proposed with validation criteria listed in Table 3, for reliable interpretation of the measure of unbound fraction. The first is that the plasma half-life of the test compound should be equal or greater than the dialysis incubation period (8 hours or more for a typical 4-hour dialysis). If this is true, then degradation due to error is less than 10%, satisfactory for most drug discovery applications. If the half-life is in the range of 4-8 hours, the degradation error will be approximately ten to twenty-five percent and will have to be corrected by time zero extrapolation. If the half-life is below 4 hours, the degradation error is over twenty five percent and standard dialysis is not recommended, a different dialysis method (rapid ultrafiltration with stability monitoring) or equilibrium dialysis at 4°C followed by temperature correction may be utilized.
Secondary criteria include that >80% of the parent peak be present after dialysis (unless specifically designed as an active metabolite) and that no degradation peak be present in the sample unless it was intentionally used as an active metabolite, at levels >10% of the total peak area. The following criteria are suggested as a starting point for discussions within the industry and to provide guidance for regulators to draw regulatory conclusions, depending on the relationship between fu measurement error and clinical dose prediction for different therapeutic areas.

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Table 3: ***Validation criteria for combined assays***

Validation Parameter	Acceptance Criterion	Action / Interpretation
Drug stability during dialysis	≤ 20% degradation	If >20%, calculate kdeg and extrapolate fu to time zero
Degradation kinetics fit	R^2 ≥ 0.95 (first-order model)	If poor fit, repeat stability profiling with improved sampling
Mass balance recovery	85–115% total recovery	If outside range, evaluate adsorption losses and handling errors
Membrane non-specific binding (NSB)	≤ 10% loss	If >10%, use low-binding membranes or apply NSB correction
Equilibrium achievement	Final two timepoints within ±10%	If not achieved, extend dialysis duration or optimize conditions
Replicate precision	CV ≤ 15%	If CV >15%, repeat assay and improve experimental control
Temperature control	37 ± 0.5°C (or specified)	Repeat experiment if temperature deviates
pH stability	pH change ≤ 0.2 units	If exceeded, improve buffering system or reduce assay time

Validation Parameter	Acceptance Criterion	Action / Interpretation
Sample integrity	No hemolysis or turbidity	Exclude compromised samples and repeat with fresh plasma

7.3. Degradation factors for IVIVE, which were used for ex vivo sampling

Degradation of the sample during ex vivo handling and analysis can be significant and bias the clearance prediction from in vitro to in vivo. Even when using chilled tubes and rapid processing, some degree of ex vivo degradation is inevitable for highly labile compounds. This can be obtained by comparing the degradation rate observed in the processed plasma sample with the intrinsic degradation rate that is observed in plasma samples under conditions in which ex vivo degradation is reduced, such as immediate protein precipitation or the inclusion of an excess amount of esterase inhibitor. The correction factor correction is applied to the intrinsic clearance values fed to physiologically based pharmacokinetic model. In the case of compounds with half-lives of less than half an hour, however, the degradation in plasma can exceed 50% during the time between blood draw and plasma separation, making correction for ex vivo degradation difficult. If this is the case, use of whole blood stability tests and the inclusion of reversible esterase inhibitors in collection tubes e.g. sodium fluoride or paraoxon is recommended. The correction factor approach needs to be validated for every class of drug and matrix, due to the different activity of the residual plasma esterases, with different dilutions, temperature or presence of organic solvents used during protein precipitation.

8. Therapeutic Performance and Clinical Relevance

8.1. How integrated assessment improves prediction of drug drug interactions

It is important to recognise that the interactions of drugs with protein binding displacement have been exaggerated in the past, with many predicted interactions not actually occurring clinically because of compensating factors, including increased clearance of the unbound drug. But if degradation is present as well, the interaction landscape gets more complicated. To increase the degradation rate of a labile drug, it is necessary to increase the unbound fraction of the drug, which can be accomplished by using a displacer drug. The overall drug concentration change is the result of displacement and enhancement of the degradation reaction.

For highly bound labile drugs, the impact of displacement can be paradoxical, and may largely counterbalance the effect of increase in free concentration as predicted by integrated models that include binding displacement effects and degradation rates. In contrast, drugs that inhibit plasma esterases may increase the half-life of co administered ester containing drugs by an effect that is independent of protein binding displacement. Traditional study designs of drug drug interactions measure only total drug concentrations and assume drug clearance by cytochrome P450 (CYP). Integrated assessment prior to preclinical development can help identify drugs which are especially prone to such interactions, and inform the design of clinical studies for assessment of interactions at appropriate time points and with stability controls.

8.2. Impact on therapeutic window for unstable high binding drugs

Therapeutic window of a drug is the ratio of minimum effective concentration to minimum toxic concentration. The free concentration time profile can experience significantly more variation than the total concentration time profile, particularly when the drug is highly bound and is plasma labile, for the reasons that the free drug is most likely degraded, whereas the bound drug provides a 'protected pool'. The free concentration can be sufficiently high to be toxic if the dose is not carefully chosen at early times after dosing. In later times, free concentrations can become inadequate even though total concentrations

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appear to be sufficient because of bound but inactive drug. For instance, some ester-based local anesthetics have wide therapeutic ranges and yet are be limited by their clinical efficacy because peak free drug concentrations after rapid intravenous injection may lead to central nervous system and/or cardiovascular toxicity due to insufficient degradation to free drug. The ratio of peak to trough free concentrations is more accurately predict by integrated stability binding assessment than the ratio based on total concentrations, which is a better predictor of therapeutic window. Extended release or continuous infusion may be necessary to achieve safe and effective levels in compounds with a peak to trough free ratio >5.

Figure 4 shows the predicted free drug plasma concentration-time profile of a moderately unstable drug, with a true unbound fraction of zero point 02 and an intrinsic degradation half-life of two hours, in the absence of binding. The effects of three situations are compared for the next 24 hours after an injection. The black solid line represents the true free concentration profile that takes into account both plasma protein binding and plasma elimination by degradation; the effective plasma half-life is 2 hours/zero point zero two = one hundred hours, suggesting that plasma protein binding is a significant factor in protecting the free drug from degradation. Without considering degradation, the red dashed line represents the plasma profile expected to be observed if the drug is stable in plasma; this plasma profile suggests that plasma drug levels remain above the assumed therapeutic range of 1 nanogram per milliliter for the full 24 hours.

In the absence of a stability correction, the standard protein binding measurement would predict the profile shown in blue dotted lines with zero point zero eight as the fu – giving a predicted 2 hours / zero point zero eight = twenty five hours effective half-life. The corrected profile indicates that the concentration of the free species is below the therapeutic window after about 12 hours, while both the stable and uncorrected profile indicate once-daily dosing. This simulation shows that the stability binding interplay is ignored when the therapeutic coverage is overestimated and the possibility of underdosing arises, but the integrated correction does correct this.

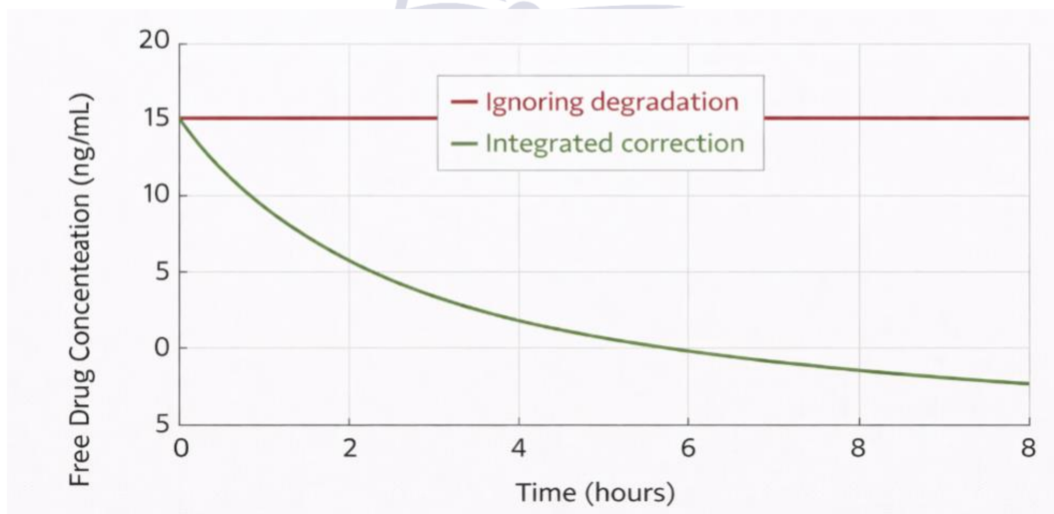

Figure 4: ***Free drug profiles with/without integration***

8.3. Special populations with altered stability and binding

Patients with altered plasma protein concentrations or enzyme activity should be considered with special attention to the stability binding interplay; protection provided by binding will be dependent on the concentration and the binding affinity of the primary binding proteins. Albumin binding of acidic drugs, which are mostly bound to albumin, is decreased by lowering of the

concentration of albumin in the plasma, as seen in liver disease, nephrotic syndrome, malnutrition, and critical illness. Hypoalbuminemia will increase the fu of a labile drug whose protection against degradation is dependent on binding to albumin and, as a result, increase the degradation rate and possibly shorten the effective half-life by the same amount.

However, during acute inflammation/infection, the alpha 1 acid glycoprotein levels increase, which increases the ability of basic drugs to bind to them and provide greater protection from degradation, resulting in a longer half-life. Integrated assessment is needed to predict overall free drug exposure in hepatic impairment because protein binding is reduced due to decreased albumin production, but metabolic stability is also decreased due to a decrease in metabolizing enzyme production. Renal impairment also affects binding because of the build up of uremic toxins which compete for binding sites; renal impairment may also affect the activity of plasma esterases by unknown mechanisms. For these special populations, drug plasma stability binding studies may help determine whether the drug needs to be dosed differently in these patient populations, as well as the design of clinical trials in these vulnerable populations.

9. Computational and In Silico Approaches

9.1. Quantitative structure activity relationship models for stability and binding

Modelling of plasma stability and plasma protein binding have been developed separately and have shown reasonable success; however no models with combined predictions of the interaction of plasma stability and plasma protein binding have been well developed. Different models employ different molecular descriptors, including lipophilicity, polar surface area, no. of rotatable bonds and the presence of specific functional groups which are prone to hydrolysis. The best models have a cross validated correlation coefficient between zero point seven and zero point eight for predicting the half-life within a factor of three for compounds in a narrow chemical series for plasma stability. Models based on lipophilicity or charge state are equally well or somewhat better for protein binding. When the compound is unstable and degrades during the binding assay, however, it will display different apparent binding data to the model than will its actual binding.

This will lead to systematic bias in training sets containing unstable compounds. Future combined model should involve descriptors related to both properties and should be trained on stability binding data from integrated stability binding and degradation experiments with known true fu and true degradation rate, respectively. These models might be used to determine structural features that allow high binding and good stability, with synthetic efforts focused on the most interesting parts of chemical space.

9.2. Physiologically based pharmacokinetic models with plasma degradation compartment

The typical physiologically based pharmacokinetic models describe the metabolism and renal elimination of drugs, assuming that the plasma is a distribution compartment, and not the site of elimination. For drugs that have significant plasma degradation, however, there should be a separate compartment representing first order plasma degradation as well. The extended model structure in Figure 5 includes a plasma degradation \clearance term (CL deg) which is equal to the degradation rate constant times the central compartment volume. This pathway is in addition to hepatic and renal clearance and its relative contribution to overall body clearance will be greater when degradation rate constant is higher and fu is lower (degradation of the bound drug is generally negligible). The implementation of plasma degradation in physiologically based pharmacokinetic models yields better prediction of free drug concentrations for labile drugs, especially at later time points when the drugs have been cleared from blood after hepatic and renal clearance. In vitro measurement of kdeg must be performed in undiluted plasma with intact protein binding (see seven), and scaling factors are needed to allow for the possibility of in vitro/in vivo differences in degradation rate resulting from flow or from the distribution of enzymes.

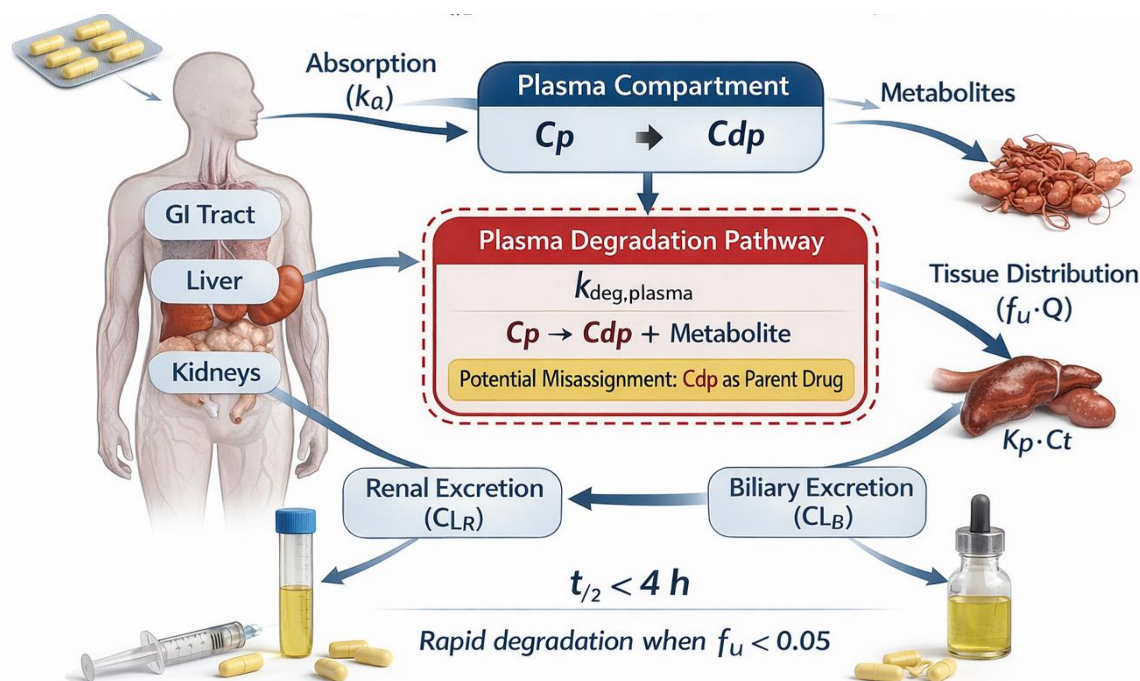

Figure 5: *PBPK model structure showing separate "plasma degradation" clearance pathway alongside hepatic and renal clearance*

9.3. Machine learning for predicting degradation binding interplay

However, machine learning methods have the promise of predicting coupled degradation and binding without explicating the underlying degradation mechanisms. Several prediction methods have been used to predict pharmacokinetic parameters from molecular structure, such as neural networks, random forests and gradient boosted trees, although the performance of each of these techniques has differed. The stability binding interplay is a very hard prediction to make because good quality integrated datasets reporting both parameters under controlled conditions are few. There are many measurements of stability and binding in public databases, e.g., ChEMBL and PubChem, but these are not typically the same experiment on the same sample, so they are of limited use for training interplay models.

The creation of such data sets for thousands of diverse compounds would be extremely costly, but would allow development of predictive models that would screen virtual libraries prior to synthesis. Near future solutions might include transfer learning approaches where large amounts of stability and binding data are first pre-trained and then fine tuned on a small amount of combined data. Machine learning models of the interplay could then be used in drug discovery pipelines to prioritize compounds predicted to have the right mix of stability and binding for specific therapeutic uses once synthesized.

10. Future Perspectives and Unanswered Questions

10.1. Need for standardized combined stability protein binding guidelines

There are no current regulatory guidance documents that call for integrated stability and protein binding evaluation of an investigational new drug (IND) application and there are no consensus guidelines in the industry to recommend best practices. The European Medicines Agency and Food and Drug Administration both give guidance on protein binding methodology but do not mention the degradation artifact or even call for stability

monitoring of the protein during binding experiments. The regulatory omission permits differences in practice between companies and academic laboratories, making it hard to compare the results of different studies and enabling compounds with unknown stability problems to progress to clinical trials.
To establish standard protocols for combined assessment, it is recommended to form a working group within IFP or AAPS, to be composed of experts, which should be able to set incubation times, criteria of stability during dialysis, requirements for reporting degradation products, and recommendations for corrective mathematical procedures. With such guidance, the quality of data could be enhanced, regulatory review would be easier and late stage failures due to uncharacterized stability binding interactions would be minimized.

10.2. Role of microsomal versus plasma esterases in tissue specific integration

For most drugs, the relative importance of plasma esterases compared to tissue-bound esterases in terms of their contribution to overall drug degradation is not well defined. In fact, microsomal esterases in the liver and the intestine have different substrate specificities and inhibitor sensitivities than plasma esterases and, as such, a compound can be stable in plasma and have a rapid metabolism in liver or the other way around. Tissue specific integration of degradation pathways involves stability measurements in subcellular fractions from several organs and physiologically based pharmacokinetic modeling to determine the relative contribution of each of these sites to the total clearance.
New data indicate that plasma degradation becomes less than a 20% of total clearance for many ester-containing drugs, and can become relatively more important for drugs in which the protein-bound fraction is very high (bound drug is unavailable for hepatic extraction). Future research should establish the esterase activity profiles of human plasma, liver, intestine, kidney and lung in a systematic manner by using standardized panels of substrates to provide a more accurate prediction of the specific tissue degradation of new chemical entities.

10.3. Nanocarriers and reversible binding in the context of stability

The use of nanocarrier drug delivery systems adds a new dimension to the stability binding interaction because the actual carrier can bind to plasma proteins to affect the circulation time and degradation profile. Surface modification of liposomes, polymeric nanoparticles and lipid nanoparticles for nucleic acid delivery can optimize the attachment of proteins that regulate the release of the drug; however, the correlation between the attachment of proteins to the carrier and the release of the drug remains unclear. A nanoparticle which is highly bound to albumin may have an extended circulation time; however, it is possible that the amount of free drug released from the carrier during the time may be significantly different from the expected profile from the carrier alone. Nanoparticles, which can be degraded by enzymes themselves, can also release the drug in a controlled fashion, depending on the binding state of the nanoparticles and the enzyme concentration. To direct rational design of these complex formulations, integrated experimental systems that probe the binding of nanoparticles, the release of drugs, and the degradation of drugs in plasma are necessary.

10.4. Real time monitoring technologies for simultaneous assessment

New analytical technologies represent the promise to enable real-time monitoring of drug stability and protein binding without taking discrete samples and conducting offline analysis. Surface plasmon resonance can allow for continuous monitoring of binding events, and can be modified to measure degradation by measuring changes in binding signal over time. Nuclear magnetic resonance spectroscopy can identify parent compound and degradation products of a drug without separation and can be used in conjunction with flow through the dialysis cells to keep track of both compartments. The process of microscale thermophoresis only uses nano-liter

sample volumes, and it is possible to measure binding affinity directly in plasma with little degradation artifact and minimal incubation time. Although these technologies do not yet meet the high throughput screening requirements of instrumentation cost and throughput, they provide extremely valuable tools for mechanistic studies and for validation of higher throughput methods. With the technologies developing and becoming more widely available, they could be used to replace traditional equilibrium dialysis for the unstable compounds and provide a true simultaneous measurement of binding and instability without artifacts that have long plagued the field.

11. Conclusion

This dimension of drug stability and plasma protein binding, which has been largely overlooked in the past, plays a pivotal role in drug failure in late stages and suboptimal dosage regimens and has been one of the most important causes of late-stage failures. We have shown that routinely using standard degradation/equilibrium dialysis protocols to assess these two parameters alone will give systematic errors when both properties are present in the same molecule—degradation during the equilibrium dialysis and binding protection from degradation that can increase the values for effective half-life by more than ten fold. Once this relationship is understood, it is easy to see how these parameters relate to each other mathematically; the implications for experimental design are deep. Conventional four to six hour dialysis without stability monitoring should not be relied upon for compounds with a plasma half-life less than 8 hours, since there will be no data on the stability during the dialysis period, which must be shortened or time course sampling with correction to time zero must be conducted.

For drug discovery teams, practical suggestions are the early adoption of integrated stability binding screening during the lead optimization phase, time course sampling of plasma, and the adoption of internal acceptance criteria (S2 ≥ 2*D). If a compound is suspected as being borderline stable, further characterization with rapid ultrafiltration or low temperature dialysis might be necessary. Integrated assessment is crucial for understanding the structure to binding to plasma proteins to degradation rate relationship of soft drugs and prodrugs deliberately designed to be labile in plasma.

Finally, we request regulatory guidance and standards that set forth stability requirements for protein binding and reporting of degradation product formation during protein binding experiments. Integrated assessment should be an integrated and routine preclinical parameter, alongside metabolic stability and permeability screening, embraced by the entire pharmaceutical sciences community. The fact that stability and binding are not independent but mechanically coupled, will enable us to achieve more accurate in vitro to in vivo extrapolation, avoid unexpected pharmacokinetic failures and provide better medicines to patients. The technology and the knowledge is available, and now it's just a matter of commitment to altering laboratory practices to more physiologically relevant integrated approaches

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