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METABOLISM KINETICS

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19.1 INTRODUCTION

It is common to apply kinetic models to *in vitro* data in an attempt to determine parameters, such as intrinsic clearance, that can be used to make correlations to the *in vivo* situation. These estimations are generally predicated on the data following a hyperbolic profile such that the Michaelis–Menten equation can be applied. This

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allows determination of V_m and K_m and thus the determination of intrinsic clearance. However, it is increasingly being recognized that, for many substrates, these "typical kinetic" conditions may not exist and "atypical kinetics" may be observed. Unfortunately, application of the Michaelis–Menten equation to these "atypical" situations results in misestimation of V_m and K_m and, subsequently, intrinsic clearance. Thus, it is important to understand the assumptions behind typical kinetics in drug metabolism reactions, the different types of atypical kinetic profiles that may also be observed, and what models and equations can be used to estimate kinetic parameters from each of these types of data.

19.2 TYPICAL KINETICS

19.2.1 Michaelis–Menten Equation and Its Use in Drug Metabolism

One of the most widely taught and used biochemical principles is embodied in an equation, which has remained the very foundation of enzymology for nearly a century. This equation allows quantitative descriptions of many enzyme-catalyzed reactions in terms of rate and concentration and therefore helps us describe the molecular workings of the cell. Enzymology has aided in the diagnoses of diseases as well as the development of therapies used to treat them, and at the heart of enzymology is the Michaelis–Menten equation:

$$v = \frac{V_m \cdot [S]}{K_m + [S]} \tag{19.1}$$

where reaction velocity, v, is described as a function of substrate concentration, [S], multiplied by a limiting rate, V_m (also known as V or V_{max} whose subscript is not to be confused with that of K_m), and divided by the sum of [S] and constant K_m, whose subscript refers to its name, the Michaelis constant.

Following up on the theory of A. J. Brown and V. Henri that enzymatic catalysis results from the formation of an intermediate enzyme–substrate complex, Michaelis and Menten reaffirmed Henri's basic assumption.[1] Mainly, the effect of substrate concentration on velocity could be described by an equilibrium between enzyme and substrate and that their complexation can lead to product.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \tag{19.2}$$

Derivation of the rate equation for Eq. 19.2 based on the initial concentration of enzyme and substrate, and the definition of the substrate dissociation constant, K_S, gives

$$v = \frac{k_2 \cdot E_0 \cdot [S]}{K_S + [S]} \tag{19.3}$$

[1]The following details were compiled largely from Cornish-Bowden [6], and for further details, consultation of this book is highly recommended.

where E_0 is the initial enzyme concentration. By avoiding any assumption about equilibrium in the first step of Eq. 19.2 (i.e., substrate binding to enzyme), Briggs and Haldane actually made the final contribution to the Michaelis–Menten equation with their steady-state treatment. Their steady-state assumption was that the concentration of the enzyme–substrate intermediate would become constant soon after initiation of the reaction. Using the same scheme (Eq. 19.2), K_S in Eq. 19.3 is substituted with the Michaelis constant:

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{19.4}$$

using the steady-state assumption as shown in the derivation available in most biochemistry textbooks. This is probably the preferred treatment unless specific details regarding substrate binding are known that enable the use of K_S. Whether K_m or K_S is used, the form of the equation is the same:

$$v = \frac{k_{cat} \cdot E_0 \cdot [S]}{K_m + [S]} \tag{19.5}$$

with the substitution of k_2 for k_{cat} (the observed rate[2] of reaction) so that no assumption is made about the number of steps that occur between substrate binding and the chemistry described by $k_{cat} \times E_0$. In other words, k_2 in Eq. 19.2 does not reflect the rate of catalysis except for the simplest two-step mechanism such that k_{cat} is used to represent the observed rate of reaction if there are more than two steps, even if the true number of steps is not known. For the same reason K_m is often more complex than shown in Eq. 19.4. E_0 and k_{cat} are then multiplied to produce the parameter V_m to yield the familiar Eq. 19.1.

Equation 19.2 does not consider the reversibility of the k_2 step, which would result in the subtraction of the reverse rate in the numerator ($k_P \times E_0 \times [P]$) and a term that includes a K_m for product P in the denominator. Even so, the Michaelis–Menten equation in its commonest form (Eq. 19.1) adequately describes the kinetics of metabolism for many drugs by enzymes like the cytochromes P450. Ideally, the rate of reaction at each substrate concentration would follow the method of *initial rates*, meaning several measurements over a limited amount of time (i.e., less than a few minutes) are subjected to linear regression to determine a rate. This prevents excessive amounts of product from building up and reversibly (or irreversibly) inhibiting the enzyme, or undergoing significant reversal back to substrate. However, this is often not feasible if time-expensive methods like liquid-chromatography separation or even extraction into organic solvent are needed to measure metabolized drug. In addition, even an incubation carried out with recombinant P450 is

[2] k_{cat}, the *catalytic constant* or *turnover number*, describes the number of turnovers an enzyme can accomplish in a given unit of time. Determination of k_{cat} requires accurately estimating V_m, therefore requiring the use of a sufficient range of substrate concentration to ensure saturation, and knowledge of E_0 ($k_{cat} = V_m/E_0$). V_m is determined preferably by nonlinear regression (although certain linear plots have been used for estimates). The initial enzyme concentration, E_0 or E_{total}, is in the same concentration units as V_m (e.g., μM/time) so that the unit of k_{cat} is time^{-1}.

much more complex than most may realize. Using the steady-state treatment, a P450 incubation may be thought of as possessing a K_m not only for drug, but for oxygen, P450 oxidoreductase (which has a K_m for NADPH), and possibly any other partially rate-limiting steps required for catalysis. Fortunately, most of these variables are kept constant and/or saturating. Considering the number of variables in P450–drug incubations and some of the difficulties involved in analyzing the products of such reactions, the Michaelis–Menten equation nevertheless remains remarkably useful.

Therefore, although proper controls must be carried out (see below), triplicate end-point measurements and their analysis with the Michaelis–Menten equation are probably the most fundamental and useful experiments in drug metabolism.

19.2.2 The Case of Multiple Substrates

Cofactors are heavily relied upon by drug metabolizing enzymes. The UDP-glucuronosyltransferases (UGTs), glutathione *S*-transferases (GSTs), flavin monooxygenases (FMOs), sulfotransferases (more correctly, sulfonyltransferases), *N*-acetyltransferases (NATs), and thiopurine *S*-methyltransferase (TPMT) all utilize nucleotide-containing cofactors for oxygen activation (e.g., FMO) or for conjugation (e.g., all others). FMOs can remain poised for oxidation without drug substrate ever binding as long as saturating levels of NADPH are present. UGTs, on the other hand, require a ternary complex of enzyme and two substrates (UDP-glucuronic acid and the aglycone substrate to be conjugated) to form before catalysis can occur.

Unfortunately, the number of parameters in rate equations expands substantially when describing models involving additional reaction steps. For instance, take the example of the kinetic mechanism of UGT that generally occurs through the ordered binding of two cosubstrates [1]. If the reverse reaction is ignored and any amount of product formed during the course of the reaction is considered negligible, the rate equation requires concentration information and estimation of K_m values for both substrates:

$$v = \frac{V_m[A][B]}{K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]} \tag{19.6}$$

where A and B represent the two substrates each with their own K_m (K_{mA} and K_{mB}) and K_{iA} is the dissociation constant for the EA complex. Even with the aforementioned assumption, a substantial number of data points would be needed to fit this rate equation due to the number of parameters involved. However, if substrate B is always present in saturating concentrations and care is taken to avoid the buildup of products, Eq. 19.6 *reduces eventually to the Michaelis–Menten equation* (Eq. 19.1). In fact, this experimental and analytical simplification is probably the standard and preferred method for determining V_m as well as the K_m for each substrate. With further analysis this method also has the benefit of revealing features of the kinetic mechanism such as whether substrate binding is random or ordered, or if the first substrate is turned over and leaves before the second substrate can be turned over (i.e., Ping-Pong mechanism) [2].

19.2.3 Use of the Michaelis–Menten Equation and Its Parameters

Most are familiar with the rectangular hyperbola that results from plotting reaction velocity (y-axis) versus substrate concentration (x-axis) and that these plotted data can be fit to the Michaelis–Menten equation using nonlinear regression (although the curve itself was never used by Michaelis and Menten and therefore is *not* called a Michaelis–Menten curve). Therefore, the parameters V_m and K_m, as best fit by the nonlinear version of the equation (Eq. 19.1), are reviewed.

At low [S], Eq. 19.1 demonstrates that velocity becomes proportional to [S]:

$$v \approx \frac{k_{cat} \cdot E_0 \cdot [S]}{K_m} = \frac{V \cdot [S]}{K_m} \tag{19.7}$$

The middle expression of Eq. 19.7 is important because a very useful parameter is obtained from the ratio of k_{cat}/K_m known as the *specificity constant.* k_{cat}/K_m (units of time^{-1} × concentration^{-1}) is an excellent evaluation of substrate specificity for an enzyme. Imagine two substrates for the same enzyme that can each be turned over without the other present. The substrate with the highest specificity constant will correctly predict that it will be turned over faster than the other substrate if both were present at any equimolar concentration. Even if one substrate has a larger k_{cat}, a substrate with a higher k_{cat}/K_m will be turned over faster if the two are compared at the same concentration.

Low substrate concentrations (i.e., much lower than K_m) are also relevant to *in vivo–in vitro* correlations. If the therapeutic dosage of a drug results in sub-K_m plasma concentrations (the most common situation), where kinetics are largely first order for the enzyme(s) metabolizing it, Eq. 19.7 can be defined as the intrinsic clearance ($CL_{int} = V_m/K_m$, units time^{-1}), which is the clearance of drug from a tissue via metabolism independent of blood flow, protein binding, and other restrictions. Although the units are different from that for k_{cat}/K_m, V_m/K_m is still a specificity constant and the comparison of CL_{int} values for different drugs with the same enzyme may prove useful in predicting the reduced CL_{int} for *each* drug involved in a drug–drug interaction or polypharmacy.

Next, when the substrate concentration is equal to K_m, K_m can be defined operationally as the *concentration of substrate where velocity equals* $\frac{1}{2}V_m$. Often, K_m in this sense is used as a measure of the affinity of substrate for enzyme as if they were in equilibrium (K_S); however, without additional information regarding rate constants, K_m should never be assumed to be anything more than an estimate of K_S with no guarantees attached. Finally, when substrate is said to be saturating so that every enzyme molecule is bound to substrate, K_m becomes negligible and $v \approx V_m$. In other words, the rate is limited by the amount of enzyme present, and hence, V_m is perhaps better described as a limiting rate rather than a maximum.

19.3 ATYPICAL KINETICS DISPLAYED BY DRUG METABOLIZING ENZYMES

Atypical kinetics is a term that has come to be used to describe any *in vitro* drug metabolism kinetics that do not fit the hyperbolic function seen when velocity is

plotted versus substrate concentration. *Allosterism* is another term used frequently in the P450 literature that refers to the ability of a ligand (a.k.a. effector) other than the substrate being turned over to bind in a distinct, noncatalytic site on an enzyme and affect the rate of metabolism of the substrate. The effector can be a second (or third or fourth, etc.) substrate molecule binding to the same enzyme or it can be a molecule that is structurally dissimilar from the substrate. Several lines of evidence suggest that some of the allosteric sites in P450s 3A4 and 2C9 are really distinct sites within the same binding pocket that substrate occupies; however, we think allosterism is still a suitable term to define this phenomenon since the P450s most likely do not bind every substrate in the same orientation or subregion of their active sites anyway.

Much attention has been given to the atypical kinetics of P450s in drug metabolism, but it is becoming apparent that phase II enzymes and drug transporters may occasionally display atypical kinetics likely stemming from the simultaneous occupancy of a single protein by multiple ligand (in addition to the normal substrates, if there are multiple substrates required for catalysis). In fact, almost all of the theoretical mechanisms that could give rise to atypical kinetics are plausible with drug metabolizing enzymes whether it occurs via one substrate (*homotropic*) or combinations of substrate and effector (*heterotropic*). Using P450s as an example, it is completely reasonable to hypothesize atypical kinetics arise from effector binding that modifies P450–P450, P450–P450 reductase, or P450–cytochrome-b_5 interactions, where the site can be in the same binding pocket as substrate or remote and be either homotropic or heterotropic.

19.3.1 Sigmoidal Kinetics with a Single Substrate

Sigmoidicity in substrate–velocity plots usually brings to mind cooperativity, which is generally thought of as arising from multimeric enzymes, like the textbook example of hemoglobin. Here, the sites where substrate binds are equivalent on each enzyme so cooperativity, not allosterism (e.g., effect of BPG on O_2 binding by hemoglobin), is used to describe the kinetic phenomenon. Evidence, however, now suggests P450s may display homotropic activation (or homotropic cooperativity), where a monomeric enzyme displays sigmoidal kinetics (Fig. 19.1) presumably through substrate binding to both a catalytic site and an effector site [3–5]. In any case, a useful expression called the Hill equation (Eq. 19.8) is adequate for describing many cases of this type of kinetics with just a few parameters:

$$v = \frac{V_m[S]^n}{K^n + [S]^n} \tag{19.8}$$

where V_m and K are analogous to the parameters in the Michaelis–Menten equation (but the parameter[3] K is *not* called the Michaelis constant) and n is the Hill coefficient. The Hill equation is empirical with respect to the parameter n because while

[3]It is not essential that K be to the power n except that since n is often not a whole integer, K as the substrate concentration that gives the half-maximal velocity will have the same units as [S], but not be directly relatable to it.

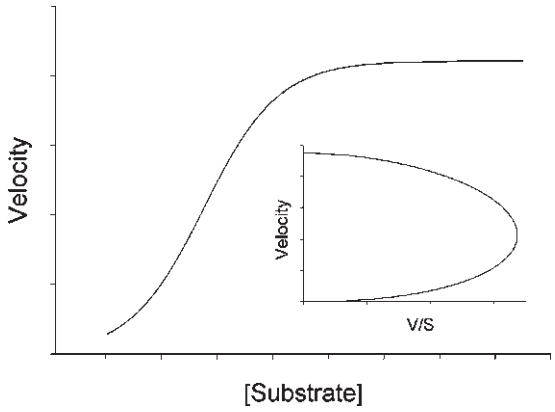

FIGURE 19.1 Graphical representation of a kinetic profile exhibiting sigmoidal (autoactivation) kinetics. Inset demonstrates the Eadie–Hofstee plot of a sigmoidal kinetic profile.

it measures relative cooperativity, it does not necessarily equal the number of binding sites for substrate because it is often not an integer. A more biochemically logical description of multiple binding sites is obtained with the use of the Adair equation (described in Cornish-Bowden [6]); however, the parameters of the Hill equation remain practical for most applications, including the study of drug metabolizing enzymes. For example, substrates (e.g., testosterone, carbamazepine, pyrene) that induce sigmoidal kinetics by CYP3A4 can all be meaningfully compared to each other using the Hill equation to determine which ones have the highest efficiency (V_m/K) and cooperativity (n).

When cooperativity is caused by an effector other than the target substrate in a monomeric enzyme (a.k.a. *heterotropic activation*) [7, 8], for instance, during inhibition screening, the Hill equation can still be used. Or, to better define the V_m and K_m parameters for substrate when it is known that the effector turnover rate is insignificant or much less than it is for substrate, Eq. 19.9 may prove useful by giving K_m and V_m in the absence or presence of effector (subscripts 1 and 2, respectively):

$$v = \frac{\dfrac{V_{m1}\cdot[S]}{K_{m1}} + \dfrac{V_{m2}\cdot[S]^2}{K_{m1}\cdot K_{m2}}}{1 + \dfrac{[S]}{K_{m1}} + \dfrac{[S]^2}{K_{m1}\cdot K_{m2}}} \tag{19.9}$$

Equation 19.9, although working under the assumption of equilibrium, will also give the K_m and V_m of a substrate metabolized in two distinct subsites within the substrate binding pocket, each with different binding affinities and velocities. More complex rate equations have been developed for sigmoidal kinetics in drug metabolism and more rare mechanisms behind cooperativity can be found in Cornish-Bowden [6], and these sources should be consulted for a full description.

19.3.2 Biphasic Kinetics (Nonasymptotic)

As defined here, a biphasic kinetic profile does not follow saturation kinetics and has two distinct phases. (*Note that sigmoidal kinetics may also be biphasic but exhibits saturation.*) At low substrate concentrations, the kinetic profile exhibits curvature similar to that observed with hyperbolic kinetics; however, at high substrate concentrations, the velocity of the reaction continues to increase in a linear fashion (Fig. 19.2), as opposed to becoming asymptotic as would be expected with hyperbolic kinetics. This type of profile most commonly occurs when the metabolism of a substrate is carried out by more than one enzyme in a multienzyme system. However, when using a single, purified enzyme source, it is most likely the result of substrate binding in a productive orientation to more than one region within the active site, but these binding regions exhibit different affinities for the substrate and also differential turnover rates. In this case, a single enzyme behaves as if it were a multienzyme system. The enzyme thus exhibits a low K_m/low V_m component (responsible for the semihyperbolic nature of the profile) and another high K_m/high V_m component (producing the linear portion of the profile). In order to model this type of kinetic profile, one must use equations that describe the kinetics of one substrate interacting with two binding sites within the same enzyme species, such as Eq. 19.10.

$$v = \frac{(V_{m1} \cdot [S]) + (CL_{int} \cdot [S]^2)}{K_{m1} + [S]} \tag{19.10}$$

The parameters V_{m1} and K_{m1} are estimated from data representing the curved portion of the plot at lower substrate concentrations and are the estimates of V_m and K_m for the low V_m, low K_m site, respectively. CL_{int} describes the linear portion of the plot exhibited at higher substrate concentrations and is the ratio of V_{max2}/K_{m2} (i.e., the high V_{max}, high K_m component of the profile). Because this upper portion of the plot is linear, one cannot estimate the actual V_m and K_m parameters for this portion of the profile since saturation is not achieved. Thus, CL_{int} is the slope (rate) for this linear portion of the kinetic profile.

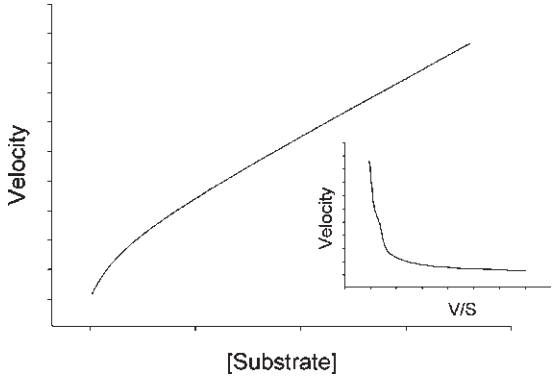

FIGURE 19.2 Graphical representation of a kinetic profile exhibiting biphasic kinetics. Inset demonstrates the Eadie–Hofstee plot of a biphasic kinetic profile.

19.3.3 Hyperbolic (Nonessential) Activation

The most straightforward kind of activation in terms of fitting K_m and V_m values to velocity versus [S] plots is probably hyperbolic activation. The name refers to the replot of K_m/V_m against reciprocal activator concentration, which is hyperbolic, not linear. (These plots for sigmoidal and biphasic kinetics are also not expected to be linear.) It is nonessential, because the effector is not necessary for catalysis to occur. While V_m and often K_m change for a substrate in the presence of an activating effector, the shape of the velocity vesus [S] curve remains a hyperbola. Therefore, the term hyperbolic activation has a more important meaning in drug metabolism kinetics because it can also refer to homo- or heterotropic activation that results in hyperbolic curves rather than sigmoidal or biphasic curves. As one might expect, changes in K_m and V_m can be fit at each activator concentration using the Michaelis–Menten equation. One of the best documented cases of this heterotropic activation is the activation of flurbiprofen (S) hydroxylation by the effector dapsone (B) in P450 2C9 and a mechanistic scheme has been proposed (Fig. 19.3). A graphical representation of the kinetic profile for this hyperbolic activation is shown in Fig. 19.4.

The equation (Eq. 19.11) for fitting multiple curves representing different activator concentrations has a manageable number of parameters including the usual K_m (which is assumed to be an estimate of K_S in this equilibrium model) and V_m for substrate in the absence of activator:

$$v = \frac{V_m \cdot [S]}{K_m \dfrac{1+[B]/K_B}{1+\beta[B]/\alpha K_B} + [S]\dfrac{1+[B]/\alpha K_B}{1+\beta[B]/\alpha K_B}} \tag{19.11}$$

where [B] is the concentration of activator, α is the factor by which K_S for substrate changes, and β can be thought of as the factor by which V_m changes (see Fig. 19.3 for further explanation of parameters).

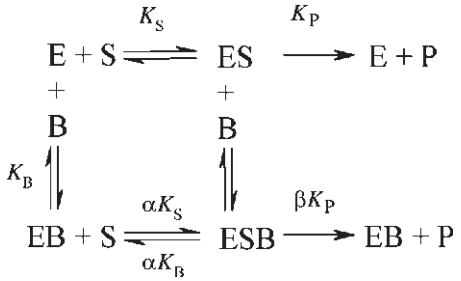

FIGURE 19.3 Kinetic mechanism for nonessential activation [2] demonstrates that the effector, B, is not essential for product formation. Furthermore, it can be seen that this scheme is flexible as α and β can be greater or less than unity. In the case of flurbiprofen, metabolism in the presence of effector dapsone results in a decrease in α, indicating an *increase* in affinity of P450 2C9 for flurbiprofen. This was further supported by equilibrium binding experiments. Parameter β was increased, indicating the velocity for oxidation of flurbiprofen has increased. Activation could also be achieved with $\alpha > 1$ but this would require higher substrate concentrations to be observed. Alternatively, if $\alpha < 1$, but $\beta < 1$, activation could result at low substrate concentrations and then result in inhibition at higher substrate concentrations.

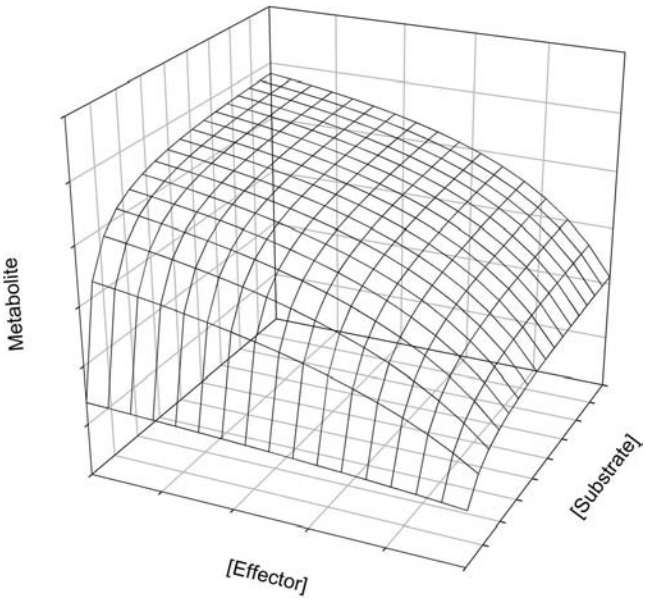

FIGURE 19.4 Graphical representation of a kinetic profile exhibiting heterotropic cooperativity (activation).

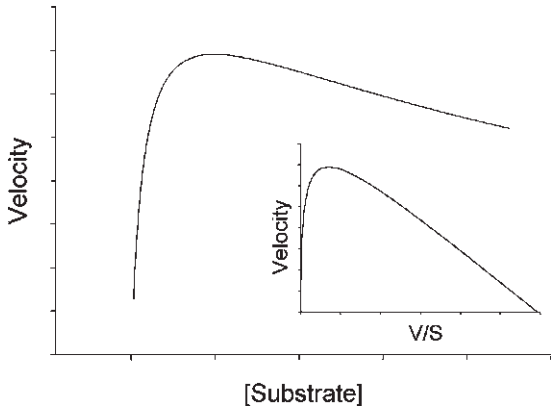

FIGURE 19.5 Graphical representation of a kinetic profile exhibiting substrate inhibition kinetics. Inset demonstrates the Eadie–Hofstee plot of a substrate inhibition kinetic profile.

19.3.4 Substrate Inhibition

In the case of substrate inhibition kinetics, as the concentration of substrate is increased, to determine V_m an inflection point in the velocity versus [S] curve occurs so that rather than plateau, the velocity starts to decrease and continues to do so as [S] is raised further (Fig. 19.5). Assuming the reactions were carried out appropriately (see Section 19.3.6), two likely scenarios arise when substrate inhibition is noted:

1. If the experiment is conducted with P450, the most likely cause is analogous to uncompetitive inhibition where two or more ligands (in this case, two substrate molecules) binding to the enzyme decrease its ability to catalyze substrate oxidation.
2. If the experiment is conducted with UGT, inhibition is likely the result of aglycone substrate binding right after a glucuronide conjugate is released from a previous round of catalysis, thereby trapping UDP and forming an inactive ternary complex. For many UGTs there appears to be a preference to bind UDP-glucuronic acid first, followed by aglycone, suggesting an ordered ternary complex mechanism.

In either case, K_m and V_m can be estimated via use of the appropriate equation. It should be noted, however, that the derived parameters (e.g., V_m and K_m) are not truly equivalent to the usual situation. Because V_m is never truly reached (or to simulate it unless the inhibition is very weak), the true K_m is also not discernible. In the past, researchers have incorrectly fit these types of data by either eliminating the points showing decreasing velocity or simply applying the Michaelis–Menten equation to the entire dataset. Lin and colleagues [9] have demonstrated the necessity to fit the entire dataset to the proper equation and the hazards of not doing so. For substrate inhibition occurring through promiscuous binding of a second substrate (case 1), the following rate equation (Eq. 19.12) is useful. It is derived from the uncompetitive inhibition model using an extra substrate molecule instead of inhibitor, therefore leading to a squared term in the denominator when multiplied out:

$$v = \frac{V_m[S]}{K_m + [S] + \dfrac{[S]^2}{K_{si}}} \tag{9.12}$$

However, in the course of kinetic analyses one may wish to gain additional information from the available kinetic data, allowing for estimation of additional kinetic parameters that may provide more insight into the processes taking place. Thus, more complicated equations for kinetic modeling of substrate inhibition kinetics have been derived (e.g., Eq. 19.13) that allow for the estimation of interaction factors and additional kinetic parameters.

$$v = \frac{V_m\left(\dfrac{1}{K_S} + \dfrac{\beta \cdot [S]}{\alpha K_i K_S}\right)}{\dfrac{1}{[S]} + \dfrac{1}{K_S} + \dfrac{1}{K_i} + \dfrac{[S]}{\alpha K_S K_i}} \tag{9.13}$$

In the case of Eq. 19.13, $K_S \approx K_m$, K_i is the dissociation constant of substrate binding to the inhibitory site, α is the factor by which the dissociation (K_S and K_i) of substrate at both sites changes when a second substrate is bound, and β is the factor by which V_m changes when a second substrate is bound. Because of the number of parameters being estimated in Eq. 19.13, modeling with this

equation will generally require substantially more data points than required for Eq. 19.12.

For substrate inhibition mechanisms involving ordered ternary complex, such as the UGTs (case 2), Eq. 19.14 is applicable:

$$v = \frac{V_m[A][B]}{K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]\left(1 + \frac{[B]}{K_{SiB}}\right)} \tag{19.14}$$

where K_{SiB} is *not* a dissocation constant.

19.3.5 Explanations for Atypical Kinetics in P450 Enzymes and Their Detection

Reports on the actual mechanism(s) behind the activation kinetics of drug metabolizing enzymes (mainly P450) are few compared to the kinetic models that have been devised to fit nonhyperbolic velocity data [10–12]. The approaches that have been taken to understand the interplay of substrate, effector, and enzyme are, however, diverse and include isotope effects, fluorescence spectroscopy [13], and nuclear magnetic resonance [14, 15] in addition to assays for distinct steps in the P450 catalytic cycle [16]. One of the most influential hypotheses behind the action of effectors that prompted the use of these varied techniques is that the effector binding site could be located in the active site with the substrate. Over the past few years evidence for simultaneous ligand binding to the same active site has now been provided for at least two microsomal enzymes, human CYP3A4 and CYP2C9 , and three bacterial enzymes, CYP102A1 (BM3) , CYP107A1 (eryF) [17], and CYP158A2 [18] as discussed below. Of course, allosterism can result from binding at remote sites or through protein–protein interactions.

Presence of Multiple Ligands Temporally Bound in the Same Active Site In the case of 3A4, the substrate pyrene could be used to demonstrate multiple ligand binding to the same enzyme active site during catalysis. Pyrene induces a sigmoidal kinetic response with increasing concentrations, and its planarity, coupled with its multiring fused aromatic structure, gives it a large surface for intermolecular pyrene–pyrene pi-stacking (a.k.a. excimer formation). After showing binding of pyrene to 3A4 by visible difference spectrophotometry and changes in fluorescence of pyrene and the enzyme, differences in the excitation spectra (absorbance of monomer and excimer in the ground state) gave multiple indications that there are excimers in the presence of enzyme distinct from those that form in solution. Crystal structures of 3A4 do demonstrate active volumes sufficiently large enough (>1000 Å) for multiple ligands in their closed conformations [19]. Some investigators have even suggested triply occupied active sites [20], but defining the nature of atypical kinetics in terms of different subsites still remains a challenge due to the dependency on the structure of both substrate and effector.

Shortly after the 3A4/pyrene report, a series of isotope effect experiments with P450 BM3 suggested that multiple fatty acids could bind to the same enzyme active site simultaneously. This explained why the F87A mutant produced a different metabolic profile of hydroxylated products in the presence of laurate. Mutation of

F87 to alanine results in an increase in hydroxylation of the ω-1 position as laurate concentrations are raised. Since the alanine substitution gives a smaller side chain, and therefore more space, it is unclear why the ratio of metabolites would change unless the presence of a second substrate or the collapse of the enzyme constricts the available volume. This is where isotope effects appear to be capable of detecting multiple ligand binding without the need for fluorescent molecules. The theory is that given two enzyme-bound ligands in rapid equilibrium, mixing protio substrate (all hydrogens) and substrate substituted with deuteriums (the deuterium–carbon bond possesses a lower vibrational energy and requires more energy for abstraction) at the site of metabolism will favor metabolism of the nonlabeled substrate. Hence, a bias in the rate of labeled and un labeled substrate metabolism is produced and it favors the hydrogen-containing substrate (i.e., k_H/k_D ratio is greater than unity). In this case, the bias for ω-1 hydroxylation of palmitate was increased even more when deuterated laurate was added. Further validation of this technique is awaited for its use as a powerful tool with possibly untapped potential.

There are now crystal structures for two of the three major families of human microsomal P450s, 2 and 3 [21], but none have captured multiple ligands in the same pocket. However, crystallography has provided convincing electron density for multiple ligand binding in the same pocket for two bacterial enzymes. While not exactly a high throughput technology, even with the use of robotics, crystallography has generated structures that are invaluable for the predictive modeling of drug–drug interactions. P450 eryF in complex with two molecules of 9-aminophenanthrene and, more recently, P450 158A2 with two molecules of flaviolin have been reported. Both structures show one ligand above the heme with the second ligand lying above the first. Interestingly, both ligands are aromatic and planar, possibly owing some of their binding to each other. As of now, there are no examples with two different ligands bound, nor does a single structure indicate the order of their binding or the extent at which they move about inside the enzyme.

With the help of crystallography, nuclear magnetic resonance spectroscopy (NMR) has the capability to provide more details about the sequence and location of binding without the need for assigning every resonance. Yoon et al. [15] have reported on the use of protein NMR spectroscopy to study the binding of 9-aminophenanthrene to P450 eryF using enzyme expressed with ^{15}N-phenylalanine so that all phenylalanine residues would have detectable amide nitrogen nuclei. Titration of 9-aminophenanthrene to levels that are substoichiometric to enzyme altered the signal intensity for Phe residues that are different from those whose intensity changed in the presence of higher levels of 9-aminophenanthrene ([substrate] > [enzyme]). Further arguments regarding the time scale of on and off rates and their interpretation in terms of the mechanism of cooperative binding were also drawn from this study. Surely, the ability to distinguish two binding events at different locations inside the enzyme will greatly complement other analytical methods, and studies with human P450s will no doubt bring a more detailed understanding to drug–drug interactions induced by atypical kinetics.

An additional use of NMR involves the enhanced relaxation rate of substrate nuclei near the iron of the heme, which is paramagnetic in the ferric state [22]. Using P450 2C9, Hummel et al. [14] studied the proton relaxation times of the substrate flurbiprofen in the absence and presence of heteroactivator dapsone. Time-averaged distances of each substrate proton can be estimated by carrying out a T_1 relaxation

experiment both with the ferric enzyme and the ferrous carbon monoxide complex, which is not paramagnetic. T_1 refers to the longitudinal relaxation and refers to the time it takes for the irradiated substrate protons to recess back to alignment with the magnetic field. The site of hydroxylation (4 -proton) was found to be closer to the iron of the heme group in the presence of heteroactivator, suggesting that its hyperbolic activation may result from increased collision frequency of substrate with the reactive iron-oxene or enhanced spin state conversion via heme dehydration.

Another dimension to the action of dapsone on flurbiprofen metabolism by P450 2C9 was provided by a study that compared the coupling of NADPH consumption to metabolite formation versus shunting to the side products superoxide, H_2O_2, and water. Catalytic coupling can be defined as the fraction of metabolite formed per NADPH consumed and is thus a measure of enzyme efficiency distinct from k_{cat}/K_m. In addition to increasing the rate of flurbiprofen metabolite formation, the effector dapsone was found to stimulate the consumption of NADPH, but decrease the formation of H_2O_2. Overall, the coupling of flurbiprofen metabolism was increased. Therefore, the distance of a substrate relative to the heme iron as altered by effectors appears to have multiple consequences as shown for P450 2C9. Possible effector-mediated changes in the position of substrate could displace the water that occupies the sixth coordination position of the iron to increase its reduction potential, keep the substrate closer to the active oxidant, and therefore decrease uncoupling of the catalytic cycle.

19.3.6 Artifactual Sources of Atypical Kinetics

During the conduct of *in vitro* drug metabolism experiments, it is prudent to be alert for the possible occurrence of atypical kinetics. However, the researcher must also be aware that there are several potential artifactual causes of atypical kinetic profiles that may lead to a misintepretation of the data. Thus, extreme care must be taken to assure the validity of the results. The comments below address several of these potential sources of error and how to minimize their impact on the kinetic profiles observed.

One of the fundamental assumptions when conducting enzyme kinetic experiments is that the substrate (and effector concentration if drug–drug interactions are being studied) remains constant throughout the experiment. For substrates and effectors that exhibit substantial turnover, one must worry about substrate/effector depletion. This will result in constantly changing substrate/effector concentrations throughout the experiment and invalidate the assumption of steady state. Related to constantly changing (and decreasing) drug concentrations are potential sources of error when the actual substrate/effector concentration is lower than believed. This can occur if either compound undergoes substantial nonspecific binding to the incubation matrix (i.e., protein) or when the drug has very low solubility. In these cases, the free drug concentration (i.e., that available to interact with the enzyme) is substantially lower than believed, resulting in overestimates of kinetic parameters such as K_m, and K_i.

Although less of an issue in today's environment of widespread LC/MS/MS availability, insufficient analytical sensitivity to accurately and reproducibly quantitate metabolite production at the extreme low end of the substrate concentration–

velocity curve may result in erroneous assignment of the kinetic profile. This is most problematic in the case of sigmoidal kinetics, where inadequate sensitivity hinders determining the less steep slope during the initial portion of the curve. One must also be cautious when using multienzyme systems (e.g., human liver microsomes) for kinetic characterization since multiple enzymes are present. This is particularly problematic with respect to observations of biphasic kinetics, since more than one enzyme may be producing the metabolite. In this case, the kinetic phenomena observed are actually a hybrid of the concerted effects of multiple enzymes and result in *apparent* kinetic constants.

Finally, the kinetic profile observed may be affected by incubation conditions and preparation. Choice of buffer salt to be used in the incubation can result in differing kinetic profiles [23] and the presence of certain organic solvents (particularly at concentrations above 2%) have also been shown to activate enzyme kinetic processes [24]. Finally, the inclusion (or exclusion) of cytochrome-b_5 can result in different kinetic profiles observed [25]. Thus, one must pay particular attention to controls and conditions when making the assignment of atypical kinetic phenomena.

19.4 CONCLUSION

Proper estimation of kinetic parameters is critical to successful extrapolation of *in vitro* data to the *in vivo* situation. In addition to standard hyperbolic kinetics, sigmoidal (autoactivation), biphasic, substrate inhibition, and heterotropic activation have all been observed in the kinetic profiles of drug metabolism reactions. The most common hypothesis for the occurrence of these types of "atypical" kinetic profiles is based on data suggesting that two (or more) substrate molecules (or a substrate and effector molecule) bind within the enzyme active site simultaneously and alter the enzyme kinetics. The occurrence of these atypical kinetic profiles complicates the determination of *in vitro–in vivo* correlations and thus requires the use of more complex equations appropriate to the type of profile observed in order to properly estimate the kinetic parameters.

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20

DRUG CLEARANCE

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Contents

20.1 INTRODUCTION

Drug discovery and development is a complex process involving various sequential stages broadly grouped as preclinical and clinical evaluation. Both phases require the characterization of the pharmacokinetics of the molecule being evaluated as a

Preclinical Development Handbook: ADME and Biopharmaceutical Properties,
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potential drug candidate. In drug discovery and development, it is of particular interest to understand the pharmacokinetic behavior of potential drug candidates as early as possible in order to select the most promising compounds for further development while not wasting unnecessary resources on those compounds that will not prove to be viable for clinical use. While many pharmacokinetic processes contribute to the viability of drug candidates, clearance processes arguably have the greatest impact on determining whether or not a compound possesses the pharmacokinetic characteristics amenable to further development of the candidate. The clearance of a drug candidate is frequently the primary factor in determining the bioavailability, dosing frequency, duration of action, and viable routes of administration for the clinical use of the compound. Thus, the rate and routes of drug clearance can be critical in determining whether or not the development of a candidate compound should move forward. It is therefore imperative that development scientists possess an understanding of the determinants and characterization of drug clearance. Our objective in this chapter is to provide an overview of clearance concepts, ranging from a description of the routes by which drugs are cleared from the body to pathophysiological conditions that may alter these processes qualitatively and quantitatively. In subsequent chapters, authors will describe the importance of drug clearance in the overall context of pharmacokinetic analysis.

20.2 ROUTES OF DRUG ELIMINATION

Avoidance of accumulation of drugs subsequent to repeated exposure necessitates varied pathways for elimination of such compounds from the body. The rate of elimination will be a key determinant of the time for which critical body sites are exposed to effective concentrations of a drug. Hence, the duration of pharmacologic effect *in vivo* is generally determined by the rate of elimination. The routes of elimination are important in determining the likelihood of altered drug disposition in various disease states, as well as the potential for drug–drug interactions. Based on the anticipated patient population for a new chemical entity, it may be preferable to have a drug that is primarily eliminated by the liver, as opposed to the renal route, as the primary pathway of elimination. The importance of such factors in assessment of compounds under development makes it imperative that development scientists possess a good understanding of the potential pathways and means for quantifying drug elimination.

20.2.1 Potential Routes of Elimination

Mechanisms of drug elimination are largely determined by the physiochemical characteristics of the ingested compounds. Small water-soluble compounds are readily eliminated unchanged by the kidney, while large molecules and lipophilic agents must undergo biotransformation in the liver prior to being excreted by the kidney. While generally quantitatively minor, elimination by alternative routes may be of clinical/practical importance.

Renal Elimination It may be argued that essentially all drugs are eliminated to some degree via the renal route. While large lipophilic molecules are not directly

eliminated by the kidney, their biotransformation products (metabolites) are generally more water soluble and subject to renal elimination. This reality is important to recognize when considering the potential impact of renal disease on drug elimination. While the elimination of a lipophilic or large molecular weight parent compound may not be altered in the presence of renal disease, the elimination of its metabolites may be altered significantly. If those metabolites exhibit pharmacologic or toxicologic effects, renal disease may alter patient response, despite the lack of alterations in the disposition of the parent compound. As it represents one of the two most important pathways of drug elimination, renal elimination will be considered in further detail in a subsequent section.

Hepatic Elimination The liver is an organ of critical importance for drug elimination. This is in part due to the fact that disease- and drug-induced alterations in metabolic capacity can have such a profound impact on the ability of the liver to metabolize drugs. It is, however, important to recognize that the liver also eliminates numerous drugs unchanged through its capacity for biliary excretion. As one of the two major organs for drug elimination (the other being the kidney), concepts of hepatic clearance are covered in further depth in a subsequent section.

Pulmonary Elimination As the general population is aware of the use of ethanol measurements in exhaled breath to approximate blood ethanol concentrations, the ability to put pulmonary excretion of xenobiotics to practical use is obvious. While pulmonary elimination is a minor route for ethanol elimination, the constant ratio of alveolar to blood ethanol concentration permits extrapolation of the latter from the former. The number of compounds for which the pulmonary route is quantitatively significant in terms of the overall elimination of the drug is small and mostly limited to gaseous anesthetics. The ability to measure $^{13}CO_2$ or $^{14}CO_2$ in excreted breath has been utilized as an indirect means of measuring drug elimination for compounds that undergo oxidative metabolism in the liver. Several such tests are commercially available as a means of phenotyping the metabolic capacity of patients via specific cytochrome P450s (e.g., CYP3A4). These tests are dependent on the one-carbon oxidation that gives rise to CO_2, which is then excreted in exhaled breath.

Other Routes of Elimination While also quantitatively minor, additional routes of elimination (e.g., tears, sweat, hair) may be of practical or clinical significance. For example, while the fraction of a dose of drug that is excreted in tears is quite small, it may have very important implications for patients. Rifampin is a compound that is eliminated in tears and is capable of staining contacts a yellow-orange color when worn by patients during therapy with this drug. Another pathway of elimination that is of minor quantitative importance is hair. However, measurement of drug concentration in hair permits assessment of drug exposure over a longer time period than possible with blood or urine. This fact is applied in the numerous commercially available kits to measure drugs of abuse in hair samples. Its noninvasive nature makes this means of exposure assessment attractive.

20.2.2 Characterization of Elimination

There are two primary ways in which drug elimination may be quantified. The first is through estimation of the *half-life* of a drug in some reference fluid (e.g., plasma).

The half-life represents the time required for 50% of the drug to be eliminated from the body. For drugs that are eliminated in a first-order fashion, this time is independent of dose or concentration. However, since many drugs are eliminated by processes that involve the direct interaction of drug with a macromolecule (e.g., an enzyme or transporter protein), large doses will result in saturation of enzymatic or transport process and give rise to altered half-lives (compared to that observed with lower doses). This is important to keep in mind in preclinical studies where large doses may be utilized. The importance of dose-dependent pharmacokinetics and the application in toxicokinetics is discussed in other chapters (see Chapters 31 and 36 in this volume).

The most useful parameter for quantifying drug elimination *in vivo* is *clearance*. Assessment of elimination using clearance is preferred over that of half-life, since the latter is a function of both clearance and distribution (see Chapters 31 and 36 in this volume). For this reason, half-life fails to provide insight into the mechanism of drug elimination and cannot be used to predict the likely impact of disease or drug interactions on drug elimination. Clearance is defined as *the volume of blood from which all of the drug would appear to be removed per unit time*. As such, it is expressed in terms of volume per unit time and may be related to real physiological volumes, such as hepatic blood flow or renal plasma flow.

An understanding of the concept of clearance can be gained by consideration of the perfusion of an isolated organ with blood containing drug (Fig. 20.1). The rate of drug entry into and out of the organ can be described as

$$\text{Rate in} = QC_a$$
$$\text{Rate out} = QC_v$$

where Q is the organ perfusion rate, C_a is the concentration of drug in arterial blood, and C_v is the concentration of drug in venous blood. Whenever $C_v < C_a$, elimination of drug occurs in the organ and it is referred to as a clearing organ. The rate of elimination can be determined as

$$\text{Rate of elimination} = QC_a - QC_v = Q(C_a - C_v)$$

A common means for describing the efficiency by which a single organ removes a drug from blood is the *extraction ratio (E)*, where

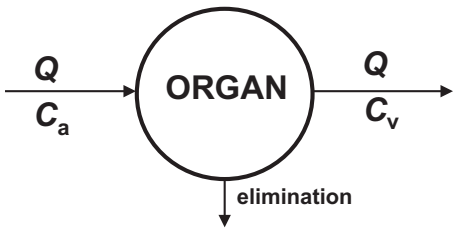

FIGURE 20.1 Schematic representation of a clearing organ. C_a designates arterial drug concentration, C_v is the venous drug concentration, and Q represents the blood flow to the organ. This schematic assumes that blood flow across the organ does not change significantly.

$$E = \frac{\text{Rate of elimination}}{\text{Rate of entry}}$$

$$E = \frac{Q(C_a - C_v)}{QC_a} = \frac{C_a - C_v}{C_a}$$

The clearance (CL) of the organ for a specific compound can be given as

$$CL = QE = Q\left(\frac{C_a - C_v}{C_a}\right)$$

The above equation reveals two potential extremes for CL. The first is when $E \rightarrow 1$, under which conditions $CL \sim Q$. Hence, for a drug that displays a high extraction ratio, the clearance is said to be *perfusion rate limited*; meaning that the rate-limiting step in the clearance is the delivery of drug to the clearing organ. Under such conditions, changes in organ perfusion rate will result in proportional changes in drug clearance. In contrast, when E is very low, changes in perfusion have little impact on drug clearance.

From the above equations, one can readily develop the means to calculate the total body clearance for a drug, CL_T, where

$$CL_T = \frac{\text{Elimination rate}}{\text{Concentration in blood}} = \frac{dX/dt}{C}$$

$$CL_T = \frac{\int_0^\infty (dX/dt)dt}{\int_0^\infty C\,dt}$$

where

$$\int_0^\infty \frac{dX}{dt}dt = \text{Total amount of drug eliminated}$$

and

$$\int_0^\infty C\,dt = AUC_0^\infty$$

where AUC is the area under the drug concentration versus time curve, X is the amount of drug in the body, and t is time. When the drug is administered intravenously, 100% of the drug enters the systemic circulation. Therefore, after intravenous administration, the total amount of drug ultimately eliminated is equal to the intravenous dose (D_{IV}). Hence,

$$CL_T = \frac{D_{IV}}{AUC_0^\infty}$$

From the above equation, it can be seen that the total clearance (sometimes also referred to as the *systemic* clearance) can readily be determined after intravenous

(IV) administration of a drug. The total clearance can be determined after non-intravenous routes of administration by accounting for the fraction of drug that reaches the systemic circulation (described in more detail in Chapter 36 in this volume).

An important principle related to the use and determination of organ-specific clearance is the additivity of clearance. Specifically, the total clearance is the sum of the individual organ clearance such that, for example,

$$\text{Total clearance} = \text{Renal clearance} + \text{Hepatic clearance}$$

This additivity arises from the fact that organs of elimination are fractionally perfused in parallel. An exception to this principle is pulmonary clearance, which arises as a result of the fact that the entire cardiac output traverses the pulmonary bed prior to being fractionally distributed to other organs of elimination (e.g., liver and kidney).

20.3 RENAL CLEARANCE

20.3.1 Basic Kidney Structure and Function

The most important functions of the kidney include regulation of body water content, mineral composition, and acidity. The kidney also plays an important role in the removal of endogenous metabolic waste products and xenobiotics and their metabolites from the body. Indeed, the major organ in mammals for the clearance of drugs and their metabolites is the kidney. Humans have two kidneys located in the upper rear region of the abdominal cavity. The functional units of kidney are called nephrons and each kidney is composed of over one million nephrons. Figure 20.2 illustrates different components of the nephron.

Each nephron consists of vascular and tubular components. The glomerulus is made up of a network of glomerular capillaries and Bowman's capsule, which is a

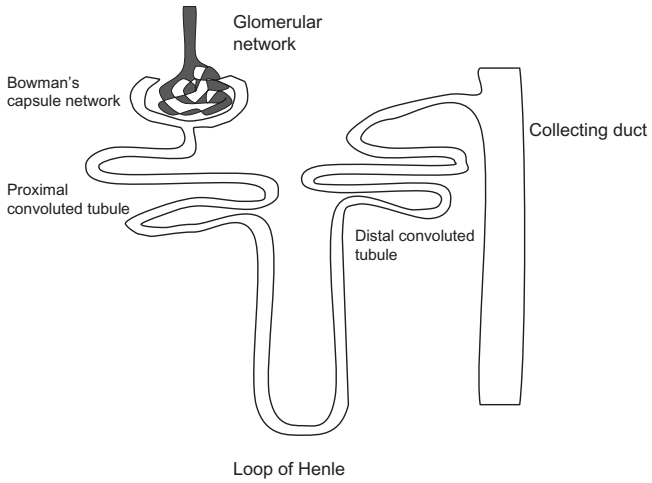

FIGURE 20.2 Components of the renal nephron.

thin double membrane that surrounds the glomerulus and functions as a filter. Blood is filtered at the glomerulus so that only protein-free filtrate is allowed to pass into the nephron. The resulting glomerular filtrate travels through the proximal convoluted tubule, where approximately 80% of the water is reabsorbed. The loop of Henle connects the proximal convoluted tubule to the distal convoluted tubule. A large collecting duct collects kidney filtrate from the distal convoluted tubules of each nephron and carries it to the bladder.

The kidney can be divided into two portions histologically: the outer renal cortex and the inner renal medulla. The renal cortex contains the glomeruli, proximal and distal convoluted tubules, the outer portion of the loop of Henle, and collecting ducts, whereas the renal medulla contains the lower ends of the loop of Henle and collecting ducts.

20.3.2 Mechanisms of Renal Clearance

The kidney excretes endogenous and xenobiotic compounds and their metabolites by three distinct mechanisms: glomerular filtration, tubular secretion, and tubular reabsorption. Glomerular filtration and tubular secretion remove drugs from the circulation, whereas tubular reabsorption can be considered as a redistribution mechanism by which drug moves from the inner tubule back into the circulation. While drugs may also be metabolized in the kidney, the quantitative contribution of this route is insignificant in terms of the overall clearance of drugs.

Glomerular Filtration Glomerular filtration is a passive process. In this process drugs and other endogenous compounds of molecular weight less than 6 kDa are filtered effectively. Drug molecules that are bound to plasma proteins, such as albumin, will not be filtered. In other words, only unbound drug in plasma water is available for glomerular filtration. The glomerular filtration rate (GFR) can be determined using marker compounds that undergo filtration only (i.e., do not undergo secretion or reabsorption) and are not bound to plasma proteins. The renal clearance of such compounds is equal to the GFR. The most common marker compounds used to measure GFR are inulin (MW 5200) and creatinine (MW 131). The rate of filtration of a drug can be given as

$$\text{Rate of filtration} = GFR \times C_{\text{fup}}$$

where C_{fup} is the concentration of free (unbound) drug in plasma. Furthermore, since $C_{\text{fup}} = f_{\text{up}} \times C$,

$$\text{Rate of filtration} = f_{\text{up}} \times GFR \times C$$

If drug is only filtered and all of the drug filtered is excreted in the urine, the rate of drug excretion will be equal to the rate of filtration. Hence

$$CL_{\text{R}} = \frac{\text{Rate of excretion}}{\text{Plasma concentration}} = \frac{f_{\text{up}} \times GFR \times C}{C} = f_{\text{up}} \times GFR$$

Thus the renal clearance of a compound that is only filtered can be determined based on a knowledge of the protein binding and the GFR (as approximated by inulin or creatinine).

The glomerular filtration rate varies from individual to individual, but in healthy subjects ranges from 110 to 130 mL/min (~180 L/day). While single-point determinations of GFR are often used to estimate drug clearance, it should be recognized that GFR is subject to circadian variation. GFR is highest in the active phase (morning) and lowest in the inactive phase (evening), with an amplitude of variation of about 20–30% [1, 2].

Tubular Secretion The kidney receives about 10–25% of the cardiac output, or about 700–1500 mL/min, assuming a cardiac output of 6000 mL/min [3], of which 60% will be the plasma flow. Approximately 18% of the effective plasma flow is filtered at the glomeruli. The remaining renal blood bathing the tubules permits access to the secretory processes in these tubules.

When the renal clearance of a drug exceeds $f_{up} \times GFR$, the drug must undergo active tubular secretion. There are two distinct systems involved in tubular secretion: one for organic anions (acids) and one for organic cation (bases). These systems are made up of multiple transporters. Both the anion and cation transport systems are located in the proximal convoluted tubule of the nephron. The most important transporters from a drug interaction perspective are the organic anion transporters. This process is thought to be localized primarily at the basolateral membrane of the proximal convoluted tubule. The transport of the organic anions across this membrane occurs through a three-step process (Fig. 20.3a).

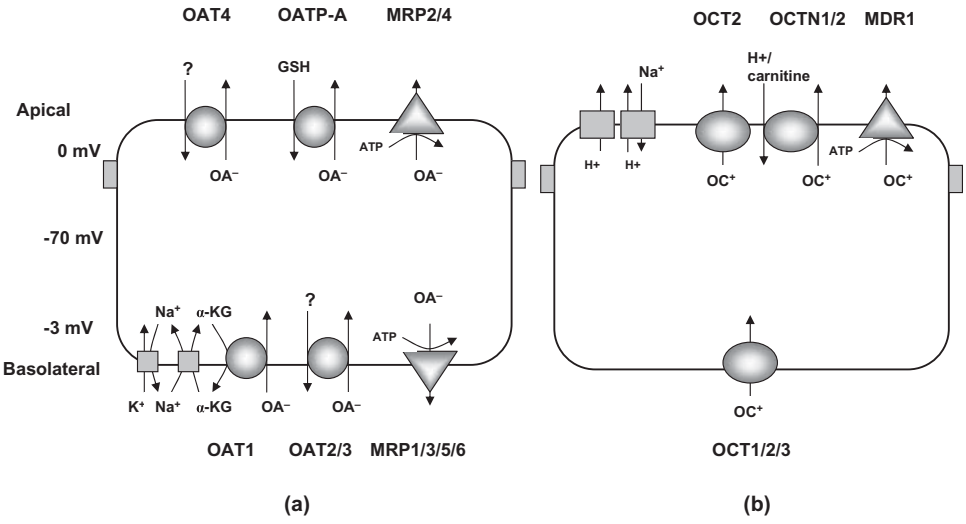

FIGURE 20.3 Functional and proposed models of organic anion (a) and cation (b) transporters in renal tubules. OA^-, organic anion; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OC^+, organic cation; OCT, OCTN, organic cation transporter; MRP, multidrug resistance associated protein; MDR, multidrug resistance P-glycoprotein. (Adapted from Ref. 19.)

The organic anion transport system (OAT1) is dependent on a cell membrane Na/K-ATPase that maintains the intracellular electrochemical gradient. Initially, ATP is hydrolyzed to drive the sodium pump. In the second step, the resulting Na^+ gradient drives α-ketogluterate (α-KG) cotransport across the basolateral membrane. The third step in this process is the exchange of α-KG with the organic anion (OA^-), as α-KG markedly stimulates the transport of organic anions. Once inside the proximal tubule cell, the organic acid is eliminated on the luminal side of the cell by a carrier-mediated transport system [4–6].

At present, it is difficult to predict whether a new drug candidate will interact with renal tubular secretion mechanisms based on structure–activity relationships alone. However, certain generalizations can be made based on transport studies using analogue compounds with different characteristics. In general, substrates with two negative charges will interact with anion transport systems, which are influenced by the molecular distance between the charges. Substances that interact with the organic anion transport system must have a hydrophobic area and two partial negative charges, a hydrophobic area and one negative ionic charge, or a large hydrophobic area and a partial negative charge [3].

Comparatively less is known about the organic cation transport system. This is partly due to the toxic side effects of the marker compounds on the cardiovascular system that could be used to study the transport process. As the organic anion transporters are responsible for the tubular secretion of organic acids, the organic cation transport systems are responsible for weak bases; often nitrogen-containing compounds that are ionized at physiological pH. Similar to anion transport, the organic cation transport also proceeds in three steps (Fig. 20.3b). In contrast to filtration, protein-bound toxicants are available for active transport. Binding to plasma proteins usually does not hinder tubular secretion of drugs because of the dynamic equilibrium that exists between free and bound drug. As free drug is removed and transported across the tubular epithelium, immediate dissociation of the drug–protein complex usually occurs [7, 8].

Since tubular secretion is an active process, there may be competitive inhibition of the secretion of one compound by coadministration of a second compound secreted via the same transporter. This can actually be taken advantage of clinically to prolong the systemic exposure to a drug readily secreted by the kidney. For example, probenecid inhibits the secretion of penicillin and coadministration of these two agents can be used to decrease the dose and frequency of penicillin that needs to be administered.

Tubular Reabsorption When the $CL_R < f_{up} \times GFR$, it is apparent that a portion of the filtered drug must undergo reabsorption. Most of the filtrate that enters the renal tubules is reabsorbed, with urine output accounting for less than 1% of the filtrate fraction that enters the tubules. This creates a large concentration gradient across the tubule and plasma, representing a driving force for passive reabsorption of solutes back into the circulation. Small molecular weight substances that are sufficiently lipophilic and un-ionized will be reabsorbed. In contrast, compounds that have large molecular weight are polar or ionized will not be reabsorbed. As many drugs are either weak bases or acids, the pH of the glomerular filtrate can greatly influence the extent of tubular reabsorption. Even small changes in pH of filtrate, either because of diet or drugs, can result in significant variations in the percentage

of drug reabsorbed or excreted. For example, the pK_a of phenobarbital (a weak acid) is 7.2 with an ionic strength equal to plasma. At a plasma pH of 7.4, the drug exists in ionized form and at pH values less than 7.2 the un-ionized form of the drug dominates and will be excreted. In the case of a drug overdose, it is possible to increase the excretion of some drugs by suitable adjustment of urine pH.

20.3.3 Determination of Renal Clearance and Mechanisms of Renal Clearance

General Model for Renal Clearance The total renal clearance of a drug is the sum of all the renal elimination mechanisms involved in the elimination of that specific drug:

$$CL_R = CL_{Fil} + CL_{ATS} - CL_{TR}$$

where CL_{Fil} is the filtration clearance, CL_{ATS} is the active tubular secretion clearance, and CL_{TR} is the reabsorption clearance [3, 9]. As indicated previously, the filtration clearance of a drug is given as $f_{up} \times GFR$, while the active tubular secretion clearance can be described as

$$CL_{ATS} = \frac{Q_{RPF} f_{up} CL_{usint}}{Q_{RPF} + f_{up} CL_{usint}}$$

where Q_{RPF} is renal plasma flow and CL_{usint} is secretory clearance of unbound drug. Since the amount of drug reabsorbed represents a fraction of that which is filtered and secreted (F_{TR}), the renal clearance can be expressed as

$$CL_R = f_{up} GFR + \frac{Q_{RPF} f_{up} CL_{usint}}{Q_{RPF} + f_{up} CL_{usint}} - F_{TR}\left(f_{up} GFR + \frac{Q_{RPF} f_{up} CL_{usint}}{Q_{RPF} + f_{up} CL_{usint}} \right)$$

The above equation represents the complex relationship between plasma protein binding, renal plasma flow, glomerular filtration rate, and intrinsic secretory activity. More complex models that take into account urine flow, pH gradient, and transport saturation are needed to fully account for the many physiological variables governing renal clearance. While this complexity may leave the reader with the impression that determination and assessment of renal clearance is hopelessly complex, such is not the case. Simple measurement of renal clearance can provide valuable insight into mechanisms when compared with the renal clearance of marker compounds, as described in subsequent sections.

Determination of Renal Clearance from Urine and Plasma Concentrations There are numerous methods available for calculation of renal clearance from measurements of plasma and urine drug concentrations. One common method is based on knowledge of the additivity of clearance. After a single intravenous dose of a drug, the fraction eliminated renally (f_R) is given as

$$f_R = \frac{Ae^{\infty}}{D_{IV}}$$

where Ae^{∞} is the total amount of drug excreted unchanged in urine. Determination of this value necessitates the collection of urine until all the drug has been eliminated from the body. If blood samples have also been obtained so that total clearance can be calculated as described previously, the renal clearance is determined as

$$CL_R = f_R \times CL_T$$

Alternatively, using the same data, one can calculate renal clearance from the relationship

$$CL_R = \frac{Ae^{\infty}}{AUC_0^{\infty}}$$

Renal clearance can also be calculated using excretion rate determinations rather than total drug excretion, where

$$CL_R = \frac{\Delta Ae/\Delta t}{C_{mid}}$$

C_{mid} represents the plasma concentration at the midpoint of the urine collection, ΔAe is the amount of drug excreted in urine unchanged over the collection interval, and Δt is the collection interval. As the renal clearance may vary somewhat between collection intervals, the best way to utilize this method is to determine the excretion rate over several different intervals. This can then be plotted against the midpoint concentration for each excretion rate. The result will provide a straight line with a slope equal to the renal clearance. Alternatively, if the drug is administered as a constant infusion until steady state is achieved,

$$CL_R = \frac{(\Delta Ae/\Delta t)_{ss}}{C_{ss}}$$

Assessment of Mechanism(s) of Renal Elimination from Renal Clearance Determinations Renal clearance estimates are commonly used to determine the mechanism of renal elimination of compounds. If the renal clearance is approximately equal to $f_{up} \times$ GFR, filtration is the predominant mechanism. If renal clearance is less than $f_{up} \times GFR$, tubular reabsorption must occur, while a renal clearance greater than $f_{up} \times GFR$ indicates that tubular secretion is occurring in addition to filtration. Since renal clearance is the sum of all renal elimination mechanisms, a renal clearance equal to $f_{up} \times GFR$ does not rule out other mechanisms of renal elimination, as it is possible that secretion and reabsorption occur to an equal extent and thus negate one another.

As indicated previously, the most commonly used markers for estimating GFR are inulin and endogenous creatinine [1]. Inulin is one of the most accurate methods to estimate GFR. However, determination of inulin clearance is not practical for routine clinical use as it requires the infusion of an exogenous substance. In contrast, estimation of GFR via creatinine does not involve the administration of an additional substance.

Experimentally, concurrent determination of the renal clearance of a drug of interest and the clearance of inulin can be used to probe the mechanisms of renal elimination for the compound of interest. Once the renal clearance values are determined, the ratio of drug clearance to inulin clearance is determined, as shown in Table 20.1. Adjustments for plasma protein binding may need to be taken into account when using this method for elucidating the mechanisms of drug elimination.

As a means of more routine estimation of GFR, clearance of the endogenous marker creatinine can be determined by

$$CL_{cr} = \frac{U_{cr} \times V}{S_{cr}}$$

where U_{cr} is the urine creatinine concentration, V is the urine flow rate, and S_{cr} is the serum creatinine concentration sampled at the midpoint of the urinary collection period.

Creatinine clearance is often considered as an ideal method to assess GFR because (1) daily creatinine production is constant, (2) serum and urinary creatinine concentrations are easily measured in the laboratory, and (3) by making the urinary collection period sufficiently long, problems associated with short collection intervals can be avoided [10, 11]. Although the results obtained by creatinine clearance estimates correlate well with estimates of GFR, some deviation from GFR is to be expected, especially at very high and very low GFR values [12]. For most applications of GFR estimated through creatinine clearance, this deviation is insignificant. Creatinine clearance can also be estimated through use of S_{cr}, body weight, height, and sex. Such estimates are particularly useful for determining initial dosage regimens for drugs in a clinical setting.

A drug that is highly extracted by the kidney, meaning it undergoes extensive active tubular secretion, will exhibit a renal clearance that approximates renal plasma flow. A widely used model compound that exhibits these characteristics is *p*-aminohippuric acid (PAH). PAH has been widely used to determine the impact of interventions of renal plasma flow and to compare the renal clearance of a compound to the renal plasma flow in the same subjects.

TABLE 20.1 Use of Clearance Ratios to Determine Mechanisms of Renal Clearance

Clearance Ratio	Probable Mechanisms
$\frac{CL_{drug}}{CL_{inulin}} < 1$	Filtration and reabsorption
$\frac{CL_{drug}}{CL_{inulin}} = 1$	Filtration
$\frac{CL_{drug}}{CL_{inulin}} > 1$	Filtration and active tubular secretion

While a renal clearance less than $f_{up} \times GFR$ is indicative of tubular reabsorption, confirmation of this mechanism can be obtained by perturbations in urine flow and pH. As the concentration gradient is the driving force for reabsorption, increasing urine flow in the presence of reabsorptive mechanisms will result in an increase in the renal clearance. While pH manipulations may alter the renal tubular reabsorption of some drugs, drugs whose ionization does not change within the range of physiologically achievable urine pH (~5–8) will not exhibit an altered renal clearance despite changes in urine pH. Hence, the observation of pH-dependent renal clearance for ionizable compounds confirms the presence of tubular reabsorption, but such changes will not be expected for neutral compounds.

20.3.4 Factors Influencing Renal Clearance

Factors that influence the ability of the kidney to eliminate drugs may cause marked changes in the pharmacokinetics of some compounds. Such factors include age, disease, plasma protein binding, drug concentration or dose, and drug interactions at the site of elimination.

Age Many functions of the kidney are not fully developed at birth. As a result, some xenobiotics are eliminated more slowly in newborns than in adults. Similarly, the aging kidney exhibits reductions both in mass and in GFR. These changes may be associated with other age-related conditions, such as atherosclerosis and hypertension, which reduce renal blood flow. An age-dependent decline in the number of nephrons also occurs. The decline in GFR is in turn responsible for the reduced renal clearance of drugs that are normally removed by the kidney. With age the capacity to excrete acid load is diminished independent of decreased GFR. The diminished capacity of older subjects to excrete acid load could be due to an insufficient decrease in the tubular reabsorption.

Drug–Protein Binding The degree of plasma protein binding affects the filtration of drugs, as protein–xenobiotic complexes are too large to pass through the pores of the glomeruli. Whether or not changes in plasma protein binding alter renal clearance is dependent on the renal extraction ratio for the drug in question. For example, furosemide exhibits a low extraction ratio and its renal clearance changes proportionally to the free fraction of drug in plasma [13]. In contrast, the renal clearance of drugs that exhibit a high renal extraction ratio, and are therefore extensively secreted, is not significantly altered by changes in protein binding. Binding to plasma proteins usually does not hinder tubular secretion of drugs because of the rapid equilibrium that exists between free and bound drug. As free drug is removed and transported across the tubular epithelium, immediate dissociation of the drug–protein complex usually occurs—freeing more drug for secretion.

Concentration or Dose As tubular secretion involves the interaction of drug with transport carriers, which are limited in number, there is the potential for saturation of these membrane transport proteins as the dose or concentration of drug is increased. In the absence of saturation, the renal clearance of a drug will be independent of dose or concentration. However, when doses are administered that give rise to concentrations that saturate the transporters, the renal clearance will decrease.

Similarly, if the tubular reabsorption is by a carrier-mediated transport process (as is the case for ascorbic acid), increasing concentrations will saturate the carriers, resulting in an *increase* in the renal clearance (since the fraction reabsorbed will be reduced and more drug will be excreted in the urine). Such saturation has practical implications for the dosing of compounds that display saturation within the range of therapeutic concentrations. It is also important for the development scientist to keep the potential for such dose- or concentration-dependent changes in mind when utilizing high doses in preclinical studies.

A limited number of drugs exhibit concentration-dependent plasma protein binding at concentrations in the range achieved after therapeutic doses. An increase in the free fraction of a drug as the dose/concentration is increased will result in an increase in the filtration clearance (since this value is equal to $f_{up} \times GFR$). In this scenario, increasing the dose of a drug may fail to give rise to the expected increases in steady-state concentration since clearance is increased with dose. Several nonsteroidal anti-inflammatory drugs, such as naproxen, exhibit this type of dose/concentration-dependent changes in renal clearance.

Drug Interactions The most well known drug–drug interactions that occur at the level of renal elimination are those in which one drug acts as a competitive inhibitor for another drug at a renal transport site. Competitive inhibitors will decrease the renal secretion of other substrates, but will not impact the filtration or extent of reabsorption that occurs passively. Such inhibition will result in a decrease in the clearance of a drug, which in turn increases the steady-state plasma concentration and results in a prolonged half-life of the drug.

Other sites of drug–drug interaction, though less common, can also affect the renal elimination of drugs. For example, antacids can alter urine pH and change the fraction of an ionizable drug that undergoes tubular reabsorption. Drugs that inhibit the renal metabolism of drugs represent another mechanism of potential interaction.

20.4 HEPATIC CLEARANCE

20.4.1 Basic Liver Structure and Function

The liver is the second largest organ in the body, weighing about 1.5 kg. The liver is located in the right upper quadrant of the abdomen, just below the diaphragm. Main functions of the liver include bile production, detoxification of waste products, storage of iron, vitamins, and trace elements, and the biotransformation of toxic compounds for excretion by the kidneys. Because of its location and ability to efficiently extract a wide variety of compounds from the portal circulation, the liver plays a central role in removing toxic materials before their entry into the systemic circulation. The extracting ability of the liver may result in a substantial decrease in the systemic availability of drugs after oral administration. In addition to its ability to biotransform a wide variety of toxicants, the liver may also directly excrete drugs and/or their metabolites into bile. Thus, the liver possesses two mechanisms for xenobiotic elimination—biotransformation and biliary excretion.

The liver is anatomically divided into two lobes, right and left. Each lobe is further divided into approximately one million lobules. These hepatic lobules are the functional units of the liver. The liver is composed of two types of epithelial cells: hepatocytes, which account for approximately 80% of the nuclear population, and cholangiocytes (epithelial cells that line intrahepatic bile ducts), which account for 3–5% of the liver cell population. A lobule is a hexagonal arrangement of plates of hepatocytes radiating outward from a central vein in the center. Roughly 75% of blood entering the liver arises from venous blood draining into the portal vein. Importantly, all of the venous blood returning from the small intestine, stomach, pancreas, and spleen converges into the portal vein, as does a portion of that which drains from the large intestine. The remaining 25% of the blood supply to the liver is arterial blood delivered through the hepatic artery (Fig. 20.4). The hepatic vascular system has several unique characteristics relative to other organs.

Terminal branches of the hepatic portal vein and hepatic artery empty together and mix as they enter sinusoids in the liver. Hence, the sinusoids are exposed to drug that originates from the gastrointestinal system as well as from the systemic circulation; and such exposure occurs simultaneously. This fact may have important implications in modeling hepatic clearance. The sinusoids are distensible vascular channels bounded circumferentially by hepatocytes. Blood flows through the sinusoids and empties into the central vein of each lobule. As blood flows through the sinusoids, nutrients and other endogenous and exogenous compounds are distributed into the hepatocytes. Hepatocytes are arranged in plates with their apical surfaces facing and surrounding the sinusoids. The basal faces of adjoining hepatocytes are welded together by junctional complexes to form canaliculi, the first channel in the biliary system. Hepatocytes secrete bile into the canaliculi, and those secretions flow antiparallel to the blood flow in sinusoids. At the ends of the

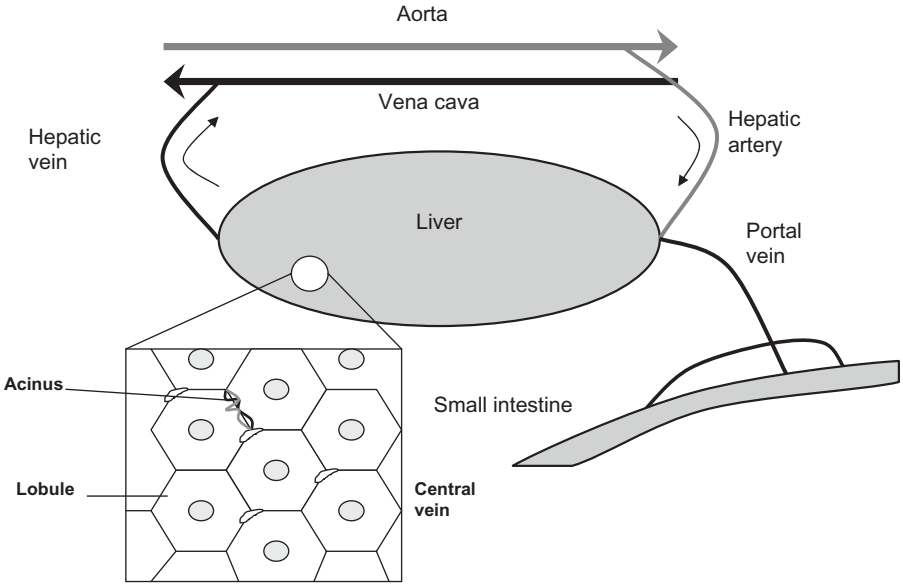

FIGURE 20.4 Hepatic vascular system and lobular structure.

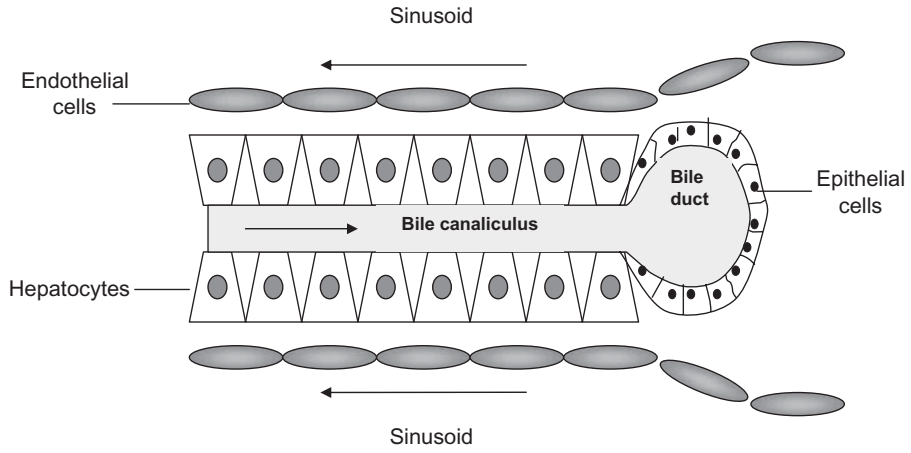

FIGURE 20.5 Representation of bile flow from bile canaliculus into the bile duct.

canaliculi, bile flows into bile ducts, which are true ducts lined with epithelial cells. Bile ducts thus begin in very close proximity to the terminal branches of the portal vein and hepatic artery.

Hepatocytes are the chief functional cells of the liver and perform a large number of metabolic, endocrine, and secretory functions. As mentioned previously, roughly 80% of the mass of the liver is hepatocytes. In three dimensions, hepatocytes are arranged in plates that are in membrane-to-membrane contact with one another. The cells are polygonal in shape and their sides are in contact either with sinusoids (sinusoidal face or apical face) or neighboring hepatocytes (basal faces). A portion of the basal faces of hepatocytes are modified, giving rise to bile canaliculi (Fig. 20.5). Bile originates as secretions from the basal surface of hepatocytes, which collect in canalicular channels. These secretions flow toward the periphery of lobules and into bile ductules and interlobular bile ducts, ultimately collecting in the hepatic duct outside the liver.

20.4.2 Mechanism of Drug Transport Across the Hepatocytes

The portal venous blood contains all of the products absorbed from the gastrointestinal (GI) tract, such as major and minor nutrients originating from ingested food, some endogenous substrates secreted into and reabsorbed from the GI tract, and drugs or other ingested xenobiotics. Drugs delivered to the liver via the portal vein or hepatic artery can be taken up by the hepatocytes and metabolized to more polar compounds. Drugs and their metabolites may then redistribute back into the sinusoids, from where they reach the systemic circulation. Alternatively, drugs or their metabolites may be secreted into the intestinal tract via the biliary system. These secreted compounds and their metabolites can be reabsorbed from the intestine or excreted through feces. Uptake of solutes from sinusoidal blood involves stepwise processes: transport, across the sinusoidal membrane, intrahepatocellular transport, and transport across the canalicular membrane.

Transport Across the Sinusoidal Membrane Once in the sinusoidal blood, drugs may be absorbed from the sinusoids by an active transport process or diffuse passively across the hepatocyte plasma membranes at the apical face. For drugs undergoing an active transport process, traversing the membrane involves several steps. The first is reversible binding of the drug to the membrane transport protein on the sinusoidal side of the cell membrane. Traversing the lipid bilayer itself could occur by a flip-flop process involving the hydrophilic region of the molecule. To aid this process, the carboxyl group of fatty acids becomes protonated by hydrogen bonding either with constituents in the membrane/water interface or constituents within the membrane itself. The uptake process is completed by dissociation of the drug at the cytosolic side of the cell membrane.

Specific plasma membrane transporters exist in the sinusoidal and canalicular membranes of hepatocytes (Fig. 20.6). These include Na^+-dependent and Na^+-independent anionic transporters, as well as cationic transporters. These plasma membrane transporters in humans include Na^+-taurocholate cotransporting polypeptide (NTCP), human organic anion transporting polypeptides (OATP1 and

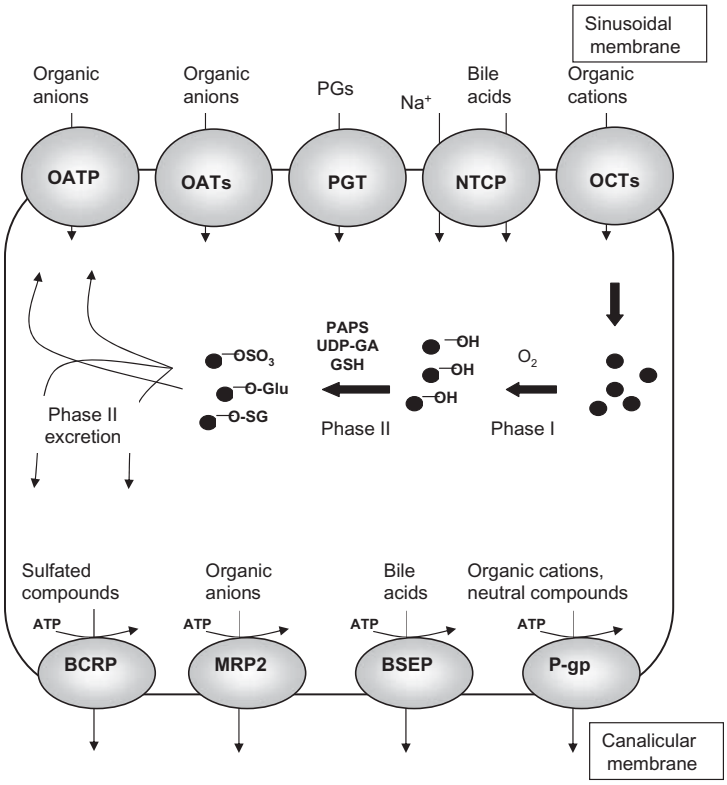

FIGURE 20.6 Uptake and efflux transporters on sinusoidal and canalicular membranes. OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PGT, prostaglandin transporter; NTCP, Na^+-taurocholate cotransporting polypeptide; OCT, organic cation transporter; BCRP, breast cancer resistance protein; MRP, multidrug resistance associated protein; BSEP, bile salt export pump; P-gp, P-glycoprotein.

OATP2), prostaglandin transporter (PGT), and various organic cation transporters; including the organic cation transporter associated with uptake of hydrophilic aliphatic and certain aromatic cations (OCT1). These membrane transporters are involved in the transport of bile acids, organic anions, and organic cations.

Intrahepatocellular Transport Intrahepatocellular drug transport may occur through several processes. These include cytoplasmic diffusion, protein mediated diffusion, cytoplasmic flow, vesicular transport, and drug transfer from intracellular membranes to intracellular proteins. Cytoplasmic diffusion and protein mediated diffusion of drugs are important in intracellular drug disposition. The transfer of solutes from the sinusoidal membrane to the canalicular membrane involves several different steps. These include lateral diffusion of the unbound ligand through the cytoplasm, lateral diffusion of the unbound ligand through membranes, vesicular transport, and transport by cytosolic binding proteins. It is also possible that transporters facilitate the uptake of some solutes by intrahepatic organelles such as lysosomes and mitochondria. Lipophilic basic drugs accumulate in the relatively acidic hepatic lysosomes as a result of the pH difference between lysosomes and the cytoplasm, but may also be due to binding to lipophilic substances and/or aggregation within lysosomes. The sequestration of cationic drugs in the liver also occurs by both slow and fast binding to proteins and membrane sites, as well as ion trapping into mitochondria and lysosomes. The accumulation of cationic lipophilic drugs in the liver, probably primarily due to trapping in mitochondria and lysosomes as well as binding, results in a slow elution and a low availability of cationic lipophilic drugs for excretion in the bile and enterohepatic transport.

Transport Across the Canalicular Membrane Transport of solutes across the canalicular membrane into the bile occurs via active transport by several membrane proteins. Mechanistically, biliary elimination of anionic compounds, including glutathione *S*-conjugates, is mediated by multidrug resistance associated protein (MRP) transporter MRP2, whereas bile salts are excreted by a bile salt export pump (BSEP). MRP2 can recognize many kinds of organic anions and is responsible for the biliary excretion of many endogenous compounds and drugs. Also expressed in the canalicular membrane is P-glycoprotein, which mediates the biliary excretion of hydrophobic, mostly organic cationic and neutral metabolites. All of the canalicular membrane transport proteins are driven by ATP hydrolysis to transport a number of drug conjugates that undergo enterohepatic recycling, including glucuronide, sulfate, and glutathione conjugates. Figure 20.6 illustrates various sinusoidal and canalicular membrane transporters involved in the uptake and efflux of solutes.

Although hepatocytes lining the sinusoids are polarized (i.e., transport compounds from sinusoidal surface to canalicular surface), there is a possibility for back-transport into the sinusoids. For instance, certain multidrug resistance associated protein (MRP) transporters (MRP1, MRP3, and MRP6) exist at the sinusoidal surface of the membrane [14]. These active transporters can transport certain drugs and metabolites from hepatocytes back into sinusoids. In some cases there is preferential excretion of the solutes back into the sinusoids rather than into the bile, thus minimizing potential enterohepatic recycling. Other drug anion conjugates are also preferentially excreted into the sinusoids by a saturable transport mechanism.

In general, transporters such as MRP1 are present in the sinusoidal membrane only at very low levels in quiescent cells.

20.4.3 Role of Liver in First-Pass Metabolism

Although every tissue has some ability to metabolize drugs, the liver is the principal organ of drug metabolism. Other tissues that display considerable activity include the gastrointestinal tract, the lungs, the skin, and the kidneys. Drugs that are administered orally are absorbed from the gastrointestinal tract, carried via the hepatic portal vein to the liver. Many drugs are absorbed intact from the small intestine and transported to the liver, where they undergo extensive metabolism by the liver before the systemic organs are exposed to the drug. This removal of a drug by the liver, before the drug has become available in the systemic circulation, is called the *first-pass effect*. First-pass metabolism can occur in the gut and/or the liver, since the entire dose of the drug absorbed after oral administration must pass through these organs prior to reaching the systemic circulation. This process may greatly limit the bioavailability of orally administered drugs, such that alternative routes of administration must be employed to achieve therapeutically effective blood levels.

20.4.4 Biliary Clearance

Xenobiotic compounds excreted into bile are often divided into three classes on the basis of the ratio of their concentration in bile versus that in plasma. Class A substances have a ratio of nearly 1 and include sodium, potassium, glucose, mercury, thallium, cesium, and cobalt. Class B substances have a ratio of bile to plasma greater than 1. Class B substances include bile acids, bilirubin, sulfobromophthalein, lead, arsenic, manganese, and many other xenobiotics. Class C substances have a ratio below 1 (e.g., inulin, albumin, zinc, iron, gold, and chromium). Compounds rapidly excreted into bile are most likely to be found among class B substances.

The mechanism by which the body directs some compounds to the biliary excretion is as yet unclear. However, it is apparent that molecular weight of the compound is a key factor in determining biliary excretion. The molecular weight threshold for humans is estimated to be 500–600. Compounds with molecular weights less than this threshold are excreted primarily in the urine. This knowledge may be useful in drug design when one objective is to achieve a lead compound exhibiting primarily biliary excretion—which obviates the need for dosage adjustment in renal disease.

Drugs that undergo biliary secretion have the potential to be reabsorbed from the GI tract and exhibit enterohepatic recycling. Enterohepatic recycling prolongs the half-life of drugs primarily excreted in bile. Interruption of this cycling through the oral administration of nonabsorbable adsorbants (e.g., activated charcoal) may enhance the clearance of affected compounds. In addition, since the gallbladder empties episodically in humans, drugs that undergo significant enterohepatic recycling may exhibit discontinuous plasma concentrations (as a portion of the excreted dose is periodically reabsorbed, resulting in increases in the plasma concentration of drug).

20.4.5 Determination of Hepatic Clearance

Determination of the hepatic clearance (CL_H) of a compound is generally achieved through applying the principle of the additivity of clearance. In particular, the total and renal clearance are determined as described previously. If there is no evidence of nonrenal/nonhepatic elimination of the drug, the CL_H is given as

$$CL_H = CL_T - CL_R$$

This means of determination for hepatic clearance obviously requires numerous assumptions that must be validated experimentally. Hepatic clearance may also be estimated from *in vitro* data, taking into account protein binding and liver blood flow.

20.4.6 Models of Hepatic Clearance

Predicting the impact of pharmacological, physiological, and pathophysiological interventions on hepatic clearance necessitates the elucidation of the determinants of hepatic clearance such that a quantitative model can be developed. Among the various mathematical models proposed to describe hepatic clearance, the venous equilibrium model (also known as the well-stirred model) represents the simplest model that appears to provide reasonably robust predictive capacity [15, 16].

Venous Equilibrium Model of Hepatic Clearance The venous equilibrium model assumes the liver to be represented as a single, homogeneous, well-stirred compartment, such that all metabolic enzymes in the liver are exposed to the same concentration of drug (Fig. 20.7). This model simplistically assumes that drug bathing the sinusoids is in equilibrium with that in hepatic venous blood (hence the name venous equilibrium). Such a model obviously does not account for various anatomical complexities present in the liver, such as its network of branching tubes each with a zonal distribution of enzymes. It is also physiologically naive to envision drug concentration as unchanging as blood traverses the sinusoidal bed. Nevertheless, despite the fact that experimental evidence suggests that the hepatic sinusoid is not

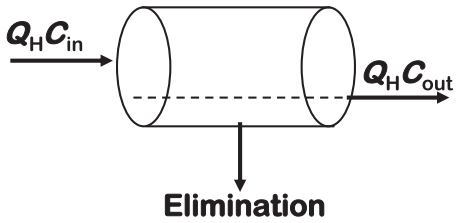

FIGURE 20.7 Schematic of the venous equilibrium or well-stirred model of hepatic clearance. This model in essence envisions drug concentration dropping immediately upon entry into the hepatic sinusoids and not declining further as blood traverses across the sinusoidal bed. Hence the drug concentration bathing the sinusoids is viewed as being in equilibrium with the concentration in venous blood. Q_H, blood flow; C_{in}, concentration of drug in blood entering the liver; C_{out}, concentration of drug in venous blood exiting the liver. Dashed line represents the drug concentration in the sinusoids. (Adapted from Ref. 20.)

well stirred [17], the model has been found to provide good estimates of the impact of various changes on hepatic clearance and drug concentration. This model identifies three independent, noninteracting determinants of hepatic clearance: hepatic blood flow (Q_H), fraction of unbound drug in blood (f_{ub}), and the unbound intrinsic hepatic clearance (CL_{uint}) The CL_{uint} represents the ability of the liver to remove drug from blood in the absence of confounding factors (e.g., protein binding and blood flow) and is determined by the ability of the liver to metabolize or secrete (via biliary secretion) the drug. Accordingly, the hepatic clearance (CL_H) and hepatic extraction ratio (E_H) for the venous equilibrium model can be expressed as [15, 16, 18, 19]

$$CL_H = \frac{Q_H f_{ub} CL_{uint}}{Q_H + f_{ub} CL_{uint}}$$

$$E_H = \frac{f_{ub} CL_{uint}}{Q_H + f_{ub} CL_{uint}}$$

This model provides important insight into the impact of changes in blood flow, protein binding, and drug metabolism/transport on hepatic clearance—particularly at the extreme values of the unbound intrinsic hepatic clearance. For example, when $Q_H >> f_{ub}CL_{uint}$, the value of CL_H will approach $f_{ub}CL_{uint}$ (i.e., $CL_H \approx f_{ub}CL_{uint}$). For such drugs, changes in hepatic clearance will be proportional to changes in protein binding or the unbound intrinsic clearance. Hence, coadministration of an enzyme inducer that increases the metabolism of such a drug will result in an increase in the hepatic clearance of the agent. Moreover, increases in the free fraction of drug will also result in proportional increases in the clearance of the drug. This indicates that the clearance of the drug is "restricted" to the free drug. Such drugs are therefore sometimes referred to as exhibiting restrictive clearance. Drugs in this category are also often classified as possessing a low intrinsic clearance. Agents that exhibit a hepatic extraction ratio < 0.3 can be viewed as possessing a low intrinsic clearance. Importantly, however, changes in hepatic blood flow will not alter the clearance of such drugs, provided the decrease is not sufficient to cause hypoxia and result in hepatocellular damage. Examples of drugs that fall into this category are diazepam, phenytoin, tolbutamide, and warfarin.

At the opposite extreme are those drugs for which $Q_H << f_{ub}CL_{uint}$, sometimes denoted as high intrinsic clearance drugs. Under these conditions, the value of CL_H will approach Q_H (i.e., $CL_H \approx Q_H$). Such drugs are referred to as exhibiting blood flow-dependent clearance (or perfusion rate limited), meaning that changes in blood flow result in proportional changes in clearance. Under these conditions, increases in enzyme or transport activity will not result in further increases in hepatic clearance, as drug is already being cleared as rapidly as it is delivered to the liver. Modest reductions in enzyme activity will also not result in significant alterations in drug clearance. However, if an inhibitor of metabolism or transport causes a magnitude of reduction such that the assumption that $Q_H << f_{ub}CL_{uint}$ is no longer true, coadministration of an inhibitor with a drug exhibiting a high intrinsic clearance may result in significant changes in drug clearance. Drugs with an extraction ratio > 0.7 are generally classified as exhibiting a high intrinsic clearance. Moreover, drug is removed so rapidly under these conditions that it is not restricted to free drug.

This model also provides important insight into factors that influence the systemic availability of drugs after oral administration. If a drug is completely absorbed after oral administration and is not metabolized by the gut, the fraction of drug that reaches the systemic circulation can be described as

$$F = 1 - E_{\mathrm{H}}$$

Since

$$E_{\mathrm{H}} = \frac{f_{\mathrm{ub}} CL_{\mathrm{uint}}}{Q_{\mathrm{H}} + f_{\mathrm{ub}} CL_{\mathrm{uint}}}$$

F can be determined as

$$F = 1 - E_{\mathrm{H}}$$

$$F = 1 - \frac{f_{\mathrm{ub}} CL_{\mathrm{uint}}}{Q_{\mathrm{H}} + f_{\mathrm{ub}} CL_{\mathrm{uint}}}$$

$$F = \frac{Q_{\mathrm{H}}}{Q_{\mathrm{H}} + f_{\mathrm{ub}} CL_{\mathrm{uint}}}$$

The relationship given above indicates that when $Q_{\mathrm{H}} >> f_{\mathrm{ub}} CL_{\mathrm{uint}}$, $F \rightarrow 1$. Such drugs will not exhibit any significant first-pass effect during passage through the liver. Hence, coadministration of an enzyme inducer or inhibitor will not significantly alter the systemic availability of drugs with these characteristics. In contrast, when $Q_{\mathrm{H}} << f_{\mathrm{ub}} CL_{\mathrm{uint}}$, $F \rightarrow 0$ and little of the drug will reach the systemic circulation after oral administration. Under these circumstances, changes in enzyme activity, as produced by inhibitors or inducers, may have profound effects on the systemic availability of drugs. Among the drugs that exhibit a high first-pass effect and low systemic availability are propranolol, verapamil, and nitroglycerin. Because of the high first-pass effect, oral doses of such drugs are substantially (sometimes an order of magnitude) greater than intravenous doses in order achieve the desired pharmacologic effect. In addition, there is generally much more inter- and intrapatient variability in the pharmacokinetics of drugs subject to extensive metabolism. As a consequence, one objective in lead optimization is often to utilize rationale drug design to eliminate first-pass metabolism.

This model of hepatic drug clearance also demonstrates the importance of understanding the role of first-pass metabolism in the interpretation of values of drug clearance obtained from the ratio of dose and area under the drug concentration versus time curve. This is most readily seen for a drug that is eliminated solely by hepatic metabolism and completely absorbed after oral administration. For such compounds, the hepatic clearance after intravenous administration can be determined as

$$CL_{\mathrm{H}} = \frac{D_{\mathrm{IV}}}{AUC_{\mathrm{IV}}}$$

where D_{IV} is the intravenous dose and AUC_{IV} is the area under the drug concentration versus time curve for that dose. Similarly, the hepatic clearance after oral administration is given as

$$CL_H = \frac{F \times D_o}{AUC_o}$$

where F is the systemic availability, D_o is the oral dose, and AUC_o is the area under the drug concentration versus time curve after that dose. Remembering that

$$CL_H = \frac{Q_H f_{ub} CL_{uint}}{Q_H + f_{ub} CL_{uint}}$$

and

$$F = \frac{Q_H}{Q_H + f_{ub} CL_{uint}}$$

one can substitute these equalities for CL_H and F, yielding

$$\frac{Q_H f_{ub} CL_{uint}}{Q_H + f_{ub} CL_{uint}} = \frac{Q_H}{Q_H + f_{ub} CL_{uint}} \times \frac{D_o}{AUC_o}$$

This relationship simplifies to

$$f_{ub} CL_{uint} = \frac{D_o}{AUC_o}$$

Importantly, note that the ratio of oral dose to AUC does not provide the value for hepatic clearance, but rather $f_{ub}CL_{uint}$. When calculated in this fashion, this is referred to as the oral clearance (CL_o). For a low intrinsic clearance drug, this value will be essentially equal to the hepatic clearance. However, for a high intrinsic clearance drug, the value will be much higher than the hepatic clearance and will exceed the value for hepatic blood flow.

This relationship also provides a means by which the unbound intrinsic hepatic clearance and hepatic blood flow can be determined experimentally. If an intravenous dose of radiolabeled drug and an oral dose of "cold" drug are administered simultaneously, the hepatic and intrinsic clearances can be calculated from the above relationships. Once these values are determined, the blood flow can be calculated from the knowledge of these two parameters. While similar determinations can be made by administering nonradiolabeled drug by both routes on different days, simultaneous administration by both routes obviates the need to account for day to day variability in hepatic blood flow or enzyme activity.

Parallel Tube Model of Hepatic Clearance The parallel tube model represents the liver sinusoids as identical parallel tubes. This model also assumes that metabolic

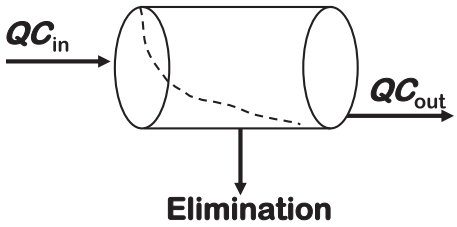

FIGURE 20.8 Schematic representation of the parallel tube model of hepatic drug clearance. This model envisions the liver as being comprised of a series of parallel tubes (one of which is shown) with drug concentration declining as blood traverses through the sinusoidal space. See Fig. 20.7 for abbreviations. (Adapted from Ref. 20.)

enzymes are uniformly distributed along the sinusoid [20]. In this model, drug concentration is envisioned as declining exponentially as drug traverses the sinusoidal tube (Fig. 20.8). This leads to different equalities for extraction ratio, hepatic clearance, and systemic availability:

$$E = 1 - e^{-f_{ub}CL_{uint}/Q_H}$$
$$CL_H = Q_H(1 - e^{-f_{ub}CL_{uint}/Q_H})$$
$$F = e^{-f_{ub}CL_{uint}/Q_H}$$

The differences between the parallel tube and venous equilibrium models in prediction of the effect of changes in blood flow, enzyme activity, and protein binding are insignificant for drugs that exhibit a low intrinsic clearance (i.e., $E < 0.3$). For intermediate drugs (i.e., $0.3 < E < 0.7$) the differences are also generally insignificant except with oral administration. The difference in model prediction is most clearly seen for drugs with high intrinsic clearance values. Using a variety of experimental models, most commonly the isolated perfused rat liver, numerous investigators have demonstrated substantial differences in the predictive capacity of these two models for such drugs. However, it should be noted that these differences are probably not of clinical significance for the overwhelming number of therapeutic agents. Therefore, the use of the most simplistic model provides important insight for the practical application of clearance concepts.

Other Models of Hepatic Clearance In addition to the two models described earlier, additional models of hepatic clearance have been proposed. The distributed model is a modification of the parallel tube model, where each tube has a distinct blood flow rate and metabolic capacity [19]. The dispersion model adds complexity via consideration of the transit time of the drug in the model. A series compartment model has also been proposed, which envisions the liver to be comprised of a series of well-stirred compartments. The review of Morgan and Smallwood [21] is recommend for readers interested in further consideration of various models of hepatic clearance.

20.5 EFFECT OF DISEASE ON DRUG CLEARANCE

From the foregoing discussion, it should be obvious that disease states that alter the functional capacity of organs of elimination may result in substantial changes in drug clearance. It is also important to recognize that the contribution of a given organ of elimination to the overall elimination of a drug may change dramatically in the face of significant pathology of another organ of elimination. Consider, for example, a drug that is eliminated 85% by the kidneys unchanged and 15% by hepatic metabolism. For such a drug, alteration of hepatic metabolism by administration of an enzyme inhibitor is unlikely to have a clinically significant impact on the disposition of the compound. In contrast, in the presence of renal disease such that hepatic metabolism becomes the predominant route of elimination, interaction with an enzyme inhibitor may be of substantial significance.

The impact of renal disease on the renal clearance of drugs has been the most widely studied disease-induced alteration of drug disposition. This is, in part, due to the ready ability to quantify kidney function via creatinine clearance. Hence, it has been possible to evaluate the impact of various degrees of functional deficit on the renal elimination of drugs. Renal diseases, mainly acute and chronic renal failure, are associated with nephron loss to varying degrees depending on the stage of the renal disease. Bricker's "intact nephron" hypothesis provides an explanation for the kidney's ability to compensate and preserve homeostasis despite a significant loss of nephron function in renal disease [22]. During renal failure, regardless of etiology, injury occurs to the nephrons in a progressive manner. Significant damage to groups of nephrons will eliminate them from contributing to the maintenance of normal renal function. The remaining intact nephrons will compensate by experiencing cellular hypertrophy. This growth process will enable them to accept larger blood volumes for clearance, thus contributing to maintenance of glomerulotubular balance and the excretion of greater solute levels, resulting in compensation. Thus, varieties of adaptations compensate for the decreased GFR and allow a new steady state of external balance to exist. However, in the process of adapting, a second component becomes disordered. The classic examples are the secondary hyperparathyroidism and uremic syndrome of chronic renal failure. Thus, renal diseases not only influence the overall elimination of drugs directly but also result in secondary abnormalities.

Progressive renal disease evolves to a multiorgan syndrome such that the disposition of drugs via nonrenal routes may be altered. For example, patients with renal disease may accumulate endogenous compounds that displace drugs from plasma protein binding sites. This has the potential to increase the hepatic clearance of low intrinsic clearance drugs. Furthermore, the accumulation of the endogenous products normally excreted in the kidney may impair the functional capacity of the liver, such that drug metabolism (and thus hepatic clearance) is impaired.

The kidney makes the major contribution to excretion of unchanged drug and also the excretion of metabolites. A range of physiological and pathophysiological states may influence the efficiency of renal clearance as described earlier. It is evident that renal drug clearance is altered to a clinically significant extent in a number of disease states. Even though the intact nephrons compensate for the decreased clearance by adaptation, they cannot completely reestablish the normal kidney function. For a drug, if the renal clearance contributes only 25–30% to

overall clearance, the renal impairment may not influence the total body clearance significantly; while for the drugs that are predominantly cleared by the kidneys, total clearance is significantly affected by renal impairment. Therefore, drug removal by artificial means (renal replacement therapy) such as hemodialysis becomes essential in subjects with substantial loss of renal function. Similar to renal clearance, the extent to which the drug is affected by dialysis is determined primarily by physicochemical characteristics such as molecular size, protein binding, volume of distribution, hydrophilicity, and plasma clearance of the drug. In addition, technical aspects of the dialysis procedure such as characteristics of dialysis membrane and dialysate flow rates may also determine the extent of drug removal [23, 24]. Drugs that are significantly cleared by the kidney often undergo substantial removal during dialysis and dosing adjustments are often required for such drugs. Only the unbound drug is available for filtration and drugs with high protein binding are poorly cleared by dialysis. The influence of renal diseases on protein binding of drugs is not well understood. It has been reported that low albumin levels are found in critically ill patients, which may increase the unbound fraction of many drugs with possible deleterious effects. These patients also often have increased levels of acid α-glycoprotein, which may increase protein binding of some drugs. Thus, the unbound fraction in healthy volunteers and in patients with renal impairment may differ substantially from the unbound fraction of drugs in critically ill patients receiving dialysis. Similarly, in critically ill patients the actual volume of distribution may differ significantly from that of healthy subjects, and it also exhibits great inter- and intraindividual variation [24, 25].

The ability of the liver to metabolize drugs depends on hepatic blood flow and enzyme activity, both of which can be affected by liver damage. Hepatic diseases can selectively modify the kinetics of drug metabolism in the liver [26]. Previous studies on the effects of hepatic disease have shown that cytochrome P450 (CYP) enzymes are more susceptible to hepatocellular injury than are NADPH-cytochrome P450 reductase or phase II enzymes, such as UDP-glucuronosyltransferases involved in drug conjugation reactions. Patients with severe liver diseases such as cirrhosis or severe hepatitis with liver failure have significant impairment of CYP enzymes. Moreover, in patients with liver damage, expression of some CYP isoforms remains unaffected while other isoforms are significantly reduced. For instance, in liver cirrhosis expression of CYP1A2 and CYP3A4 is decreased, while in cirrhosis with cholestasis expression of CYP2C and CYP2E isoforms is reduced [27, 28]. Therefore, a thorough knowledge of the particular enzyme involved in the metabolism of a drug and the impact of hepatic damage on that enzyme is essential to provide a reasonable basis for dosage adjustment in patients with hepatic impairment. In addition, liver failure can influence the binding of a drug to plasma proteins. These changes can occur alone or in combination; when they coexist their effect on drug kinetics can be synergistic. The kinetics of drugs with a low hepatic extraction are sensitive to hepatic failure rather than to liver blood flow changes. While the kinetics of many drugs are altered by liver disease, quantifying the required dosing changes remains a challenge. At present, there is no satisfactory test that provides a quantitative measure of liver function, with which drug clearance is highly correlated.

Liver diseases may also impair the biliary excretion of drugs. Cholestasis is a hepatic abnormality in which there is stagnation of bile flow, which may arise from

a physical obstruction of the biliary tree (extrahepatic cholestasis) or a decrease in the secretion of the bile by the hepatocytes (intrahepatic cholestasis). The impairment of biliary secretions may result in the accumulation of metabolites, such as glucuronide conjugates, that may be transported into blood. Under these conditions, the overall time that a compound remains in the body increases considerably. There are other clinical conditions in which the metabolism of drugs is altered, including heart failure and other liver diseases.

It is also important to recognize that chronic renal failure can significantly affect the disposition of both low and high hepatic extraction drugs, which are cleared predominantly by the liver. Renal diseases have also been suggested to affect biliary clearance through the accumulation of some P-glycoprotein substrates in the plasma, which may in turn inhibit the secretion of some drugs [9, 29].

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21

IN VITRO METABOLISM IN PRECLINICAL DRUG DEVELOPMENT

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Contents

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21.1 INTRODUCTION

In drug therapy, some important questions concerning the efficient and safe use of a drug are the following: (1) What is the size of the dose? (2) How often should it be given? (3) Is there interference from other simultaneously used drugs or other chemicals? (4) Are there important idiosyncratic reactions due to a drug? (5) Are there important patient characteristics to be considered? [1]. The first two questions relate to basic pharmacokinetic characteristics of a drug, principally to its bioavailability and clearance. The third question relates to potential interactions. The fourth concerns the possibility of metabolism-related toxicities, and the fifth relates to endogenous, host-related and genetic features of an individual patient to whom a drug is administered. At the basic level, we need to know about the drug substance (1) its clearance and factors contributing to it, (2) major and minor, including potentially toxic, metabolites (i.e., metabolite profile), and (3) drug-metabolizing enzymes catalyzing (at least) rate-limiting routes, and their inhibition and induction and physiological and pathological behavior.

In this chapter, metabolism in preclinical drug development is the principal topic. However, before going into details, some more general concerns during drug discovery and development should be expressed. Drug development is the optimization of not a single feature but multiple features, even regarding metabolism and kinetics [2, 3]. Consequently, optimization is always a trade-off between several independent or interdependent features. Thus, there are no preset answers to the optimization problem; instead, it is a continuous cross-talk between various optimization tasks. It is therefore difficult to say that one *in vitro* test is more important than another, because the relative importance depends on the individual substance under study and its unique characteristics. However, this is not to say that we do not need a general strategy to study drug metabolism and pharmacokinetics by *in vitro* methods. We need a general strategy, but it has to be modified depending on the substance, its stage of development, and many other considerations.

In Table 21.1, an attempt has been made to put metabolism into a wider perspective in pharmacokinetic (PK) processes. Optimization of metabolic features in the context of pharmacokinetics is divided into characteristics, which should be pre-

TABLE 21.1 Metabolic Processes, Characteristics, and Factors that Should Be Predicted on the Basis of *In Vitro* Screening Assays or Assumed During the *In Vitro–In Vivo* Extrapolationa

PK Process	Characteristic	Factors	Assays
Systemic exposure			
Hepatic clearance	Hepatic blood flow	Protein binding permeability	Animals *in vivo*
	Hepatic metabolic clearance	CYP enzymes, phase II enzymes	*In vitro* and cell based (see text)
	Biliary clearance	Transporters	Liver perfusion
Bioavailability			
First-pass clearance	Intestinal clearance	Efflux transporters and intestinal metabolism	Caco-2
	Hepatic clearance	See above	
Interactions			
Metabolism	Inhibition	CYP enzymes	*In vitro* microsomes, recombinant CYPs or cell based
		Phase II enzymes	as above
	Induction		Hepatocytes, receptor-binding
Metabolic activation	Reactive metabolites, oxygen radicals, etc.	Producing enzymes	*In vitro* systems

[a]For some illustrative examples concerning various PK aspects, see chapters in a book by Pelkonen et al. [6].

dicted on the basis of *in vitro* information, factors (i.e., biological phenomena or constituents mediating them), and assays or screens (i.e., systems available to measure a factor or a characteristic). Furthermore, an additional entry depicts metabolic activation, which is thought to be of importance for "idiosyncratic" reactions (see Section 21.5).

One more introductory note: the literature on subjects in this chapter is very large and making proper acknowledgments to all important contributions is impossible. Selection of references thus reflects our somewhat personal view, but hopefully contains the most useful ones, from which the reader can go further.

21.2 *IN VITRO* BIOLOGICAL SYSTEMS TO CATALYZE METABOLISM

The questions posed at the beginning of this chapter will be definitively and unequivocally answered during the clinical phase, including postmarketing pharmacovigilance, of drug development. However, this is too late from the early drug development point of view and thus, to avoid failures and withdrawals later on, the basic information for answering these questions should preferably be produced in the nonclinical phase of development. In the current paradigm of nonclinical drug development,

in vitro screening systems are used for producing the appropriate answers through correlation and extrapolation, which actually are absolute requirements for the judicious use of *in vitro* studies to support drug development. Because of interspecies differences and extrapolation uncertainties, human-derived or humanized *in vitro* systems are preferably used. Important goals for using various human liver derived *in vitro* systems are the following: (1) to elucidate and determine principal metabolic routes of a new chemical entity (NCE) and to tentatively identify principal metabolites; (2) to identify phase I enzymes, especially cytochrome P450 (CYP), catalyzing the principal oxidation routes (primary metabolites) and phase II enzymes catalyzing the conjugated metabolites, and to gain some quantitative data on their significance for the overall metabolic fate of an NCE; and (3) to provide useful background information for characterizing potential interactions and physiological, genetic, and pathological factors affecting the kinetics and variability of an NCE in the *in vivo* situation.

21.2.1 Metabolic Competence of the *In Vitro* System

When screening potential drug candidates, one is faced with a question. How large or restricted should the set of enzymes be to cover a reasonable number of potential drug-metabolizing enzymes; that is, what is the optimal range of enzymes to be screened? In the end, each drug is an individual, with its characteristic physicochemical properties, metabolic reactions, metabolizing enzymes, kinetics, and so on. Regarding metabolism, thus far the main focus has been on CYP enzymes. This is natural, because the CYP enzyme superfamily is responsible for approximately 70–80% of the rate-limiting phase I metabolism of drugs [4]. However, there are a large number of other important enzymes participating in the metabolism of drugs. For example, should we routinely screen for various UGT or SULT enzymes and at which stage of the development? Table 21.2 gives an overview of drug-metabolizing enzymes, which should be considered when studying the metabolic competence of a given *in vitro* screening system [5, 6]. Although it is impossible to give unequivocal recommendations for how comprehensive metabolic competence should be employed in the *in vitro* test system, it is always useful to consider this matter and explicitly state objectives of the specific test and reasons for selecting the specific biological preparation, with a specified set of enzymes.

21.2.2 From Recombinants to Perfusions

Enzyme sources (i.e., biological preparations), are another concern. Most important possibilities are briefly described in Table 21.3, which presents an overview of current biological preparations for use as enzyme sources and some of their advantages and disadvantages. Liver microsomes have been the preferred source of enzymes for *in vitro* metabolism screening. Recombinant expressed enzymes have become more and more usable, because of easy availability and lack of variability. Isolated and/or cultured cells have a larger complement of enzymes in more natural cellular surroundings. These aspects have been very thoroughly covered recently [13, 14]. There are strong arguments in favor of using human primary hepatocytes [15], but obviously their availability for early and extensive screening is rather difficult. There are only a few studies in which several enzyme sources have been

TABLE 21.2 Drug–Metabolizing Enzymes (Preferably Human) that Should/Could/Might Be Included in the *In Vitro Assays*

Enzyme Classes/Enzymes	(Types of) Drugs Metabolized (Examples)	Sources Available	Need of Incorporation into *In Vitro* Systems
Cytochrome P450 enzymes (about 10–13 drug-metabolizing enzyme forms)	Practically 90% of all drug substances	Hepatocytes most versatile and comprehensive; other tissues and cell types selectively	Incorporation of P450-competent enzyme source is necessary for most purposes
An example: CYP1A1	Few pharmaceutics, principally polycyclic aromatic hydrocarbons and other carcinogens	Recombinant enzyme; mainly extrahepatic expression (e.g., placenta from smokers)	Induction usually required for expression; incorporation not necessary routinely
An example: CYP3A4	A majority, >50%, of all clinically used drugs	Hepatocytes, recombinant	Incorporation necessary for comprehensive screening
Flavin-monooxygenases (FMOs) (five forms)	Compounds with secondary and tertiary amines or sulfydryl groups (chlorpromazine, desipramine, methimazole)	Hepatocytes most versatile and comprehensive	Incorporation comes automatically with hepatocytes; specific incorporation only in special circumstances
Prostaglandin H synthase	PAH-diols, aflatoxin B1, aromatic amines	Liver, kidney, bladder (microsomes)	Incorporation advisable for toxicity screening?
Alcohol/aldehyde dehydrogenases and oxidases	Various compounds with alcohol and aldehyde functions (ethanol)	Many tissues; cytosol	Some incorporation comes automatically with hepatocytes or hepatic homogenate; specific incorporation only in special circumstances
Monoamine oxidase	Selegiline, moclobemide	Many tissues; mitochondria	Incorporation advisable in specific situations

TABLE 21.2 ***Continued***

Enzyme Classes/Enzymes	(Types of) Drugs Metabolized (Examples)	Sources Available	Need of Incorporation into *In Vitro* Systems
Esterases/hydrolases/peptidases	Compounds with cleavable ester/amide bond (procaine, succinylcholine, lidocaine)	Many tissues including blood and blood cells	At least some activity present when liver preparation is incorporated into the test system
Reductases	Many substances with azo, nitro, and carbonyl functions (chloramphenicol, naloxone); importance not well characterized	Present in hepatocytes (cytosol, some in microsomes)	Activity needs special circumstances; need of incorporation not adequately defined
UDP-glucuronosyltransferases (UGTs); glucuronide conjugation	Most drugs with suitable O-, S-, and N-functional groups (morphine, diazepam, paracetamol)	Hepatocytes most versatile and comprehensive (microsomes); subcellular systems need UDPGA	Incorporation of UGT-competent enzyme source is advisable for most purposes
Sulfotransferases (SULTs); sulfate conjugation	Phenols, alcohols, aromatic amines (paracetamol, methyldopa)	Hepatocytes most versatile and comprehensive (cytosol); subcellular systems need PAPS	Incorporation not adequately defined
GSH transferases (GSTs); glutathione conjugation	Epoxides, arene oxides, nitro groups, hydroxylamines (ethacrynic acid)	Hepatocytes most versatile and comprehensive (cytosol, microsomes)	Incorporation not adequately defined
Acyl-CoA glycinetransferase; amino acid conjugation	Acyl-CoA derivatives of carboxylic acids (salicylic acid)	Hepatocytes (mitochondria)	Incorporation advisable only in special cases
N-acetyltransferases (NATs); acylation	Amines (sulfonamides, isoniazid, clonazepam, dapsone)	Hepatocytes (cytosol)	Incorporation advisable only in special cases
Methyl transferases; methylation	Catecholamines, phenols, amines (L-dopa, thiouracil)	Various tissues (cytosol)	Incorporation advisable only in special cases

Sources: Adapted from Refs. 5 and 41.

TABLE 21.3 Comparison of *In Vitro* Enzyme Sources Used in Preclinical Research

Enzyme Sources	Availability	Advantages	Disadvantages
Liver homogenates [7]	Relatively good; commercially available	Contains basically all hepatic enzymes	Liver architecture lost; cofactor addition necessary
Microsomes [7]	Relatively good; transplantations or commercial sources	Contains most important rate-limiting enzymes; relatively inexpensive; easy storage	Contains only phase I enzymes and UGTs; requires strictly specific substrates or antibodies for individual DMEs; cofactor addition necessary
Recombinant CYP enzymes [8]	Commercially available	Can be utilized with HTS substrates; role of individual CYPs in the metabolism can be easily studied	The effect of only one enzyme at a time can be studied
Primary hepatocytes [9, 10, 15]	Difficult to obtain; relatively healthy tissue needed; commercially available	Contains the whole complement of DMEs cellularly integrated; induction effect of an NCE can be studied; cryopreservation possible	Requires specific techniques and well established procedures; levels of many DMEs decrease rapidly during cultivation
Liver slices [11]	Difficult to obtain; fresh tissue needed.	Contains the whole complement of DMEs and cell–cell connections; induction effect of an NCE can be studied; cryopreservation possible	Requires specific techniques and well established procedures; limited viability
Immortalized Cell lines [12]	Available at request; only a few adequately characterized cell lines exist	Nonlimited source of enzymes	Expression of most DMEs is poor; genotype/phenotype instability

Sources: Adapted from Refs. 5 and 41.

rigorously compared [16–18], but it seems that the currently used enzyme systems—recombinant enzymes, human liver microsomes and/or homogenates, liver slices, and hepatocytes—all give fairly reliable results, if their inherent restrictions are taken into consideration.

21.2.3 Current Types and Components of *In Vitro* Systems

During the development of an NCE, according to the current paradigm, several types of investigations are usually carried out, the earlier the better. The enzyme sources in these studies are usually human-derived systems. A summary of the major *in vitro* methods is provided in Table 21.4. The most important objectives of these studies are the elucidation of metabolic stability of an NCE, identification of metabolites and metabolic routes, and identification of CYP forms metabolizing an NCE. The following paragraphs summarize briefly these approaches. More details are given in subsequent sections.

1. The metabolic stability of an NCE determines its future as a drug candidate. By determining the time and concentration dependence of disappearance and/or metabolite formation *in vitro* in an appropriate system, its hepatic clearance *in vivo* can be predicted [23, 24].
2. Metabolite identification, at least at the tentative level, can be developed from incubations with human liver cells and other preparations, for example, homogenates or microsomes (see Section 21.4).

TABLE 21.4 *In Vitro* Studies for the Characterization of Metabolism and Metabolic Interactions of Potential Drugs[a]

In Vitro Test	Preparations	Parameters	Extrapolations
Metabolic stability	Microsomes, homogenates, cells, slices	Disappearance of the parent molecule or appearance of (main) metabolites	Intrinsic clearance, interindividual variability
Metabolite identification	Same as above	Tentative identification of metabolites, for example, by LC-TOF-MS and LC-MS-MS	Metabolic routes, qualitative (semiquantitative, if possible) metabolic chart
Identification of metabolizing enzymes	Microsomes with inhibitors or inhibitory antibodies; recombinant individual enzymes	Assignment and relative ability of enzymes to metabolize a compound	Prediction of effects of various genetic, environmental, and pathological factors; interindividual variability
Enzyme inhibition	Microsomes, recombinant enzymes	Inhibition of model activities by a substance	Potential drug–drug interactions
Enzyme induction	Cells, slices, permanent cell lines (if available), constructs	Induction of model activities (or mRNA); receptor binding (e.g., PXR or CAR)	Induction potential of a substance

[a]For some salient background data and examples, see Refs. 15 and 19–22.

3. After characterizing the metabolic stability and metabolic routes of an NCE, the *in vivo* prediction requires clarification of the enzymes that participate in the *in vitro* biotransformation of the NCE. After determining the initial velocity conditions and enzyme kinetic parameters for identified rate-limiting pathways, some of the main tools and approaches used in enzyme assignment are CYP selective chemical inhibitors and antibodies, cDNA expressed CYPs, correlation analysis, and measures of affinities of an NCE for CYPs (by inhibition).

Numerous compounds have been characterized for their inhibitory potency against different CYPs. Many of them are selective for the desired enzyme only at relatively low concentrations. Today, there are several commercial sources for CYP-specific inhibitory antibodies. Inhibitory antibodies raised specifically against a certain CYP form are a good tool in distinguishing between CYP forms.

cDNA expressed enzymes are convenient tools when a specific activity or a selective chemical inhibitor cannot be used in metabolic studies. Recombinant enzymes are used to ascertain the role of a certain CYP in the metabolism of an NCE. Still, the biotransformation of an NCE by a single CYP does not necessarily mean its participation in the reaction *in vivo*.

For correlation analysis, a well characterized bank of human liver samples is needed. In correlation analysis, the measured CYP-specific activities are correlated against the rate of the metabolic pathway of an NCE in every individual liver sample. Correlation analysis gives information about the possible extent of the contribution of certain CYPs to the reaction under study.

4. The effect of an NCE on characteristic CYP-selective activities is studied by coincubating series of dilutions of an NCE with a specific substrate. By comparing the effects of an NCE on the CYP-specific activities to the respective effects of diagnostic inhibitors, a tentative prediction of the *in vivo* situation can be made.

21.2.4 Measuring Induction Potential *In Vitro*

Induction of xenobiotic-metabolizing enzymes is an adaptive cellular response that usually leads to enhanced metabolism and termination of the pharmacological action of drugs. A majority of the drug-metabolizing CYP enzymes are inducible, including the most important CYP3A4. Induction of human CYP enzymes is difficult to study because there are no human liver cell lines that express the full complement of CYP enzymes or reproduce the induction observed *in vivo* [25]. Thus, there is increasing interest in the development of mechanism-based test systems for CYP induction, and proof of principle has been demonstrated for the nuclear receptors CAR, PXR, and PPARα. The main *in vitro* methods used are direct and indirect binding assays as well as cell-based reporter gene protocols [26, 27].

Because these systems are based ultimately on the inducer/receptor interaction, they cannot detect inducers that require *in vivo* transformation to active species or inducers that act via an alternative mechanism. For instance, dexamethasone may increase the expression of the PXR and CAR receptors and thus induce CYP enzymes or synergize with other inducers. Even though the mechanism-based induc-

tion screens may not detect every CYP inducer, they are still expected to be valuable as preliminary screens.

21.2.5 Species Considerations

Species differences in drug-metabolizing enzymes are large and more often than not unpredictable. Ortologous enzymes can be rather closely related in terms of sequence homology and still display large quantitative and qualitative differences toward potential substrates and inhibitors. A much cited example is concerned with mouse hepatic Cyp2a4 and Cyp2a5, which differ in only 11 amino acids and still there is an almost complete reversal of substrate specificity concerning testosterone and coumarin [28]. On the other hand, metabolic reaction of a given compound may be catalyzed by enzymes belonging to different subfamilies in different species (see examples in Ref. 29). Currently, it is practically impossible to predict species differences in metabolism. Thus, it is advisable to perform some comparative studies in the preclinical phase. For example, metabolic stability studies give at least a tentative view about differences in clearance rates between different species. Metabolite identification in hepatic preparations from different species may point to important considerations when trying to extrapolate results from *in vivo* animal studies to human risk assessment. The earlier these types of comparative studies are being performed, the better their results can be taken into consideration, for example, in the selection of appropriate species for certain types of toxicity studies.

21.3 ASSIGNMENT OF METABOLIZING ENZYMES

In terms of potential clinical consequences, the assignment of metabolizing enzymes is perhaps the most important single task in *in vitro* metabolic studies. For the identification of the CYP (or basically any other) enzyme responsible for the metabolite formation, a number of different and complementary approaches are used. The indirect method utilizes specific CYP enzyme inhibitors, which are each added in turn into the incubation with the study compound in the presence of liver microsomal preparation, and the formation of the metabolite is monitored quantitatively. The decreasing metabolite formation indicates that the CYP responsible for the metabolite formation is being inhibited. Different inhibitor concentrations can be used to calculate the IC_{50} value of the inhibitor toward the metabolite formation, but often only single inhibitor concentration (high enough for good inhibition of certain CYP but low enough to keep the inhibition CYP-selective) is used. The use of CYP-specific antibodies is carried out similarly to the inhibitors. Another often used and complementary method is the use of recombinant CYP enzymes, where the involvement of a certain CYP in the metabolite formation can be directly detected as a metabolite peak in LC-MS. In addition, CYP activity phenotyped liver preparations are used to reflect the formation of the metabolites. The increasing metabolite formation paralleling the increasing activity of a certain CYP enzyme in the different liver preparations suggests also a probable involvement of that selective CYP isoform in the biotransformation.

21.3.1 Affinity (Inhibition) Studies

Inhibition of CYP enzymes is the most common cause of drug–drug interactions and has led to the removal of several drugs from the market during the past few years [30, 31]. Thus, studying the inhibitory effect of NCEs is extremely important; an inhibitory profile would also give an idea about the affinity spectrum of the compound.

The most common method is to compare metabolite formation of the known CYP-selective biotransformation in a liver microsomal incubation with and without the study compound. The probe substrate in a biotransformation reaction is chosen so that its monitored metabolite is known to be formed specifically via one certain CYP enzyme. These studies are often carried out as a cocktail-type approach using a number of different CYP-specific probe substrates (3–10) simultaneously in the one incubation, and the formed metabolites are usually analyzed with a single LC/MS-MS method developed for the simultaneous analysis of all the different metabolites (see Ref. 32). The cocktail approach is especially good for the screening of possible CYP interactions of an NCE, as the one incubation and one LC/MS run give information of all studied CYP enzymes in a time-efficient manner. If high inhibition potential toward certain CYPs in a cocktail-type screening for an NCE is detected, then the more accurate IC_{50} values can be evaluated using single-probe metabolite reactions and also using metabolite spiked standard samples for more accurate quantitation in LC/MS.

21.3.2 Enzyme-Selective Substrates, Inhibitors, and Antibodies

Selection of model drugs and reactions is an important consideration in many *in vitro* testing systems. For example, recent surveys [33, 34] give lists of CYP-selective substrates and inhibitors and some potential alternatives for situations in which preferable probes cannot be used. A validation study of several CYP substrates has also been published [35]. Without going into extensive details here, one important consideration is clinical relevance: probe substances should preferably be clinically used drugs for which relatively good and comprehensive *in vivo* data is available [36]. This would make *in vitro–in vivo* extrapolations more reliable, because these probes could then be used as comparators for molecules under study. Table 21.5 compiles currently used substrates and biotransformations as well as inhibitors, which display at least some selectivity toward specific CYP enzymes.

In the metabolizing enzyme identification studies employing diagnostic inhibitors, the approach is essentially the same as in the CYP interaction studies, but the detected analytes are the metabolites of the studied compound, and the concentration of the diagnostic inhibitor in the incubation is varied. The use of diagnostic inhibitors has become rather routine in the early screening of promising candidate drugs.

21.3.3 Recombinant Enzymes

Isolated heterogeneous human CYP enzymes, expressed as single enzymes at a time from cDNA in bacterial, yeast, and mammalian cells, have been commercially avail-

TABLE 21.5 Summary of Human Hepatic Drug-Metabolizing CYP Enzymes and Their Selected Probe Substrates and Inhibitors Used in *In Vitro* and *In Vivo* Studies

CYP	Percentage (%) in Liver[a]	Substrate	Inhibitor	Other Characteristics
1A2	~10	Ethoxyresorufin Melatonin Caffeine Phenacetin	Furafylline Fluvoxamine	Inducible Polymorphic
2A6	~8	Coumarin Nicotine	Tranylcypromine	Inducible Polymorphic
2B6	~2	Bupropion Efavirenz Cyclophosphamide	Thio-Tepa Ticlopidine	Inducible Polymorphic
2C8	~5	Paclitaxel Amodiaquine Rosiglitazone	Montelukast Quercetin	Polymorphic
2C9	~20	*S*-warfarin Diclophenac Tolbutamide Losartan	Sulfaphenazole	Polymorphic Inducible
2C19	~2	Omeprazole *S*-mephenytoin Proguanil	Fluconazole	Polymorphic Inducible
2D6	~2	Dextromethorphan Debrisoquine Bufuralol Propranololol	Quinidine Paroxetine	Polymorphic
2E1	~15	Chlorzoxazone Ethanol	Pyridine Disulfiram	Inducible
3A4	~40	Midazolam Testosterone Simvastatin Nifedipine Erythromycin	Ketoconazole Itraconazole	Inducible

[a]Relative and absolute amounts of hepatic P450 proteins vary highly among people. Rounded values are based on a meta-analysis by Rowland Yeo et al. [45].

Sources: Data adapted from Refs. 37–44.

able for several years. Recombinant CYPs have been adopted as frontline tools in early drug development. These systems can be utilized to ascertain whether an NCE is a substrate for a particular CYP form and what metabolite is generated by that specific enzyme. Moreover, recombinant enzymes can be used as small-scale bioreactors to generate usable amounts of metabolic product [46–48]. They have been used also for clearance predictions and drug–drug interaction studies [49]. It should be kept in mind, however, that recombinant expressed enzymes are not in their natural microsomal environment and consequently there is a concern about the extent of applicability of the findings.

21.3.4 Enzyme Kinetic Characterization of Principal Metabolic Reactions

It is of importance to determine enzyme kinetic characteristics of at least the principal and rate-limiting reactions of a substance for scaling up and predicting *in vivo* kinetics of an NCE. If there is only one rate-limiting reaction catalyzed by a single enzyme, this investigation is easier, but if there are several reactions and multiple enzymes, the task is obviously more difficult and the prediction may become more imprecise. The importance of enzyme kinetic characterization becomes apparent in the extrapolation process: the first step in the scheme is determining "enzyme efficiency or enzyme intrinsic clearance," essentially reflecting V_{max}/K_m, which is then scaled to liver unit weight.

21.4 IDENTIFICATION AND QUANTIFICATION OF METABOLITES

Analytical tools are at the heart of metabolic studies during early drug development. Metabolism studies start with analyses of the parent compound in simple metabolic stability studies, in which the substrate loss is being determined, and continue to more complex structural analyses on the identification of major and minor metabolites produced by the catalysis of oxidative and conjugative enzymes, and ending with development of routine analytical methods for the identification of the respective enzymes and also addressing other drug interaction-related metabolism studies.

Although the measurement of the disappearance of an NCE in human liver preparations (i.e., "metabolic stability") sounds rather simple, it is actually a rather complicated undertaking, including the development of an assay for the parent compound, and contains a lot of caveats, starting with chemical stability and binding problems. However, if this assay is expanded with the identification of metabolites produced, it gives very useful information for drug development and for planning of subsequent experiments. With current mass spectrometry (MS) techniques and the use of liver preparations, microsomes, the S9 supernatant, or homogenate, fortified with all appropriate cofactors, a tentative understanding of metabolites and metabolic routes can be obtained. On this basis, educated guesses about the involvement of potential metabolizing enzymes can also be made. Current analytical repertoire makes it relatively easy to devise appropriate routine assays for measuring metabolism of a compound. Naturally, it depends on the results of the identification of principal metabolites for a compound, but if a compound is metabolized, a routine method has to be developed for the identification of metabolizing enzymes.

21.4.1 First Incubations—Tentative Identification of Metabolites

The approach used for elucidating the *in vitro* biotransformation of an NCE is dependent on the needed specificity of the data to be obtained [50]. For an NCE with no known metabolites or any information about metabolic behavior, the easiest and time-efficient way to identify the *in vitro* metabolites is to produce all oxidative and conjugative metabolites with a single incubation with cultured hepatocytes or liver homogenates that include all CYP enzymes, as well as the principal conjugative enzymes, at least UGT, SULT, GST, and NAT (Table 21.2). After incubating the drug

substance with cultured hepatocytes or in liver homogenate together with the cofactors NADPH, UDPGA, GSH, and PAPS, needed for function of the enzymes, the metabolites formed are identified usually by liquid chromatography–mass spectrometry (LC/MS) techniques [51–53]. If some more specific information concerning the metabolism is needed, other types of liver preparations can be utilized, and the number of added cofactors can be varied. For example, if only phase I oxidative reactions need to be studied, liver microsomal fraction is used instead of the homogenate, as it contains all the oxidative CYP enzymes but lacks the GST and SULT enzymes. However, glucuronide conjugation may also occur with microsomal fraction, if cofactor UDPGA is used in incubation (for more detailed description, see Sections 21.1 and 21.2).

After the metabolite peak has been found from the chromatographic (LC/MS) data, the biotransformation can be identified according to the mass spectrum obtained for the metabolite. With modern LC/MS instruments equipped with atmospheric pressure ion sources, most commonly electrospray (ESI) [52, 53], usually only molecular ions are detected in the spectrum. This enables the elucidation of the shift in the molecular weight during the biotransformation from the substrate to metabolite. Some common shifts in molecular weights caused by metabolic reactions are given in the Table 21.6. Depending on the MS instrument type used, fragment ion MS data can also be obtained from the metabolite, enabling the elucidation of the biotransformation site in the substrate. With mass spectrometers capable of high resolution and good mass accuracy, accurate mass data from the metabolites

TABLE 21.6 Changes in Molecular Mass Due to the Most Common Metabolic Reactions

Biotransformation	Mass Change (u)	Charasteristic Fragmentation[a] (NL = Neutral Loss)
Dehydrogenation (oxidation)	–2	
Demethylation	–14	
Desethylation	–28	
Oxidative desulfuration	–32	
Hydrogenation (reduction)	+2	
Methylation	+14	
Hydroxylation	+16	
N/S-oxidation	+16	
Epoxidation	+16	
Acetylation	+42	
Sulfation	+80	–80 u (NL of SO_3)
Glucuronidation	+176	–176 u (NL of $C_6H_8O_6$)
Glutathionation	+305[b]	–129 u (NL of $C_5H_7NO_3$)
		–275 u (NL of $C_{10}H_{17}N_3O_6$, aryl-GSH)
Amino acid conjugation		
Glycine	+57	m/z 76 (Gly + H^+), m/z 74 (Gly – H^+)
Taurine	+107	m/z 126 (Tau + H^+), m/z 124 (Tau – H^+)

[a]The fragments of phase I metabolites are typically the same as for the substrate, or differ equally from the molecular weight.

[b]The glutathionation may lead to a number of different molecular mass changes; the addition of 305 u is the most simple case where glutathione replaces hydrogen atom in the substrate.

can be obtained, enabling differentiation in the biotransformation with a different molecular formula but the same nominal mass change, for example, distinquishing simultaneous demethylation and hydroxylation from hydrogenation, even though both reactions lead to an increase in molecular weight of two mass units. Additional H/D exchange studies can be conducted to distinguish different types of oxidations, for example, hydroxylation versus N-oxidation, giving the same mass shift but completely different biotransformation [54].

21.4.2 Absolute Structures—Reference Metabolites and NMR Studies

In some cases the data obtained by LC/MS methods as above does not give a clear enough picture about the structure of the formed metabolite, or the obtained tentative structure of the metabolite has to be elucidated in a more detailed manner. In these cases, the most simple and unambiguous approach is to synthesize the possible structures obtained for the metabolite (after LC/MS), and the LC/MS behavior of the synthesized compounds is compared with the unknown metabolite to confirm/exclude the elucidated structure. This of course may take more time for the chemists to synthesize the desired structure(s). To reliably conclude that the synthesized compound and the metabolite have the exact same structure, more than one single chromatographic method (different columns, different eluent pH) should be used to verify the similar LC/MS behavior.

An alternative possibility is to isolate and purify the metabolite from the incubation matrix, and to elucidate the exact structure using nuclear magnetic resonance (NMR) methods [55, 56]. However, the sensitivity of the NMR is not even close to the level of mass spectrometric methods, and about 100 nanograms of sample has to be obtained from purification to be able to acquire definitive NMR data in sensible time (i.e., overnight), and orders of magnitude more for more insensitive two-dimensional NMR experiments. Fortunately, in many cases, the most sensitive simple NMR measurement (i.e., basic one-dimensional ^{1}H NMR) may already help in confirmation of the structure. Also online coupled LC/NMR instruments [57] are now available to avoid the purification step, but the chromatographic separation in these systems always suffers in comparison to more easily coupled LC/MS techniques.

21.4.3 Quantitative Studies

Besides the qualitative LC/MS studies in metabolite identification, quantitative data is also needed from a number of different studies with *in vitro* liver preparations. Among these, the most typical are the P450 enzyme interaction studies, direct drug–drug interaction studies, metabolizing CYP enzyme identification studies, and the estimation of metabolic stability (already mentioned). To save time, these analyses are often carried out semiquantitatively, that is, without spiked standard samples, and the results are reported as relative LC/MS peak areas of each detected compound in different samples, and these areas are expressed in percentages by comparing them to reference incubation samples (e.g., without an inhibitor or zero time incubation). In this stage of drug development, this sort of approach usually gives the accuracy needed, as the main purpose is to see if there are interactions or not,

or to evaluate the general level of metabolic stability. Also, in this phase the metabolites themselves are usually not available to be used as external standards for quantitation (for creating a calibration curve), and so if any spiked standard samples are used, the metabolites are quantified as "substrate equivalents." This in turn may sometimes lead to large differences between the results obtained and the real metabolite concentrations, as the mass spectrometric response between the drug and its metabolite may vary a lot due to different ionization and fragmentation properties, especially if the biotransformation occurs in the same functional group of the compound where the ionization occurs in the ionization process of the LC/MS analysis.

The main issue in the analyses of early drug development is to have good specificity, to be sure to monitor the correct compound (metabolite), and to have good linearity of detection response, so that the relative peak areas, obtained without calibration curves created by spiked standard samples, are representative of the concentration differences in different samples. When the need for high sensitivity is added to the list of requirements, the usual analytical system of choice is LC/MS-MS with triple quadrupole mass spectrometers.

21.4.4 Requirements for Analytical Instrumentation

Although the range of modern analytical instrumentation commercially available for metabolism studies is very large, today's metabolism studies are carried out using high performance liquid chromatography coupled online with mass spectrometry (LC/MS), and additionally some other detector types, such as UV-diode array (DAD, PDA), fluorescence, or radioactive detectors, are used. The additional detectors are solely used for "detecting purposes"—not for identification of the compound but for its quantitation or detection if the mass spectrometric response of the analyte is poor. The fluorescence detector can be used as a very sensitive detector for certain types of fluorescing compounds if suitable excitation and emission wavelengths are chosen, and the radioactive detector can be used for screening and quantification of the metabolites of the radiolabeled substrate. However, these are rarely needed in *in vitro* studies; the LC/MS instruments can give many types of data from a single run, including detection and identification of the metabolites, and at least semiquantitative concentration estimates.

Generally, any kind of mass spectrometer with HPLC compatible ion source can be used in metabolite idenfication and quantitation, but unfortunately, none of the mass spectrometer types is the optimal instrument for all kinds of studies (qualitative vs. quantitative), and thus at least two different types of instrument are required if all the studies are to be conducted at a state-of-the-art level. The strengths and drawbacks of the most common types of mass spectrometer studies are given in Table 21.7. The table includes only the time-of-flight/QTOF [58, 59], ion trap [60, 61], and triple quadrupole [62, 63] type instruments, but recently a number of hybrid instruments containing features from many instrument types have been introduced by manufacturers. Of these new instrument types, most useful in the analysis of small drug-like molecules are the triple quadrupole/linear ion trap instrument, where the last quadrupole of a traditional triple quadrupole instrument is replaced by a linear ion trap to increase the full scan sensitivity [64], and the ion-trap-TOF instrument, where the collision cell of QTOF is replaced by an ion trap to enable MS^n

TABLE 21.7 Applicability of the Most Common Type Mass Spectrometers for *In Vitro* Drug Metabolism Studies (in LC/MS)

Parameters	Time-of-Flight (TOF-MS)	Ion Trap (IT-MS)	Triple Quadrupole (QQQ-MS)	Quadrupole-TOF (QTOF-MS)
Strengths	• High full scan sensitivity • High resolution for exact mass measurement • Very fast data acquisition (for HTS) • Easy to operate	• High full scan sensitivity • CID MS/MS and even MS^3 possibility	• Very high sensitivity for known analytes (MRM) • High linear range • High quality CID MS/MS, also with precursor ion and neutral loss scanning • Very fast data acquisition (in MRM mode)	• High full scan sensitivity • High resolution for exact mass measurement • Very fast data acquisition (for HTS) • CID MS/MS
Drawbacks	• Poor linear range • No real MS/MS possibility (only "in-source" MS/MS)	• Poor linear range • No exact mass possibility • No precursor ion or neutral loss scanning MS/MS • Slow scanning speed	• No exact mass possibility • Poor full scan sensitivity (for screening)	• Poor linear range • No precursor ion or neutral loss scanning MS/MS
Optimal use	Excellent for metabolite screening and identification of biotransformations	For metabolite screening and their tentative identifications; identification of biotransformation site	Excellent for quantitative analysis of known analytes; identification of biotransformation site; HTS applications	For metabolite screening; identification of biotransformations and their sites; HTS applications
Price	200,000–300,000€	100,000–160,000€	160,000–300,000€	350,000–600,000€

experiments with high resolution for fragment ions [65]. For higher mass resolution studies, mostly needed when working with biomolecules, the Orbitrap [66] and ion cyclotron resonance [67] mass spectrometers offer superior performance.

The basic rule of thumb is that the time-of-flight mass spectrometers are the instruments of choice for a screening type of analysis (biotransformation screening and tentative metabolite identification), whereas the triple quadrupole instruments are superior in quantitative work. For metabolite screening the instrument needed should have good sensitivity with wide scan range to detect all unexpected metabolites simultaneously; also, it should be able to give qualitative data for at least tentative identification of the metabolites. The time-of-flight instruments have all this, the qualitative data coming from easy operation, accurate mass measurements, and in-source fragment ion data.

21.5 ACTIVE/REACTIVE/TOXIC METABOLITES

It would be very useful to know whether metabolism is needed for the biological action (pharmacologic or toxicologic) of drugs. Metabolic activation, which may be pharmacologically useful (prodrug metabolism) or toxicologically adverse, is an established primary mechanism of action for many drugs and toxicants [68–71]. Especially during early drug development, a screening system able to detect the formation of reactive drug metabolites would provide crucial information for the development program. In toxicity risk assessment, a robust and validated screening system is urgently needed, because metabolism is often needed for tissue toxicity, immunotoxicity, genotoxicity, and/or carcinogenicity to be initiated. In Table 21.8,

TABLE 21.8 Potential *In Vitro* Screening Systems for Detecting the Formation of Reactive Metabolites

Type of Assay	Rationale	Example
Inhibition of drug metabolism	"Suicide" binding of a reactive metabolite with the enzyme	Tienilic acid
Formation of GSH conjugate	Binding of a reactive metabolite with GSH	Paracetamol
Covalent binding	Detection of bound reactive metabolite by radioactivity or by MS	Many carcinogens and mutagens
Lipid peroxidation	Detection of reactive oxygen species and/or peroxides by thiobarbituric acid reagent	Many halogenated substances
Target cell toxicity	Toxicity, for example, cell viability of metabolites produced either in the target cell itself or exogenously	Practically all substances are ultimately toxic to cells at high enough concentrations; mediation of toxicity by metabolism has to be incorporated into the test

several potential screening systems are summarized. Generally, the generation of short-lived reactive metabolites is relatively straightforward to observe in various *in vitro* systems, although, it has to be stressed, none of them are adequately validated [72]. Because reactive metabolites are usually short-lived and unstable, they cannot usually be detected as such, but only after binding to trapping agents, cellular macromolecules, or other cell components. However, it is difficult to predict whether the formation of metabolites capable of binding with trapping agents or macromolecules will ultimately result in serious toxicity *in vivo* [73].

During early drug development, assays for mechanism-based inhibition are perhaps more widely used than other assays for detecting reactive metabolites. Mechanism-based inhibition can occur via the formation of metabolite intermediate complexes or via the strong covalent binding of reactive intermediates to the protein or heme of the CYP. Mechanism-based inhibition is terminated by enzyme resynthesis and is therefore usually long-lasting [74, 75]. In some cases, the metabolic product inactivates the enzyme completely. This is referred to as suicide inhibition. The most important phenomenon of mechanism-based inhibition is the time-, concentration-, and NADPH-dependent enzyme inactivation [2, 74]. Classical mechanism-based inhibitors include furafylline (CYP1A2) [76, 77] and gestodene (CYP3A4) [78]. It is worth noting that many compounds display both mechanism-based and competitive modes of inhibition; one such example is ticlopidine [79].

Although the need to detect and identify reactive metabolites is widely recognized for drug development, there are large gaps in our knowledge and also uncertainties about which kinds of strategies to employ. Detecting reactive metabolites does not necessarily mean that tissue toxicity or immunotoxicity will ensue. Metabolic activation seems to be behind many immunotoxic manifestations, but unequivocal evidence has been difficult to produce [80, 81]. One of the more important problems has been the selectivity of binding of reactive intermediates. Previously, it was thought that binding of reactive metabolites with cellular macromolecules is essentially a nonselective process. Now it has become more and more apparent that the process is primarily a selective process and even slightly different reactive species display large differences in their binding targets [82]. Development of screening methods that would differentiate toxicologically significant reactive metabolites from inactive ones would be a significant advance. Interesting analyses for future developments in research on metabolic activation from the industrial perspective are provided by Evans et al. [83] and Baillie [73].

21.6 TESTING CONFIGURATIONS

With the advent of combinatorial chemistry and large libraries of chemicals, a need for high throughput screening (HTS) has become ever more pressing in the pharmaceutical industry. Most major drug companies make use of various HTS configurations for efficient screening. Regarding the metabolic properties of NCEs, screenings of metabolic stability and drug–drug interactions have advanced to a considerable extent [22]. Often, rather sophisticated detection systems based on fluorescence are used in HTS systems. In the following, MS detection techniques are described.

21.6.1 Sample Preparation

Because of relatively defined incubation conditions with liver preparations or hepatocytes, sample preparation for LC/MS can usually be kept very simple. Usually the simple centrifugation of the incubation sample and careful pipetting of the supernatant into an HPLC autosampler vial is enough for an LC/MS compatible sample. However, when performing quantitative analyses, even for relative peak areas without external standards, the use of an internal standard is preferable. If long sample lists are acquired (e.g., overnight from an autosampler), the high buffer and salt concentrations together with other biomolecules or high concentration incubation media components may contaminate the mass spectrometer ion source, leading to general decrease in the sensitivity level toward the end of the sample list. By using an internal standard in the samples and using the relative peak area between the analyte and the internal standard as a result, instead of a plain analyte peak area, the effect of ion source contamination on the results can be avoided (as long as the sensitivity level stays acceptable).

21.6.2 Single Enzyme/Activity Systems

Many *in vitro* metabolism studies—such as interaction studies with CYP-selective inhibitors and antibodies, inhibition screens using studied NCEs with CYP-selective substrates, and studies with recombinant enzymes—are relatively routine and robust from the biochemical and analytical point of view, and there is a wide literature background for using these tools in various *in vitro* settings. Detailed descriptions of the methodological aspects of the more widely used CYP-selective substrates and inhibitors can be found in recent articles [35] (see also Table 21.5).

21.6.3 Medium to High Throughput Systems

Thorough surveys of medium to high throughput systems for *in vitro* absorption, distribution, metabolism, and excretion (ADME) have recently been published [84, 85]. When identifying the metabolizing or interacting CYP enzymes for larger numbers of compounds, the use of recombinant CYP enzymes and diagnostic CYP inhibitors are both suitable for HTS mode. With diagnostic CYP inhibitors, only one inhibitor concentration is used, low enough to be specific for inhibition of the target CYP but high enough for adequate inhibition if the study compound is metabolized via the same CYP. In the HTS approach the LC/MS analysis time is a crucial factor, and therefore very specific analytical methods for detecting only the monitored metabolites in a very short time are needed. The study compounds may also be incubated as mixtures of compounds, and the LC/MS method is adjusted to detect all the desired metabolites from each compound in a single analysis, again increasing the analytical challenge. However, the possible interaction with the simultaneously incubated compounds may affect the results obtained; especially with recombinant enzymes, the formation of monitored metabolite is a sign of the involvement of the certain CYP enzyme in the metabolite formation.

For fast screening of P450 inhibition, it is possible to employ a cocktail-type analysis of known CYP-selective biotransformations in liver microsomal incubations. A number of assays for different CYP enzymes have been developed and

published [22]. The most simple cocktails contain only a few important CYP isoforms, whereas the most complete assays contain all nine of the most important CYPs and also more than one probe reaction for some isoforms [32]. If a HTS for a large number of study compounds is needed, usually only one concentration per compound is needed to give a rough estimate of the level of interaction with each CYP. However, the development and use of the cocktail approach is much more challenging than the use of single-substrate incubations, as the substrate concentrations have to be optimized so as not to cause any interactions between each other; also the reliable LC/MS analysis of many chemically different metabolites with a single run is quite challenging. If very high throughput is needed, the studied compounds may be delivered into the incubations as cocktails of several compounds, and the go or no-go decision is then given to all of the compounds tested together. However, this approach contains a risk of losing good lead candidates, as only one potent inhibitor in the mixture of simultaneously tested compounds may lead to wasting the other, noninhibiting, compounds.

21.6.4 Analytical Considerations

From the analytical point of view, the issues covered in Section 21.4 are all valid here as well. The quantitative analyses in these studies are usually carried out as relative LC/MS peak areas, without any spiked calibration standard samples. The excellent linear range and specificity of triple quadrupole mass spectrometers makes them the best choice for LC/MS instrumentation, enabling very sensitive detection of the low metabolite concentrations. Also, when detecting metabolites from several compounds incubated simultaneously in the same well/vessel, the triple quadrupole instruments provide the most specific detection for each analyte. Even structural analogues with the same molecular weight and the same retention times can still be separated, as long as the compounds have different fragmentation pathways in the collision-induced dissociation. However, with HTS applications and constantly changing analytes, the time-of-flight mass spectrometers (or ion trap instruments) may offer a good alternative for instrumentation, as the excellent sensitivity for acquiring data over a high mass range enables the detection of all different analytes, without the need to adjust the detection parameters before the analysis, as is the case with triple quadrupole instruments. Therefore, with LC/TOF-MS and LC/QTOF-MS, the same generic method may be used for a large set of completely different compounds in HTS applications [86]. With QTOF, the MS/MS option in data acquisition is available. However, if the application is for P450 inhibition screening, where detected compounds remain the same all the time, the triple quadrupole mass spectrometer is the best possible instrument for the analysis.

When the aim in analyses is to produce as much data as possible from a single LC/MS run, many software packages can control the data acquisition with so-called data-dependent operations [87], which may be particularly useful with MS/MS instruments, as the detection of high abundance ions with the certain m/z in the spectrum of a high abundance ion may trigger some other type of data acquisition using this ion as a precursor mass—such as product ion scanning and selected reaction monitoring. This way, both qualitative and quantitative data can be acquired simultaneously. If the molecular weights of the studied metabolites are known

before analysis, the same acquisition functions can of course be set manually, which usually provides better quality data.

21.7 PRIORITIES OF *IN VITRO* METABOLISM STUDIES AND *IN VITRO–IN VIVO* EXTRAPOLATION

One of the important questions concerning the starter *in vitro* test of metabolism is: Which one is best as a first test? Because each molecule is rather unique in terms of metabolism and kinetics, we would need a rather comprehensive test, which should cover as many salient features of a compound as possible. But this requirement is contradictory to simplicity, rapidity, and economy, which are needed for early testing.

There is no fixed single strategy for performing metabolic studies during early drug development. Rather, major drug companies employ their own slighly variable schemes. Table 21.9 presents one possible strategy of metabolism studies in early drug development, which is described below. Whatever strategy is adopted, assessment of metabolic stability is very important to perform early on in the drug development program, because it determines, despite many caveats inherent in current testing systems, to a large extent the usefulness of the molecule and gives an important value for *in vitro–in vivo* extrapolation.

21.7.1 Priorities of *In Vitro* Metabolism Studies

Determination of basic physicochemical properties is the prerequisite for every subsequent property determination. When developing adequate analytical methods for NCEs, it is important to know solubility, pK_a, lipophilicity, and also chemical

TABLE 21.9 Example of a Rough Priority Scheme for *In Vitro* Studies Related to Metabolic Properties of an NCE

Properties of an NCE	Remarks
Solubility, lipophilicity, pK_a, chemical stability, possible inpurities, plasma protein binding (free fraction)	These data are absolutely required before metabolism studies to prevent unpleasant surprises; free fraction (plasma protein binding) needed for proper extrapolation
Metabolic stability in microsomes/ homogenates or hepatocytes	A crucial piece of information for the extrapolation of hepatic clearance
Metabolite identification in microsomes, homogenates, or hepatocytes	Human hepatocytes are an enzyme source of choice if available
Inhibitory interactions in human liver microsomes	Also help pinpoint enzymes with affinity toward the studied compound
Identification of metabolizing CYPs	Primarily employed: diagnostic inhibitors, recombinant enzymes, correlation analysis (if a liver bank is available)
Induction of drug metabolism	Preferably in human hepatocytes if available
Metabolic stability and identification of metabolites in animal liver preparations	To search for appropriate species for certain toxicity studies

stability. A cost-effective approach is to integrate method development, simple degradation study, and physicochemical property characterization together with plasma protein binding and nonspecific binding. It is feasible to start *in vitro* metabolism and permeation studies after the physicochemical, degradation, and binding properties of an NCE are already known. It is easier to incubate, to analyze, and most importantly to predict the *in vivo* situation, if possible interfering problems are dealt with before starting the metabolism experiments.

Metabolism data using human liver preparations are irreplaceable if the drug candidates are intended for human use. Since a rat is not human, it is not feasible to start metabolism studies with rat or any other animal liver preparations; their time is later, when animal models for toxicological studies are selected. A good practice is to start metabolism studies with human and several obvious animal models, but resources might be wasted if the compound is rejected. The first human *in vitro* metabolism studies should be conducted with a system involving all the possible drug-metabolizing enzymes and cofactors for the enzymes. High initial concentrations of parent compound should be used and all the possible (predicted and unpredicted, major and minor) metabolites should be identified. Special attention needs to be addressed to those metabolites potentially associated with toxicity, such as acyl glucuronides, epoxides, glutathione pathway metabolites, and hydroxyl amines, because their presence indicates a need for lead optimization. This primary metabolism screen for tentative metabolic stability and metabolite identification could best be conducted with a LC-TOF-MS or LC-QTOF-MS instrument. The preferred liver preparation would be human liver homogenate (pool of several individuals) due to its relatively easy access compared to hepatocytes. A permanent hepatocyte-like cell line would be highly desirable, but no such cell line has yet been found or developed.

For the compounds that survive the primary metabolism screen, several second tier studies are needed. First, kinetic metabolic stability and metabolite formation studies are needed for proper *in vivo* prediction. This is usually done in human liver microsomes, unless the metabolites found in the primary screen indicate homogenate, S9, or cytosol has to be used, with several time points, concentrations, and replicates. Second, rate-limiting CYP enzymes, or other enzymes if indicated by primary screen, should be identified to assess potential drug–drug interactions and also kinetic aspects. Human liver microsomal incubations of an NCE together with CYP-specific inhibitors or antibodies give a clear indication of which CYP enzymes are involved. If the picture is completed by incubations with recombinant CYP enzymes and both tests point to the same CYP enzyme, an involvement of a certain CYP enzyme in a particular biotransformation can be relatively reliably confirmed. Third, a microsomal study to be conducted at this stage is CYP enzyme inhibition screen. A simple cocktail approach with one concentration of NCE aiming at "% activity inhibited" will usually do the trick. If something risky is observed, more thorough investigations including mechanistic studies should be done.

In the third round and for the molecules that remain, one should study CYP induction in human hepatocytes. Once the hepatocytes are employed, it will be cost efficient to study the other metabolic properties on hepatocytes also: confirmation of the previous investigations in microsomes/homogenates/S9. Thus, one should again study metabolic stability, metabolite identification, CYP inhibition and induction, and metabolizing CYP enzymes. A good add-on would be a study of accumula-

tion of the parent and metabolites in hepatocyte cells, because this information may be crucial for the interpretation of *in vitro–in vivo* extrapolation.

Finally, the rat (or mouse or dog or monkey) becomes relevant. If the candidate drug passes the *in vitro* tests with human preparations, a multispecies metabolism study would be appropriate to find out the most relevant species for toxicological investigations. Special attention should be given to the metabolites formed—not metabolic stability—as every relevant human metabolite should be found from the toxicological species.

21.7.2 *In Vitro–In Vivo* Extrapolation

Metabolic clearance (intrinsic clearance, CL_{int}) is an important determinant for extrapolation purposes. CL_{int} is a direct measure of the efficacy of an enzyme to metabolize a substrate. Knowing that the drug concentrations in *in vivo* situations are usually far below their K_m values (i.e., under linear velocity conditions), CL_{int} is equal to the ratio of V_{max}/K_m [88, 89]. When the contribution of multiple enzymes to the metabolism is assumed, the net *in vitro* CL_{int} in the whole study system can be expressed as the sum of each metabolic pathway [89, 90].

Several elements limit the reliability of predicting CL_{int} on the basis of *in vitro* studies. The most important factor to be taken into account is the nonspecific binding of the substrate to the microsomal protein fraction. Generally, for drugs with high protein binding affinity, underestimations with CL_{int} can occur if protein binding is taken into account. However, inclusion of both microsomal and plasma protein binding usually results in good agreement between extrapolated and actual clearance values [91, 92].

Altered hepatic blood flow may have an impact on the accuracy of the *in vitro–in vivo* extrapolation. On the basis of their hepatic clearances, drugs can be classified either as low clearance or high clearance compounds. In the first case, the hepatic blood flow has a minor effect on the total clearance of the drug (enzyme-limited clearance), whereas in the latter case, changes in the hepatic blood flow will have a drastic effect on the total clearance of the drug (flow-limited clearance). However, the clinical outcome is hard to predict, as conditions affecting portal blood flow usually involve concurrent complications [2, 93].

In general, *in vitro–in vivo* extrapolations are performed with two objectives in mind: prediction of the intrinsic clearance (CL_{int}) of an NCE and prediction of potential drug–drug interactions. Other pharmacokinetic parameters, such as plasma half-life, volume of distribution, and oral bioavailability, have also to be considered for the estimation of *in vivo* kinetics on the basis of *in vitro* studies. Many *in vivo* factors affect the results of extrapolation. One of these is the binding to plasma and tissue proteins and, ultimately, the distribution volume of the drug. Details of various models and equations can be found in recent reviews [94, 95].

21.8 VALIDATION OF *IN VITRO* SYSTEMS

The main purpose of *in vitro* tests used in preclinical departments of drug companies is to aid drug development, not registration. Naturally, companies are interested in

the precision and accuracy of *in vitro* tests, but there is no requirement of formal validation. Consequently, validation is done primarily in-house. If the results of the tests are being used in regulatory applications, then a certain amount of validation—even if not formal—is required. FDA, EMEA, and authorities in Japan have all published some guidelines under which circumstances of *in vitro* drug interaction results can be used to support marketing authorization. The current view is that prediction of drug–drug interactions on the basis of *in vitro* studies is most advanced from the validation and reliability points of view [1, 96–98].

In vitro systems are generally useful in identifying compounds with severe potential liabilities, such as metabolic instability, very high affinity to a principal metabolizing enzyme, or metabolism by a polymorphic enzyme (e.g., CYP2D6). Whether false positives in these respects are rare or common, is not known. Also, the frequency of false negatives is not known. Discussions with company scientists about their unpublished data have led to a view that the predictive power of various *in vitro* tests tends to weaken when the number and structural variability of tested substances increase. There is no consensus about what is the acceptable frequency of false positives or negatives, or more precisely, what are the acceptable tolerances for findings. The only remedy for these uncertainties is validation according to the established principles [99].

21.9 CONCLUSION

Metabolism is a major determinant governing both pharmacokinetics and clinical response of the majority of drugs and a great deal of effort is now directed at assessing key metabolic parameters in the early stages of drug development. Several *in vitro* methods are now available for determination of metabolic features, often yielding data that reasonably well predict *in vivo* behavior of the studied drug molecules [1]. Further development and refining of these methods will provide us with methods having increased precision and robustness, allowing for highly reliable analysis and prediction of metabolic features. In addition, analogous methodology will be employed on predicting absorption, organ uptake, and efflux mediated by various transporter systems, plasma protein binding, and cellular determinants of intrinsic clearance. As an outcome of this development, we are already witnessing a diminishing number of drug candidates being withdrawn from clinical studies (or the market) due to major kinetic problems, such as strong metabolism induction or interaction potential.

An important consideration concerns the integration of results from various *in vitro* tests. Pharmacokinetics is actually an integrated whole, and different pieces are valuable only in the context of the whole. Development of modeling and simulation tools, for example, physiologically based pharmacokinetic models, will aid in incorporation of the results from *in vitro* studies into realistic models and in understanding genetic, physiological, and pathological factors affecting the behavior of an NCE when a substance is administered for the first time to a human being. Although much has already been done, there is still a need for reliable and robust pharmacokinetic models that would incorporate various factors and processes affecting the fate of a molecule. With the help of these *in silico* models, information from *in vitro* tests becomes continuously integrated into the database.

The use of human-derived biological systems seems rather natural, because we are interested primarily in human metabolism and kinetics. Human hepatocyte assay is regarded as the gold standard for metabolic screening, but there are several problems in this approach. There is an urgent need to develop a renewable, immortal hepatocyte-like permanent cellular model, which would express all the necessary enzymes and transporters at the level resembling *in vivo* hepatocyte. One promising candidate is a recently developed hepatic cell line, HepaRG, which expressed most enzymes and transporters at a relatively high level [100, 101].

An important drawback, at least in some academic laboratories that work to develop *in vitro* techniques to measure drug metabolism, is the absolute necessity of expensive mass spectrometric techniques for analytical work. However, we feel that rational and productive research on *in vitro* drug metabolism tests is not possible without up-to-date analytical techniques. Analytical chemistry is at the heart of early drug development.

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